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Determination of alkylphenol metabolites in fish bile using solid-phase analytical derivatization (SPAD) and gas chromatography–mass spectrometry in electron ionization mode (GC–EI–MS)

Grete Jonsson • Atle Nævdal • Jonny Beyer

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International Council for the Exploration of the Sea
Conseil International pour l'Exploration de la Mer

H. C. Andersens Boulevard 44–46
DK-1553 Copenhagen V
Denmark
Telephone (+45) 33 38 67 00
Telefax (+45) 33 93 42 15
www.ices.dk
info@ices.dk

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Contents

Abstract

This document provides advice on the analysis of alkylphenol (AP) metabolites in fish bile. APs are released to aquatic environments from many different sources related to human activities, such as offshore oil production. The method for determination of APs includes enzymatic deconjugation of fish bile followed by solid-phase analytical derivatization (SPAD) with bis(trimethylsilyl)-trifluoroacetamide (BSTFA). The derivatized APs are separated, then quantified using gas chromatography–mass spectrometry in the electron ionization mode (GC–EI–MS). Quality control measures should be implemented to ensure good performance of the method. This GC–EI–MS method allows for a selective and sensitive analytical detection of APs in fish bile.

Keywords. Fish, alkylphenols, biomonitoring, offshore oil industry, produced water, review.

1 Introduction

The issue of alkylphenolic chemicals (APs) as environmental contaminants in aquatic ecosystems is a general concern, because of the endocrine disrupting potential of some APs. APs are released to aquatic environments from many different sources related to human activities. One example is offshore oil production where APs are released to the sea in produced water discharges that could result in AP contamination of fish exposed to the contaminated water. Detection of AP metabolites in fish bile will offer a more sensitive AP exposure marker than the detection of AP parent compounds in tissues, such as liver. Discrimination of low-concentration exposed fish populations from non-exposed populations has relevance for the environmental monitoring and ecological risk assessment of produced water, and AP contaminants in particular.

Currently in North Sea oil fields, the total annual discharge of produced water is more than 400 million tonnes and of this the Norwegian share is approximately 160 million tonnes (OSPARCOM, 2009). APs are common in produced water and the annual discharge from North Sea oil fields contains hundreds of tonnes of APs (Utvik, 1999; Boitsov *et al*., 2007). The concentration of phenolic compounds in produced water varies typically between 0.6 and 10 mg l^{-1}, depending on time of sampling and production field (Brendehaug *et al*., 1992; Røe, 1998; Utvik, 1999). Phenol allied with C1–C3 APs constitute more than 95% of the total phenols in produced water, whereas APs with a higher degree of alkylation (butyl through heptyl, C4–C7) are present in lower concentrations, 2–237 µg l^{-1} (Brendehaug *et al*., 1992; Røe, 1998; Boitsov *et al*., 2004). In seawater samples obtained around offshore installations, environmental concentrations of C1–C4 APs have been determined at levels of 20–140 ng l^{-1} (Riksheim and Johnsen, 1994; Harman *et al*., 2009).

A large dilution at offshore sites mitigates high concentration effects of produced water and its constituents in the downstream area. Ecological risk assessment studies have so far failed to produce evidence of AP contamination effects, such as reproductive disturbance, in North Sea fish populations as a consequence of the APs released in produced water streams (Myhre *et al*., 2004; Beyer *et al*., 2012). Nevertheless, there should be a continued effort to improve the analytical tools for detecting AP exposure in fish at low environmental concentrations, because more sensitive exposure markers would strengthen the environmental risk assessment of produced water discharges at offshore fields.

Alkylphenols are readily taken up by fish, mainly through the gills (Sundt *et al*., 2009). After distribution within the fish, the APs are metabolized to a large degree in the liver. The metabolic products then accumulate in the gall bladder before elimination. Analyses of alkylphenols in biological matrices are generally more challenging than analyses in aqueous samples. Although a variety of metabolites might be formed in different fish tissues, the glucuronic acid conjugate has been identified as the major metabolite excreted in the bile following alkylphenol exposure (Thibaut *et al*., 1998; Smith and Hill, 2006; Jonsson *et al*., 2008b). In addition, lower concentrations of the sulphate conjugate and traces of parent alkylphenols have been detected in bile. The hydrophilic group is attached at the hydroxy moiety of the molecule (Figure 1). Conjugated AP metabolites will be considerably more hydrophilic than the parent compounds, and hence they are easier for the fish to excrete. Because conjugated alkylphenol metabolites are not commercially available, the alkylphenol constituents of the bile must be subjected to enzymatic hydrolysis

(deconjugation) prior to analytical determination. There has also been some debate regarding the best fish tissue for the determination of exposure to APs. Chemical analysis of APs in fish liver may not be an optimal approach for providing AP exposure data, because tissue distribution studies reveal that only a small fraction of APs is retained in the liver tissue and other internal tissues compared with the fraction retained in bile fluid (Sundt *et al*., 2009). For a number of years, surveys at oil fields in the North Sea have analysed fish liver or muscle tissue to assess AP exposure, but no conclusive evidence of increased AP contamination has been reported from these studies. Based on the results of recent studies (Sundt *et al*., 2009), it is likely that analysis of fish bile would be a more sensitive indicator of possible AP contamination.

