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Quantification of the xenoestrogens 4-*tert.*-octylphenol and bisphenol A in water and in fish tissue based on microwave assisted extraction, solid-phase extraction and liquid chromatography–mass spectrometry

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Abstract

Extraction methods were developed for quantification of the xenoestrogens 4-*tert.*-octylphenol (tOP) and bisphenol A (BPA) in water and in liver and muscle tissue from the rainbow trout (*Oncorhynchus mykiss*). The extraction of tOP and BPA from tissue samples was carried out using microwave-assisted solvent extraction (MASE) followed by solid-phase extraction (SPE). Water samples were extracted using only SPE. For the quantification of tOP and BPA, liquid chromatography mass spectrometry (LC–MS) equipped with an atmospheric pressure chemical ionisation interface (APCI) was applied. The combined methods for tissue extraction allow the use of small sample amounts of liver or muscle (typically 1 g), low volumes of solvent (20 ml), and short extraction times (25 min). Limits of quantification of tOP in tissue samples were found to be approximately 10 ng/g in muscle and 50 ng/g in liver (both based on 1 g of fresh tissue). The corresponding values for BPA were approximately 50 ng/g in both muscle and liver tissue. In water, the limit of quantification for tOP and BPA was approximately 0.1 μg/l (based on 100 ml sample size).

Keywords: Rainbow trout; 4-*tert.*-octylphenol; Bisphenol A; Xenoestrogens; Alkylphenol

1. Introduction

It has recently been proposed that many high-volume industrial chemicals, already found in high concentrations both on land and in the aquatic environment, may have adverse effects on the endocrine systems in humans and animals [1,2]. Among these high-volume chemicals are the alkylphenolpolyethoxylates (APEOs) including their degradation products, the alkylphenols, and bisphenol A. Both the alkylphenols and bisphenol A have been shown to possess estrogenic activity in in vitro and in vivo screening systems [3–7]. The APEOs belong to one of the largest groups of non-ionic surfactants and are used both as detergents and in many formulated products such as herbicides, pesticides and paints. Bisphenol A is used in the production of polycarbonate, epoxy resins, flame retardants and many other products. The alkylphenols, mainly 4-nonylphenol and 4-*tert.*-octylphenol, and bisphenol

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A have been detected in marine and freshwater habitats around the world at considerable concentrations [7,8]. Thus studies on dose–response relationships for these compounds are needed together with identification of the internal threshold concentrations in tissues that elicit estrogenic effects. In particular the determination of internal threshold concentrations has been shown to be difficult, because of the lack of selective and sensitive methods for assessing tissue distribution and concentrations of relevant chemicals. Furthermore, the determination of dose–response relationships in studies using water-borne exposure to chemicals requires fast and reliable methods for assessing the actual water concentration of the compound(s) in question.

The aim of the present work was to develop selective and sensitive methods for the determination of 4-*tert*.-octylphenol and bisphenol A in water, and in liver and muscle tissue from a relevant aquatic fish species. There has previously been reports on the determination of phenolic compounds in the aquatic environment (for an example see Puig et al. [9]), which support the use of APCI mass spectrometry. The two compounds used in the present study can be considered as representative of para-substituted phenolic estrogen-like compounds, and the methods are therefore also applicable to other compounds with similar structures.

2. Experimental

2.1. Reagents and materials

The compounds 4-*tert*.-octylphenol (tOP) (97%), 4-*tert*.-butylphenol (tBP) (99%), bisphenol A (BPA) (99%) and bisphenol A-d_{16} (BPA-d_{16}) (98%) were obtained from Aldrich (Steinheim, Germany). Stock solutions (100 μg/ml) were prepared in HPLC grade methanol from Rathburn (Walkerburn, Scotland) and used for further dilutions. The solutions were stored in the dark at 4°C to prevent photochemical degradation. Zorbax Eclipse XDB-C_{18} 150×2.1 mm reversed-phase HPLC column (5-μm particle size) was obtained from Hewlett-Packard (Palo Alto, CA, USA). Solid-phase extraction cartridges, Sep-Pak NH_2 (500-mg packing material) and Sep-Pak C_{18} (500-mg packing material) were obtained from Waters (Milford, MA, USA). Water used as HPLC solvent was purified with a Milli-Q water system (Millipore, Bedford, MA, USA). Solvents used for the extraction of fish: dichloromethane (for ultra trace analysis) from Scharlau and methanol (HPLC grade) from Rathburn.