The combination of solid-phase analytical derivatization (SPAD) with gas chromatography–mass spectrometry with selected ion monitoring (GC–MS–SIM) analysis for quantitative determination of APs of produced water origin in fish bile was recently reported by Jonsson and co-workers (Jonsson *et al*., 2008a, 2008b). These studies included characterization of metabolites by high-performance liquid chromatography fluorescence (HPLC-F) and development of a sensitive analytical method for isolation and detection of the APs. The recommended analytical approach is to treat fish bile with β-glucuronidase and sulphatase followed by solid-phase analytical derivatization (SPAD) with bis(trimethylsilyl)-trifluoroacetamide (BSTFA). The corresponding trimethylsilyl (TMS) protected phenols formed are then analysed by gas chromatography–mass spectrometry in the electron ionization mode (GC–EI–MS; Jonsson *et al*., 2008a). Combining solid-phase extraction (SPE) and derivatization (SPAD) increases the derivatization efficiency for sterically hindered APs and improves sample purity, because excess reagents are evaporated from the sorbent prior to elution. The evaluation and characterization of the method included analyses of 21 AP compounds with a degree of alkylation ranging from methyl (C1) to nonyl (C9). The GC–EI–MS-based procedure resulted in overall limits of detection (LODs) in the range of 5–18 ng g^{-1} bile for 19 out of 21 investigated APs; analytical recoveries ranged from 83 to 109%.

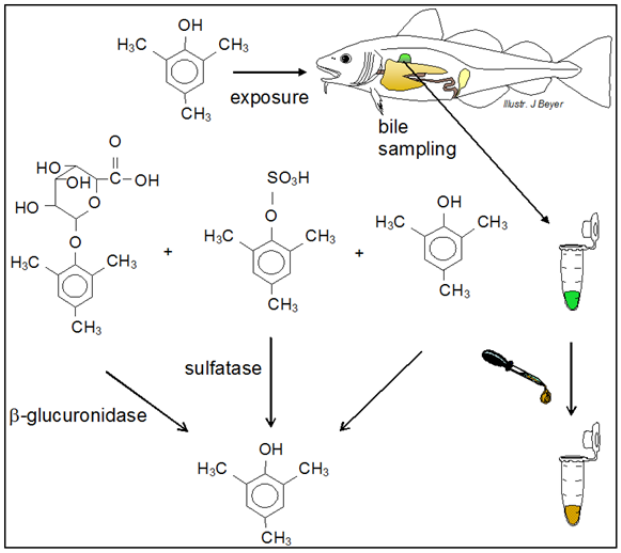

Figure 1. In fish, the major metabolite of AP, in this case 2,4,6-trimethylphenol (2,4,6-TMP), is the glucuronide conjugate, whereas minor concentrations of the sulphate conjugate and unconjugated AP can also be detected. Following enzymatic treatment, both the glucuronated and sulphated APs are converted to the original AP. The sum of deconjugated and unconjugated AP are then derivatized and analysed by GC–MS.

Based on the method testing and development work of Jonsson and co-workers, several standard operating procedures (SOPs) were prepared for different parts of the total analysis procedure. It was also decided to provide a further dissemination of these technical procedures to other users through the platform of *ICES Techniques in Marine Environmental Sciences*. Therefore, in this report, we describe an analytical procedure that includes deconjugation of fish bile followed by solid-phase analytical derivatization with bis(trimethylsilyl)-trifluoroacetamide, then separation and quantification of derivatized APs using the GC–EI–MS methodology. The method has been used to determine AP metabolites in the bile of fish exposed in the laboratory and in fish caged close to offshore oil production platforms (Sundt *et al*., 2012).

2 Sample preparation: solid-phase analytical derivatization (SPAD)

This section describes the preparation of fish bile samples for GC–EI–MS analysis of deconjugated AP-metabolites as their trimethylsilyl (TMS) derivatives (Figure 2).

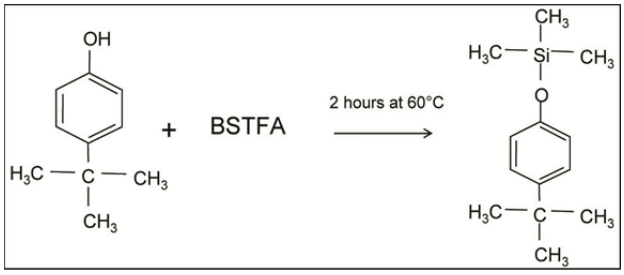

Figure 2. Preparation of trimethylsilyl (TMS) derivative of *tert*-butylphenol for GC–EI–MS analysis.

2.1 Equipment

- Solid-phase extraction vacuum manifold
- Solid-phase extraction tubes (DSC-18, 1 ml 100 mg, Sigma-Aldrich, Germany)
- 1.5 ml microcentrifuge tubes
- pipettes (10–1000 µl)
- 4 ml amber glass vials with Teflon-lined screw caps
- heating cupboard (adjustable to 40 and 60 °C)
- MS-compatible autosampler vials
- hot plate with heating manifold for SPE column
- tubing and connections for nitrogen gas drying

2.2 Chemicals

- *β*-glucuronidase with 5% sulphatase activity, Type HP-2 from *Helix pomatia* (Sigma-Aldrich, Steinheim, Germany)
- bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Varian (Morton Grove, IL, USA)
- anhydrous sodium acetate (analytical grade)
- acetic acid (analytical grade)
- acetone (HPLC grade)
- methanol (pesticide grade)

- methyl *tert*-butyl ether (MTBE, pesticide grade)
- nitrogen gas (99.999%)

2.3 Buffers and solutions

BUFFERS AND SOLUTIONS	PREPARATION AND STORAGE
sodium acetate, 0.4 M, pH 5.0	For one litre buffer, weigh 33.14 g anhydrous sodium acetate into a 600 ml beaker. Dissolve in distilled water. Adjust to pH 5.0 by adding concentrated acetic acid (approx. 11 ml). Adjust to 1 l in a volumetric flask by adding distilled water.
5% methanol in sodium acetate buffer, 0.4 M, pH 5.0	Mix 5 ml methanol with 95 ml sodium acetate buffer, 0.4 M, pH 5.0
â-glucuronidase (1:10)	Dilute the crude enzyme 1:10 with sodium acetate buffer 0.4 M, pH 5.0
AP spike solution	Laboratory-specific SOP
APIS (Internal standard)	Laboratory-specific SOP
AP–GCIS (GC internal standard)	Laboratory-specific SOP

2.4 Procedure

1) Deconjugation of bile samples

Using 1.5 ml microcentrifuge vials (write down the exact weight of added bile, APIS, and APIS spike solution):

- Pipette 300 µl β-glucuronidase (1:10) and 190 µl sodium acetate buffer 0.4 M, pH 5.0 (170 µl sodium acetate buffer 0.4 M, pH 5.0 for spike controls).
- Weigh 30 µl bile into the vial.
- (FOR SPIKE CONTROLS ONLY: Weigh 10–100 µl AP spike solution) into the vial.
- Weigh 80 µl APIS into the vial.

Mix carefully and incubate at 40 °C for 2 h. Allow the samples to reach room temperature before proceeding to the derivatization step (SPAD).

2) Solid-phase analytical derivatization (SPAD)

- Wash columns with one volume acetone and one volume methanol.
- Condition the columns with two successive volumes of sodium acetate buffer (0.4 M, pH 5.0). Do not let the column dry!
- Apply the sample and aspirate through the cartridge at a flow rate of approximately 1 ml min^{-1}. Do not let the column dry!
- Wash the column with two successive volumes of 5% methanol in sodium acetate buffer. Remove residual solvent by aspiring air through the column for approximately one minute.
- Dry the column. Remove residual solvent by drying the inside of the volume with a cotton-tip and applying three high pulses of nitrogen on top of the cartridge. Leave the column in the heater manifold (45 °C) with a gentle nitrogen stream for 10 min, and then without nitrogen for another 10 min.
- Allow columns to reach room temperature before the derivatization reagent is added.
- Pipette 90 µl of BSTFA on top of the column packing material. The volume should be sufficient to wet the packing material, but should

not be added in excess to avoid unwanted extraction and reduced recovery.

- Cover the top of the cartridge with aluminium foil and heat at 60°C for 2 h. The APs are now converted to trimethylsilyl (TMS) derivatives.

3) Sample elution

IMPORTANT! Remember to wash the vacuum manifold tubing (outside and inside) with acetone and allow the tubing to dry to remove any remaining water before elution.

- Elute derivatized alkylphenols with three times 0.5 ml MTBE into a 4 ml vial with screw cap.
- Weigh 80 µl AP GCIS into the vial and mix well.
- Transfer the sample to 0.7 ml autosampler vials for GC–MS analysis.

2.5 Notes

Sample storage

- The samples should be stored at 4°C.
- Standards have displayed good stability for six months when stored at 4°C.
- Bile should be stored at −20°C, or colder.