2.2. Water extraction

For the studies of recovery and analytical precision, sample volumes of 100 ml of tap water were chosen and spiked at three different levels with either tOP or BPA. Samples for tOP analysis were made 0.1 m*M* with sodium dodecyl sulphate (SDS) as described in Chee et al. [10]. Internal standard, 50 μl of a 10 ng/μl solution of tBP when analysing for tOP or 30 μl of a 25 ng/μl BPA-d_{16} solution when analysing for BPA, were added. The solutions were percolated through a Sep-Pak C_{18} extraction tube at a flow of approximately 1 ml/min, previously conditioned with 5 ml of methanol and 5 ml of water, and subsequently washed with 2 ml of water. After 5 min of drying under vacuum, the compounds were desorbed by 4 ml of methanol. Finally, the sample extract was evaporated to dryness using a flow of N_2 and redissolved in 1 ml methanol. Aliquots of 20 or 10 μl were used for analysis of tOP and BPA respectively.

2.3. Tissue extraction

Samples of frozen muscle or liver tissue from juvenile rainbow trout were placed in a mortar filled with liquid nitrogen and crushed. Aliquots (ca. 1g) were allowed to partly thaw and internal standard was added, 50 μl of a 10 ng/μl solution of tBP when analysing for tOP or 30 μl of a 25 ng/μl BPA-d_{16} solution when analysing for BPA. Samples for recovery and analytical precision were spiked at three different levels of tOP or BPA and then treated as normal samples.

The tissue samples were placed in the reaction vessel of a Soxwave 100 microwave extraction apparatus from Prolabo (Fontenay-Sous-Bois, France), containing 20 ml dichloromethane/methanol (2:1 v/v). Samples were extracted for 25 min at 20% power. The mixture was filtered through a paper filter and a 0.9% KCl solution was added (20% of

final volume). After 10 min of centrifugation at 1000 *g*, the dichloromethane phase was removed and evaporated to dryness. The samples were redissolved in either 1 ml cyclohexane when analysing tOP or 1 ml of methanol:hexane (1:20) when analysing BPA. Samples were applied to Sep-Pak NH_2 500-mg cartridges, previously conditioned with 5 ml methanol and 5 ml cyclohexane when analysing tOP or 5 ml methanol:hexane (1:20) when analysing BPA, at a flow of 1 ml/min. Before desorption, the cartridges were washed with either 5 ml of cyclohexane (tOP) or 2 ml of hexane (BPA). After drying, both tOP and BPA samples were desorbed by 4 ml of methanol. Finally, the extract was evaporated to dryness using a flow of N_2 and redissolved in 1 ml of methanol. Aliquots of 20 or 10 μl were used for analysis of tOP and BPA respectively.

2.4. LC–MS analysis

A Hewlett-Packard LC–MSD system consisting of a Series 1100 HPLC (solvent degasser; binary pump, autosampler, thermostatted column department) and a G1946A MSD quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionisation (APCI) LC–MS interface was used for separation and quantification of tOP and BPA. Chromatography was performed using a Zorbax Eclipse XDB-C_{18} (150×2.1 mm) reversed-phase HPLC column equilibrated with 75% solvent A (20% methanol in water) and 25% solvent B (100% methanol) when analysing tOP or 100% solvent A when analysing BPA. The alkylphenols (tOP and tBP) were eluted with a gradient of 25–60% solvent B in 2 min followed by 60–100% solvent B in 13 min. The bisphenols (BPA and BPA-d_{16}) were eluted with a gradient of 0–70% solvent B in 7 min followed by 70–90% solvent B in 6 min. The flow-rate was 0.4 ml/min.

The MSD was tuned automatically using the built-in calibrant delivery system. Operating parameters of the APCI interface were optimised in full scan mode (*m*/*z* 50–400) using flow injection analysis (FIA) of either tOP or BPA in 70% methanol. Optimum conditions for tOP/BPA were as follows: drying gas temperature 225/330°C, drying gas flow 4/5 l/min, nebulizing gas pressure 55/60 p.s.i., vaporising temperature 350/280°C, corona current 30/40 μA, capillary voltage (Vcap) 2500/3000 V. Samples were analysed at a fragmentor voltage of 80 V and 100 V for tOP or BPA respectively. However, up to three ions for tOP and four ions for BPA could be monitored for each compound by re-running suspect samples using fragmentor voltages of either 210 and 160 V. Quantitative analysis was carried out using selected ion monitoring (SIM) in negative mode for the ions *m*/*z* 149 (tBP), 205 (tOP), 241 (BPA-d_{16}) and 227 (BPA). The calibration curves consisted of five or six points at the concentrations 5, 25, 125, 625, 3125 ng/ml and 20.7, 207, 842, 2170, 4694 and 10044 ng/ml for tOP and BPA respectively. The internal standard concentrations were 500 ng/ml and 2500 ng/ml for tOP and BPA respectively. Peak area ratios of standards and samples with respect to I.S. were computed using Hewlett-Packard Chemstation software. Calibration curves were generated using linear regression analysis with all values weighted equally. The limit of detection was determined at a signal-to-noise ratio (S/N)>3. The limit of quantification for tOP was in water approximately 0.1 μg/l (based on 100-ml sample size) and approximately 10 ng/g in muscle tissue and 50 ng/g in liver tissue (both based on 1 g of fresh tissue). The corresponding values for BPA were approximately 0.1 μg/l (water) and 50 ng/g in both muscle and liver tissue.

2.5. Method application

Juvenile rainbow trout (80–120 g) were kept under a 12 h light:12 h dark photoperiod in 80-l steel tanks supplied with running freshwater. Six groups of six fish were given intraperitoneal injections of either tOP or BPA dissolved in 48% ethanol while a control group was injected with the vehicle alone. Groups of fish were injected with either 0.5, 2.5 or 12.5 mg/kg of either one of the compounds at day 0, 6 and 12 and sampled after 18 days. All injection volumes were adjusted to 1 ml/kg fish. Prior to injections the fish were anaesthetized with 0.02% phenoxyethanol. At the end of the period the fish were anaesthetised with 0.02% phenoxyethanol and sacrificed. Liver and muscle tissues were collected and stored at –80°C until analysis of tOP or BPA.

2.6. Vitellogenin in blood plasma

Vitellogenin was measured by a direct sandwich ELISA using polyclonal rabbit antibodies raised against rainbow trout vitellogenin [6].

3. Results and discussion

3.1. LC–MS

The structure of tOP is given in Fig. 1 together with a full scan (*m/z* 50–250) spectrum of a tOP standard using a fragmentor voltage of 210 V. In addition to the $[M\text{-}H]^-$ ion at *m/z* 205 two fragment ions were detected. The base peak at *m/z* 133 $[M\text{-}H\text{-}72]^-$ probably results from the loss of a C_5H_{12} group whereas the *m/z* 117 $[M\text{-}H\text{-}88]^-$ fragment cannot straightforwardly be explained. However, since the $[M\text{-}H]^-$ ion was selected for quantitative analysis, samples were run using a fragmentor voltage of 80 V, which reduced the intensity of fragment ions to less than 20% relative to the $[M\text{-}H]^-$. SIM chromatograms at *m/z* 205 of an unspiked liver, a standard equivalent to 73 ng tOP/g, and a liver spiked with 73 ng tOP/g is given in the right panel of Fig. 1. A peak resulting from the matrix at a retention time of 16.3 min is seen in chromatograms from both blank and spiked liver samples (Fig. 1A and 1C). It does not, however, interfere with the quantification of tOP. Furthermore, it is possible to detect a small peak with the retention time of tOP (15.9 min) in the blank liver sample. However, since the area of the interfering peak is less than 5% of the lowest spiked tOP standard (Fig. 1C), its influence on the quantification was insignificant. Similar chromatograms were obtained when muscle tissue samples were analysed (results not shown). In Fig. 2, the structure of BPA is shown together with a full scan (*m/z* 50–250) spectrum of a BPA standard using a fragmentor voltage of 160 V. In addition to the $[M\text{-}H]^-$ ion at *m/z* 227, three fragment ions can be detected. The *m/z* 212 fragment probably results from a cleavage of one of the CH_3 groups, whereas the *m/z*