Table 1. The AP compounds sorted according to increasing GC retention time. QIS (quantitative internal standard), GC–PIS (GC performance internal standard).

	Compound	Abbreviation	"Type of compound"
1	2-methylphenol	2-MP	Target
2	3-methylphenol	3-MP	Target
3	4-methylphenol	4-MP	Target
4	3,5-dimethylphenol	3,5-DMP	Target
5	2,4-ethylphenol	2,4-DMP	Target
6	4-ethylphenol	4-EP	Target
7	4-*n*-propylphenol	4-*n*-PP	Target
8	2,4,6-trimethylphenol	2,4,6-TMP	Target
9	4-*tert*-butylphenol	4-*t*-BP	Target
10	4-isopropyl-3-methylphenol	4-IP-3MP	Target
11	2-*tert*-butyl-4-methylphenol	2-*t*-B-4-MP	Target
12	4-*tert*-butyl-2-methylphenol	4-*t*-B-2-MP	Target
13	2,5-diisopropylphenol	2,5-DIPP	Target
14	4-*n*-butylphenol	4-*n*-BP	Target
15	4-*n*-pentylphenol	4-*n*-PenP	Target
16	4-*n*-hexylphenol	4-*n*-HexP	Target
17	4-*tert*-octylphenol	4-*t*-OctP	Target
18	4,6-di-*tert*-butyl-2-methylphenol	4,6-DtB-2MP	Target
19	4-*n*-heptylphenol	4-*n*-HepP	Target
20	4-*n*-octylphenol	4-*n*-OctP	Target
21	4-*n*-nonylphenol	4-*n*-NonP	Target
1 APIS	4-methylphenol-*d*8	4-MP-*d*8	QIS
2 APIS	3,5-dimethylphenol-2,4,6-*d*3	3,5-DMP-*d*3	QIS
3 APIS	4-*n*-propylphenol-*d*12	4-*n*-PP-*d*12	QIS

4 APIS	4-*tert*-butyl-*d*9-phenol-2,3,5,6-*d*13	4-*t*-BP-*d*13	QIS
5 APIS	4-*n*-pentylphenol-*d*11	4-*n*-PenP-*d*11	QIS
6 APIS	4-*n*-octylphenol-*d*17	4-*n*-OctP-*d*17	QIS
1 AP–GCIS	Hexamethylbenzene	HMB	GC–PIS

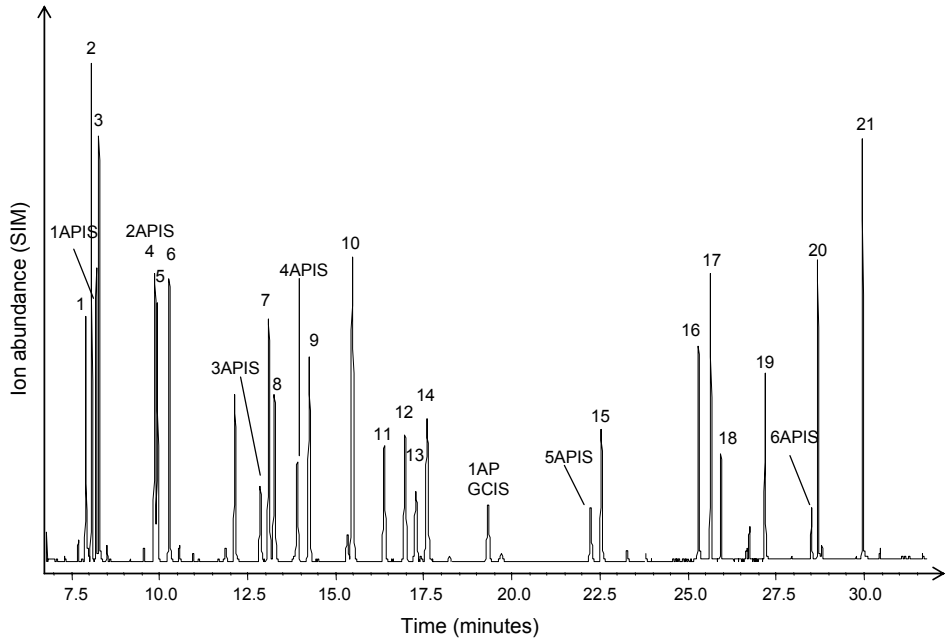

Figure 3. Reconstructed ion chromatogram of a calibration standard. Peak numbers correspond to the numbers listed in Table 1.

2.6 Quality control and method validation

Response factor curve. The response factor curve should be established by analysis of duplicates (at least) of each standard calibration level.

Calibration standards. Calibration standards (at least five covering the actual concentration range) and a blank (standard 1) should be analysed together with the sample series to ensure the validity of the response factor curves.