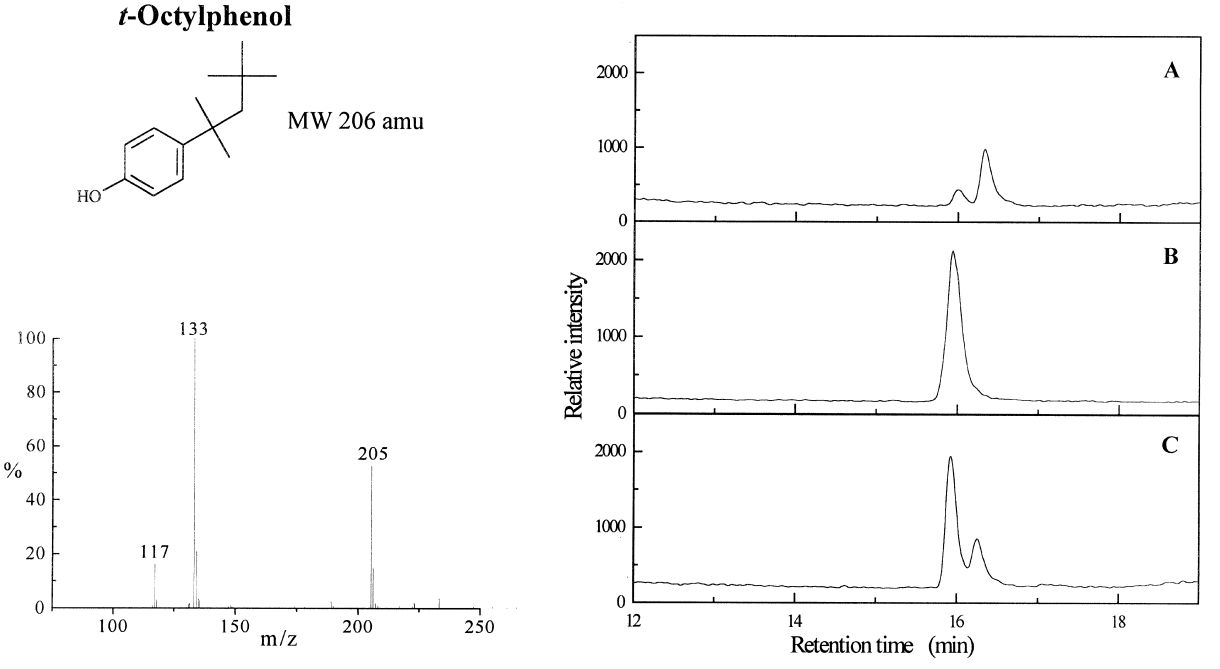

Fig. 1. Negative-ion LC–APCI–MS of *t*-octylphenol. Left upper panel: structure of tOP; left lower panel: full scan (*m/z* 50–250) spectrum of a tOP standard using a fragmentor voltage of 210 V. Right: Selected-ion monitoring chromatograms of *m/z* 205 using a fragmentor voltage of 80 V. (A) Control liver, (B) standard equivalent to 73 ng tOP/g liver, (C) control liver spiked with 73 ng tOP/g.

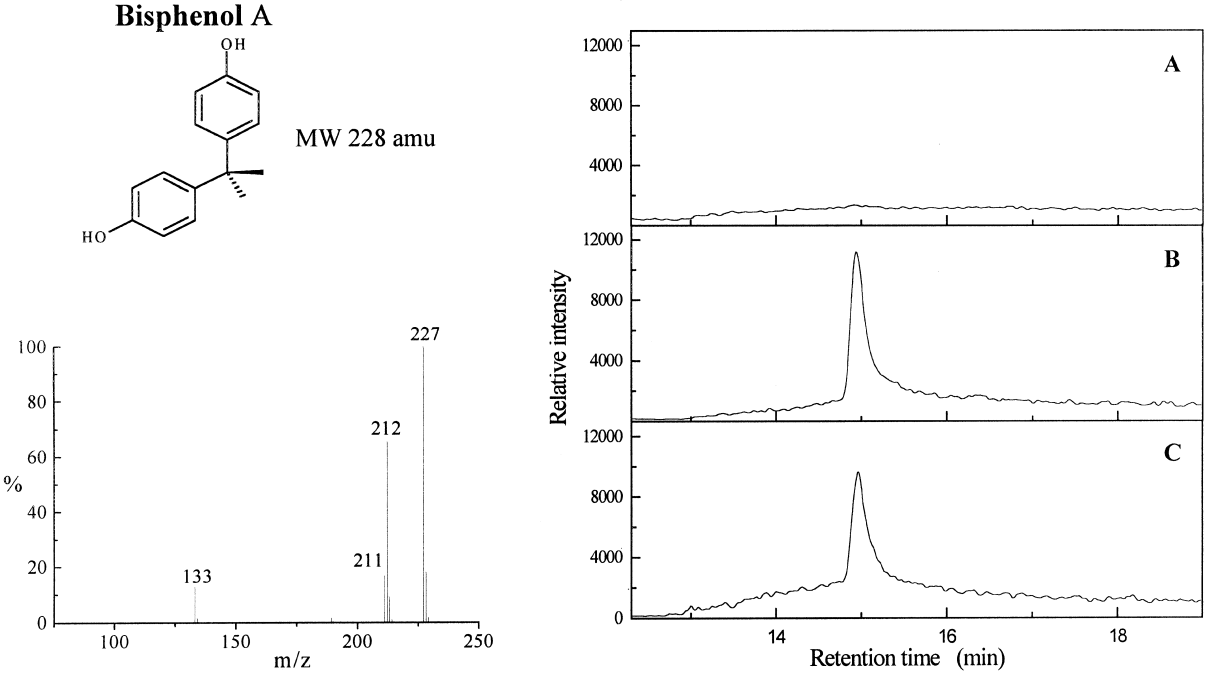

Fig. 2. Negative-ion LC–APCI–MS of bisphenol A. Left upper panel: structure of BPA; left lower panel: full scan (m/z 50–250) spectrum of a BPA standard using a fragmentor voltage of 160 V. Right: Selected-ion monitoring chromatograms of m/z 227 using a fragmentor voltage of 100 V. (A) Control liver, (B) standard equivalent to 100 ng BPA per gram of liver, (C) control liver spiked with 100 ng BPA per gram.

211 probably results from the loss of oxygen giving the following ion $[(C_6H_4)C_3H_6(C_6H_4OH)]^-$. The small peak at m/z 133 $[M\text{-}H\text{-}94]^-$ is probably the same fragment ion as the one seen for tOP (Fig. 1). Again, a lower fragmentor voltage was chosen for quantification in order to maximize the signal from the pseudomolecular ion (m/z 227). This is illustrated in the right panel of Fig. 2, where SIM chromatograms at m/z 227 of an unspiked liver, a standard equivalent to 100 ng BPA/g, and a liver spiked with 100 ng BPA/g are shown. Like for tOP, similar BPA chromatograms were obtained when muscle was used as tissue sample (results not shown).

During LC–MS analysis, carbon deposits were observed to accumulate on the corona needle of the APCI interface, resulting in a gradual reduction of sensitivity. This problem was minimized by directing the flow to waste 3 min before and after the retention times of the compounds. Furthermore, the use of internal standard also helped in correcting changes in detector response during the analyses of samples.