Non-contaminated bile. For every series of samples, a sample of non-contaminated bile should be prepared as described in Section 2.4.

Spike control. For every series of samples, non-contaminated bile spiked with known concentrations of alkylphenols should be prepared as described in Section 2.4.

House control. A house control (or controls, when available) should be analysed in addition to the spike control.

Derivatization reactions may not always result in a 100% yield, and if the losses are significant, a correction should be made. This bias will be partly compensated for if the calibration standards are also derivatized with the same reagent and under similar conditions. Nevertheless, it should be realized that the yield of such derivatization reactions may not be the same in real samples and in clean solutions, and a recovery check should therefore be carried out, for example, by spiking a "blank" bile with known amounts of AP metabolites. Alternatively, a hydroxylated compound that does not usually occur in fish bile samples could be used as the

internal standard, assuming that its yield in the derivatization step matches that of the analytes.

In many analytical schemes, it has become common practice to add a known amount of a suitable internal standard to the sample at the onset of the sample preparation step. A second internal standard is in some cases added just prior to the final separation/detection step. Ideally, an internal standard should resemble the analyte(s) as closely as possible and, therefore, all random or systematic errors that may occur during the analytical procedure (e.g. weighing/dilution errors, solvent evaporation losses, incomplete derivatization, variations in injection volume or detector response) will affect the internal standard to the same extent as the analyte. Usually, the internal standard should not be present in real samples and should not interfere with the analyte determination.

For optimization of (quantitative) detection methods and for quality assessment/quality control, laboratory reference materials (RMs) can be used. In addition to real fish bile samples, RMs can be made from pure compounds, calibration standard solutions, extracts, or spiked bile samples, all depending on the intended quality assurance (QA) or quality control (QC) use. However, once a method has been optimized, QC measurements should preferably not be performed on clean calibration standard solutions, because such samples are not representative regarding interferences. They may therefore give an overly optimistic impression of the method's performance. Instead, RMs should have a matrix as similar to real samples as possible.

To prepare a suitable RM of fish bile, a relatively large volume of bile from a group of fish exposed to a specific (and relevant) AP treatment is collected, pooled, thoroughly homogenized, split into multiple aliquots, and then analysed a number of times to establish the mean "x" and standard deviation "σ" for specific analytes. The x and σ values are essential parameters in constructing a control chart (CC). A CC based on a standard Shewhart X-bar chart type (Mullins, 1994) or a more advanced CC alternative, (e.g. Prabhu *et al.*, 1994) can be used. The RM aliquots should be distributed in separate vials or ampoules and stored under conditions of optimal stability, for fish bile preferably −20°C or colder. At regular intervals or with each analytical series, a subsample of the RM is analysed and new data are entered into the CC. Over a certain period, the long-term reproducibility of the method that is an essential quality parameter will be documented. Typical warning limits for deviance from the reproducibility are set at $x \pm 2\sigma$ levels and action limits at $x \pm 3\sigma$ levels.

3 Preparation of calibration standards and spike solution

This section describes the preparation of a multilevel calibration standard curve for the GC–MS determination of 21 different TMS derivatized alkylphenols. It also describes the preparation of a spike solution for spike controls and AP internal standard solutions (APIS) to add to bile samples.

3.1 Equipment

- Pipettes (10–1000 µl)
- 2 ml amber glass vials with Teflon-lined screw caps
- 20 ml glass vials with Teflon-lined screw caps (MS-compatible autosampler vials)
- Volumetric flasks (25 and 100 ml)

3.2 Chemicals

- Stock solution of a mixture of 21 APs (Table 2, concentrations ranging from 500 to 3000 µg g^{-1} MTBE) and six APIS (Table 3, concentrations ranging from 500 to 3000 µg g^{-1} MTBE; Chiron, Trondheim, Norway)
- bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Varian (Morton Grove, IL, USA)
- anhydrous sodium acetate (analytical grade)
- acetic acid (analytical grade)
- hexamethylbenzene (>99%)
- methyl *tert*-butyl ether (MTBE, pesticide grade)

3.3 Buffers and solutions

For preparation of sodium acetate, 0.4 M, pH 5.0, see Section 2.3.

3.4 Preparation of calibration standards

The following procedure is recommended for preparing the 1:1000 dilutions:

1) AP user solution
- Transfer the stock solution (see Table 2 for details) from one ampoule to a 2 ml amber vial with screw cap for storage.
- Dilute AP stock solution approximately 1:1000 in MTBE as follows:
- Weigh an empty 100 ml volumetric flask with stopper.
- Fill the flask with MTBE approximately to the mark and record the EXACT weight of the solvent with stopper.
- Transfer 100 µl of the stock AP solution to the 100 ml volumetric flask. WEIGH with stopper.
- Mix well and distribute to 20 ml vials with Teflon-lined screw caps.