3.2. Tissue analysis

The present methods developed for extraction of tOP and BPA from fish tissue were based on microwave-assisted solvent extraction (MASE) and solid-phase extraction (SPE). Quantitative analyses of alkylphenols in biota previously described were based on various extraction techniques, for example soxhlet extraction [11] or steam distillation [12]; these methods generally require large sample sizes, high solvent volumes and long extraction times. To our knowledge, BPA has until now never been extracted and quantified in biota. Previous studies only describe the extraction of BPA dissolved in liquid matrices such as bile fluids and the contents of food cans [13,14].

The extraction procedures chosen in the present

method considerably reduced the abovementioned problems of the more traditional extraction techniques. In a recent study using the same microwave apparatus (Soxwave 100), moisture in the sample was reported to enhance the efficiency of the extraction [15]. The application of microwave energy causes a selective heating of the matrix (more precisely the water content) over the extractant which results in a migration of the target compound(s) from the matrix to the extraction solvents. SPE has the advantage of immediate analyte enrichment, thereby reducing analysis time and the amount of solvents used for the extraction. Taken together, the two extraction techniques allow the use of small amounts of tissue (typically 1 g), low volumes of solvents (20 ml), and short extraction times (25 min), yet they still provide precise and accurate results. With respect to tOP, recoveries (60% in liver and 78% in muscle) found in the present method were comparable with recoveries (35–100%) of alkylphenols of varying chain length found in different species (algae, fish and birds) [11,12].The extraction techniques were validated by comparing the values observed from spiked tissue samples to those of expected standards made up in methanol and not subjected to extraction (Table 1). The criterion for the acceptance of data was a maximum deviation of 15% according to Braggio et al. [16]. The intra- and inter-assay precision, accuracy and recovery data, specified in Table 1, show that the performance of the extraction method is acceptable.

In order to test the methods on real samples, fish were injected three times with either 0.5, 2.5 or 12.5 mg/kg of tOP or BPA. Injection was chosen to ensure that all fish in the same group were given exactly the same dose. The yolk protein vitellogenin in oviparous vertebrates is generally accepted as a specific biomarker of estrogenic activity [17]. The relevance of the doses can be illustrated by the fact that fish injected with 0.5 and 2.5 mg/kg of tOP or BPA did not elicit an estrogenic response after 18 days based on induction of vitellogenin, while fish injected with 12.5 mg/kg did elicit a small but significant estrogenic response (results not shown). In Table 2, liver and muscle concentrations of tOP and BPA in exposed fish are shown. Regarding the lowest injected dose, tOP and BPA could be detected in neither liver nor muscle tissue. In fish exposed to 2.5 or 12.5 mg/kg it was possible to quantify tOP in both liver and muscle tissue, whereas BPA could only be measured in the liver. In the present experiment, fish were given the last injection 6 days prior to sampling. Thus the results should be compared to previous studies, reporting half-lives of 19–20 hs in

Table 1
Intra- and inter-assay precision and accuracy together with recovery for tOP and BPA in liver- and muscle samples spiked at 3 different levels ($n=6$)

Compound/ tissue	Expected amount	Intra assay			Inter assay			Average absolute recovery (%)
		Observed amount	Precision % C.V.	Accuracy % Bias	Observed amount	Precision % C.V.	Accuracy % Bias	
tOP liver	73 ng	69	6.2	5.4	76	6.9	4.1	60
	213 ng	206	5.5	3.3	202	11.6	5.1	60
	1754 ng	1864	8.8	6.3	1756	6.3	1.1	60
	Internal std	–	–	–	–	–	–	60
tOP muscle	72 ng	75	10.9	4.1	78	8.8	8.3	78
	212 ng	220	8.3	3.8	224	9.2	5.7	78
	2069 ng	2208	10.7	6.7	2099	10.1	1.4	78
	Internal std.	–	–	–	–	–	–	74
BPA liver	100 ng	97	3.1	3.0	102	4.5	2.0	49
	250 ng	239	9.2	4.4	247	3.7	1.2	49
	2500 ng	2400	10.0	4.0	2640	14.7	5.6	49
	Internal std.	–	–	–	–	–	–	47
BPA muscle	100 ng	95	8.3	5.0	103	6.3	3.0	79
	250 ng	263	8.7	5.2	252	9.0	0.8	79
	2500 ng	2634	2.7	5.4	2561	4.1	2.4	79
	Internal std.	–	–	–	–	–	–	78

Table 2
Concentrations of tOP and BPA in liver- and muscle tissue of rainbow trout injected with either vehicle (control) or three different doses of the compounds ($n=6$). For details see Experimental[a]

Compound/ tissue	Exposure	Concentration (ng/g ww±SD)
tOP in liver	Control	BLOQ
	0.5 mg/kg	BLOQ
	2.5 mg/kg	52±13
	12.5 mg/kg	85±18
tOP in muscle	Control	BLOQ
	0.5 mg/kg	BLOQ
	2.5 mg/kg	19±6
	12.5 mg/kg	90±36
BPA in liver	Control	BLOQ
	0.5 mg/kg	BLOQ
	2.5 mg/kg	135±77
	12.5 mg/kg	220±190
BPA in muscle	Control	BLOQ
	0.5 mg/kg	BLOQ
	2.5 mg/kg	BLOQ
	12.5 mg/kg	BLOQ[a]

[a] BLOQ=Below limit of quantification.

fish tissue for another alkylphenol (nonylphenol) [18]. Furthermore, elimination rates of 50 – 80% after 1 and 2 days respectively have been found in rats orally administered BPA [19].

3.3. Water analysis

In determination of dose–response relationships it is important to assess the actual water concentration of the compounds in question rather than using the nominal concentration. Therefore simple extraction methods using SPE coupled to LC–MS were developed. The method for quantification of tOP in water was based on previously published methods using SPE and HPLC coupled to either UV or fluorescence detection [10,20]. The application of mass spectrometry considerably enhanced the sensitivity of the present methods compared to the abovementioned methods (up to 40 times). With respect to BPA, only one paper describes the quantification in water using liquid extraction coupled to gas chromatography–mass spectrometry [21]. The quantification limit is however 200 times higher compared to the present method. The validation of the extraction was performed by comparing the values observed from spiked water samples to that of an expected standard made up in methanol and not subjected to SFE (Table 3). With respect to tOP, recoveries (95%) found in the present method were comparable with recoveries (83.6–96.5%) found in extractions of nonylphenol from water by Chee et al. [10]. Acceptable intra- and inter-assay precision, accuracy and recovery data for both compounds were demonstrated in accordance with Braggio et al. [16]. The sensitivity of the methods enables the use of very low water concentrations in either long term exposure studies or combination studies of the two compounds.

4. Conclusions

Selective and sensitive methods have been developed and validated for quantification of tOP and

Table 3
Intra- and inter-assay precision and accuracy together with recovery for tOP and BPA in water spiked at three different levels ($n=6$)

Compound	Expected concentration (μg/ml)	Intra-assay			Inter-assay			Average absolute recovery (%)
		Observed concentration	Precision % C.V.	Accuracy % Bias	Observed concentration	Precision % C.V.	Accuracy % Bias	
tOP	0.107	0.096	11.6	10.3	0.095	10.8	11.2	95
	11.6	12.1	7.1	4.2	11.6	13.9	0.1	95
	97	91	4.2	6.3	91	5.3	6.2	95
	Internal std.	–	–	–	–	–	–	97
BPA	0.125	0.133	19.5	6.4	0.134	11.9	7.2	104
	10	10.4	6.7	4.0	10.3	12.6	3.0	104
	100	103	6.8	3.0	109	6.4	9.0	104
	Internal std.	–	–	–	–	–	–	100

BPA in both water and fish tissues. Studies linking internal doses, obtained using different uptake routes (water, food and injection), of the two xenoestrogens with an estrogenic response are currently being carried out in our laboratory. The methods are also applicable with respect to field studies using caged rainbow trout or feral fish species. Studies linking possible estrogenic responses to xenoestrogens or naturally estrogens in fish exposed to sewage effluents in Denmark, are currently being carried out by our laboratory.

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