2) APIS user solution
- Transfer the stock solution (see Table 3 for details) from one ampoule to a 2 ml amber vial with screw cap for storage.
- Dilute the APIS stock solution approximately 1:1000 in MTBE as described for AP user solution.

3) AP–GCIS stock and user solution

AP–GCIS stock solution
- Weigh 25 mg hexamethylbenzene (HMB) into a 25 ml volumetric flask. Fill the flask with MTBE (approximately to the mark) and WEIGH the solvent with stopper.
- Mix carefully and store the AP–GCIS stock solution in 2 ml amber vials with Teflon-lined screw caps.

AP–GCIS user solution
- Dilute the AP–GCIS stock solution approximately 1:1000 in MTBE as described for AP user solution.

4) Preparation of calibration standards (use calibration standard preparation forms/spreadsheet for registration and calculation)
- Pipette and record exact weights into 2 ml amber vials with screw cap in the following order.

- MTBE containing 25% acetonitrile (vol/vol)
- AP user solution
- APIS user solution
- BSTFA

- Mix carefully and heat at 60°C for 2 h. Allow the standards to reach room temperature.
- Pipette and add 100 ul AP–GCIS user solution to each vial and record weight.
- Mix carefully and distribute to autosampler vials (ten vials for each concentration).

3.5 Preparation of spike solutions

1) AP spike user solution (for preparation of spike controls).
- Dilute AP stock solution approximately 1:1000 in 0.4 M sodium acetate buffer, pH 5.0 as described for AP user solution.

2) APIS user solution for bile samples.
- Dilute the APIS stock solution approximately 1:1000 in 0.4 M sodium acetate buffer, pH 5.0 as described for AP user solution.

3) AP–GCIS user
- AP–GCIS user is dissolved in MTBE as described above, Section 3.4 point 3, and is added to the final sample extract.

3.6 Notes

Sample storage

- The bile samples should be stored at −20°C (or −80°C).
- The standards are stored at 4°C and have displayed good stability for six months.

AP and APIS stock solution mixtures are dissolved in methyl *tert*-butyl ether (MTBE) and ordered from Chiron, Trondheim, Norway (see Tables 2 and 3).

Calibration standards. AP, APIS, and AP–GCIS user solutions to be used for preparation of calibration standards must be dissolved in MTBE.

Bile samples and spike controls. AP and APIS user solutions to be added to bile samples must be dissolved in 0.4 M sodium acetate buffer, pH 5.0. AP–GCIS user is added to the final sample extract and must be dissolved in MTBE.

3.7 Alkylphenol stock solutions

Table 2. Alkylphenol stock solution; a mixture of 21 alkylphenols, target compounds, dissolved in methyl *tert*-butyl ether (Chiron, Trondheim, Norway). The concentrations in the table might not be accurate, but the relative concentration should be approximately as indicated.

	Compound	Abbreviation	Concentration µg g^{-1}
1	2-methylphenol	2-MP	3000
2	3-methylphenol	3-MP	3000
3	4-methylphenol	4-MP	3000
4	3,5-dimethylphenol	3,5-DMP	2000
5	2,4-ethylphenol	2,4-DMP	2000
6	4-ethylphenol	4-EP	2000

7	4-*n*-propylphenol	4-*n*-PP	2000
8	2,4,6-trimethylphenol	2,4,6-TMP	2000
9	4-*tert*-butylphenol	4-*t*-BP	1000
10	4-isopropyl-3-methylphenol	4-IP-3-MP	1000
11	2-*tert*-butyl-4-methylphenol	2-*t*-B-4-MP	1000
12	4-*tert*-butyl-2-methylphenol	4-*t*-B-2-MP	1000
13	2,5-diisopropylphenol	2,5-DIPP	1000
14	4-*n*-butylphenol	4-*n*-BP	1000
15	4-*n*-pentylphenol	4-*n*-PenP	500
16	4-*n*-hexylphenol	4-*n*-HexP	500
17	4-*tert*-octylphenol	4-*t*-OctP	500
18	4,6-di-*tert*-butyl-2-methylphenol	4,6-DtB-2-MP	500
19	4-*n*-heptylphenol	4-*n*-HepP	500
20	4-*n*-octylphenol	4-*n*-OctP	500
21	4-*n*-nonylphenol	4-*n*-NonP	500

Table 3. Alkylphenol internal standard (APIS) stock solution; a mixture of six deuterated alkylphenols, Quantitative Internal Standards, dissolved in methyl *tert*-butyl ether (Chiron, Trondheim, Norway). The concentrations in the table might not be accurate, but the relative concentration should be approximately as indicated in the table.

	COMPOUND	ABBREVIATION	CONCENTRATION µG G^{-1}
1 APIS	4-methylphenol-*d*8	4-MP-*d*8	3000
2 APIS	3,5-dimethylphenol-2,4,6-*d*3	3,5-DMP-*d*3	2000
3 APIS	4-*n*-propylphenol-*d*12	4-*n*-PP-*d*12	2000
4 APIS	4-*tert*-butyl-*d*9-phenol-2,3,5,6-*d*13	4-*t*-BP-*d*13	1000
5 APIS	4-*n*-pentylphenol-*d*11	4-*n*-PenP-*d*11	1000
6 APIS	4-*n*-octylphenol-*d*17	4-*n*-OctP-*d*17	500

Table 4. GC–MS performance internal standard (AP–GCIS) stock solution.

	COMPOUND	ABBREVIATION	CONCENTRATION µG G^{-1}
1 AP–GCIS	Hexamethylbenzene	HMB	1000

3.8 Remarks

Laboratories should make their own forms during preparation of:

- Stock solutions
- User solutions for samples
- User solutions for calibration standards
- Calibration standards

4 GC–EI–MS analysis of TMS derivatized alkylphenols

This section describes the analysis of trimethylsilyl (TMS) derivatized alkylphenols by gas chromatography–mass spectrometry in electron impact mode (GC–EI–MS).

4.1 Equipment

Equipment	Supplier
GC–EI–MS with split/splitless injector., e.g. Schimadzu GC–MS–2010 EI	Shimadzu
Autoinjector, e.g. AOC-5000	Shimadzu
Column: CP–SIL 8CB LB (l: 50 m, dm: 0.25 mm, film thickness: 0.25 µm)	Instrument teknikk
Helium	Aga

4.2 Chemicals

Chemical	Supplier
Methyl *tert*-butyl ether (washing solution)	VWR

4.3 Buffers and solutions

Buffers and solutions	Preparation and storage
AP calibration standards	Determination of AP metabolites in fish bile; preparation of TMS derivatized calibration standards and spike solution

4.4 GC–EI–MS analysis

Target alkylphenols, internal standards (APIS) and GC performance standard (AP–GCIS) are illustrated in Table 5. The group for the target compound identifies the relevant internal standard. The listed retention times (RTs) will change when the column is shortened and the GC–MS acquisition windows and the solvent cut time might be changed.

Table 5. List of alkylphenols (AP target), internal standards (APIS), and GC performance standard (AP–GCIS). m/z = mass/charge (for precursor ions).

Alkylphenol information			APIS		AP TMS Ions (m/z)	
Type	Full name	Abbreviation	Group	RT	Quantifier	Identifier
AP target	2-methylphenol	2-MP	1	8.129	165.1	180.1
AP target	3-methylphenol	3-MP	1	8.294	165.1	180.1
APIS	4-methylphenol-*d*8	4-MP-*d*8	1	8.429	172.1	187.1
AP target	4-methylphenol	4-MP	1	8.507	165.1	180.1
APIS	3,5-dimethylphenol-2,4,6-*d*3	3,5-DMP-*d*3	2	10.080	182.1	197.1
AP target	3,5-dimethylphenol	3,5-DMP	2	10.101	179.1	194.1
AP target	2,4-ethylphenol	2,4-DMP	2	10.167	179.1	194.1
AP target	4-ethylphenol	4-EP	2	10.503	179.1	194.1
APIS	4-*n*-propylphenol-*d*12	4-*n*-PP-*d*12	3	13.092	185.1	219.1
AP target	4-*n*-propylphenol	4-*n*-PP	3	13.334	179.1	208.2
AP target	2,4,6-trimethylphenol	2,4,6-TMP	3	13.496	208.2	193.1
APIS	4-*tert*-butyl-*d*9-phenol-2,3,5,6-*d*13	4-*t*-BP-*d*13	4	14.147	217.1	235.2
AP target	4-*tert*-butylphenol	4-*t*-BP	4	14.489	207.2	222.2
AP target	4-isopropyl-3-methylphenol	4-IP-3MP	4	15.720	207.2	222.2
AP target	2-*tert*-butyl-4-methylphenol	2-*t*-B-4MP	4	16.620	221.1	236.2
AP target	4-*tert*-butyl-2-methylphenol	4-*t*-B-2MP	4	17.216	221.1	236.2

AP target	2,5-diisopropylphenol	2,5-DIPP	4	17.526	235.2	250.2
AP target	4-*n*-butylphenol	4-*n*-BP	4	17.846	179.1	222.2
AP GCIS	Hexamethylbenzene	HMB	5	19.595	147.1	162.2
APIS	4-*n*-pentylphenol-*d*11	4-*n*-PP-*d*12	5	22.477	181.1	295.2
AP target	4-*n*-pentylphenol	4-*n*-PenP	5	22.779	179.1	236.2
AP target	4-*n*-hexylphenol	4-*n*-HexP	6	25.523	179.1	250.2
AP target	4-*tert*-octylphenol	4-*t*-OctP	6	25.883	207.2	278.2
AP target	4,6-di-*tert*-butyl-2-methylphenol	4,6-DtB-2MP	6	26.170	277.2	292.2
AP target	4-*n*-heptylphenol	4-*n*-HepP	6	27.420	179.1	264.2
APIS	4-*n*-octylphenol-*d*17	4-*n*-OctP-*d*17	6	28.731	181.1	295.2
AP target	4-*n*-octylphenol	4-*n*-OctP	6	28.915	179.1	278.2
AP target	4-*n*-nonylphenol	4-*n*-NonP	6	30.185	179.1	292.2

4.5 GC programme

Injection volume: 1 µl

Injection mode: Splitless

Sampling time: 1 min

Injection temperature: 280 °C

Flow control mode: Linear velocity

Pressure: 147.6 kPa

Total flow: 35.6 ml min^{-1}

Column flow: 1.55 ml min^{-1}

Linear velocity: 35.0 cm s^{-1}

Purge flow: 3.0 ml min^{-1}

Split ratio: 20

Oven temperature programme

RATE (°C MIN^{-1})	FINAL TEMPERATURE (°C)	HOLD TIME (MIN)
-	50	1.0
15.0	120	0.0
1.0	135	0.0
10.0	300	3.0

Total time: 40:17 min.

4.6 MS programme settings

Ion source temperature: 240 °C

Interface temperature: 300 °C

Acquisition mode: SIM

Sampling interval: 0.20 s

Solvent cut time: 7.0 min

NB: Check solvent cut time and GC–MS acquisition windows, if the column is shortened.

GC–MS acquisition windows for determination of alkylphenols, showing which ions are determined in which time-window. (See also Jonsson *et al.*, 2008a.)

Group	1	2	3	4
Start time (min)	7.00	11.30	18.50	24.00
End time (min)	11.29	18.49	23.99	32.00
	m/z	m/z	m/z	m/z
	165.1	179.1	147.2	179.1
	172.1	185.1	162.2	181.1
	179.1	193.1	179.1	207.2
	180.1	207.2	181.1	250.2
	182.1	208.2	236.2	264.2
	187.1	217.1	247.2	277.2
	194.1	219.1		278.2
	197.1	221.1		292.2
		222.2		295.2
		235.2		
		236.2		
		250.2		

4.7 Calculation

Multilevel (nine levels, concentrations ranged from 3 to 1500 ng g^{-1}) calibration curves were determined for a mixture of 21 APs that were prepared in amber 2 ml vials with screw caps containing PTFE septa, using MTBE as solvent. Six deuterated APs (4 MP-*d*8, 3,5-DMP-*d*3, 4-*n*-PP-*d*12, 4-*t*-BP-*d*13, 4-*n*-PenP-*d*11, and 4-*n*-OP-*d*17, with concentrations ranging from 45 to 165 ng g^{-1}) were added as internal standards prior to derivatization with BSTFA (100_l) for 2 h at 60 °C. HMB (170 ng g^{-1}) was added as a GC performance standard prior to analysis. Calibration curves for quantitative analysis were fitted by least-square regression for the 21 measured TMS APs. AP metabolite concentrations are reported as ng AP metabolite g^{-1} bile.

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6 Author contact information

Grete Jonsson
Stavanger University Hospital
4011 Stavanger, Norway
Grete.Jonsson@sus.no

Atle Nævdal
International Research Institute of Stavanger (IRIS)
4068 Stavanger, Norway
Retired

Jonny Beyer (corresponding author)
International Research Institute of Stavanger (IRIS)
4068 Stavanger, Norway
Jonny.Beyer@iris.no

7 Abbreviations

AP	alkylphenol metabolite
APIS	alkylphenol internal standard
BSTFA	bis(trimethylsilyl)-trifluoroacetamide
CC	control chart
EI	electron impact
GC	gas chromatography
GC–EI–MS	gas chromatography–electron impact–mass spectrometry
GCIS	gas chromatography internal standard
GC–MS–SIM	gas chromatography–mass spectrometry with selected ion monitoring
HMB	hexamethylbenzene
HPLC-F	high-performance liquid chromatography fluorescence
LOD	limit of detection
MS	mass spectrometry
MTBE	methyl *tert*-butyl ether
m/z	mass/charge (for precursor ions)
PIS	performance internal standard
PTFE	polytetrafluoroethylene
QA	quality assurance
QC	quality control
QIS	quantitative internal standard
RM	reference material
RT	retention time
SIM	selected ion monitoring
SOP	standard operating procedure
SPAD	solid-phase analytical derivatization
SPE	solid-phase extraction
TMP	trimethylphenol
TMS	trimethylsilyl