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Analysis of alkylphenol and bisphenol A in meat by accelerated solvent extraction and liquid chromatography with tandem mass spectrometry

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Abstract

The ubiquity of alkylphenols and bisphenol A (BPA) in the environment is a worldwide scientific and public concern due to the persistence, toxicity and endocrine disrupting properties of these compounds. This paper introduces a new method based on accelerated solvent extraction, with a subsequent cleanup step using amino-propyl solid phase extraction cartridges and liquid chromatography–'electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) for the simultaneous determination of nonylphenol (NP), octylphenol (OP) and bisphenol A (BPA) in different meats. Recovery studies were performed at different fortification levels. The average recoveries of each compound ranged from 91.5% to 99.9% for BPA, 89.0% to 93.3% for NP and 97.8% to 101.3% for OP. The limits of quantification (LOQs) were 1.00, 0.20 and 0.40 μg/kg for BPA, NP and OP, respectively. Investigation of the levels in commercial samples indicated that NP was ubiquitous in different types of meat at levels ranging from 0.49 to 55.98 μg/kg, and higher concentrations of NP and BPA were found in aquicolous animals.

Keywords: Alkylphenols; Bisphenol A; Accelerated solvent extraction; HPLC–ESI–MS/MS; Meat

1. Introduction

During the last decade, there has been a worldwide scientific and public discussion about the potential consequences of long-term dietary exposure to endocrine disrupters. Among these substances, the presence of alkylphenol (AP) and bisphenol A (BPA), which has demonstrated estrogenic potency and chronic toxicity by in vitro and in vivo bioassay studies (Al-Hiyasat, Darmani, & Elbetieha, 2004; Bicknell, Herbison, & Sumpter, 1995; Blake & Ashiru, 1997; Can, Semiz, & Cinar, 2005; Funabashi, Nakamura, & Kimura, 2004; Iwata, Eshima, Kagechika, & Miyaura, 2004; Laws, Carey, Ferrell, Bodman, & Cooper, 2000), has caused more concern than natural and synthetic hormones due to their wide use, ubiquitous occurrence and persistency in the environment.

APs including octylphenol (OP) and nonylphenol (NP) are widely used as intermediates to produce surfactant (anionic and non-ion surfactants) and as stabilizers of ethylcellulose resin, oil-soluble phenol resin and esters. These compounds are also discharged into the environment as metabolites of alkylphenol ethoxylates mainly by biodegradation from sewage treatment plants (Ying, Williams, & Kookana, 2002). BPA is mainly used as a monomer in the preparation of epoxide resins, polycarbonate plastics and as an antioxidant or stabilizer in polyvinylchloride. After BPA has been released into the environment and manufactured into packaging materials, food and feed may contain some of these products as a result of (i) diffuse

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environmental pollution and direct uptake by animals via food or air and potential bioaccumulation and transfer through the food web; (ii) food processing by contact with plastics, resins, lacquers, surfactants, and paints from pipes, gaskets, and containers; and (iii) migration from packaging and bottling material, envelopes, and printer ink. Humans may also be affected through the consumption of contaminated drinking water and foods. The occurrence of OP, NP and bisphenol A has been widely studied in aquatic environments (Bolz, Hagenmaier, & Korner, 2001; Kawahata, Ohta, Inoue, & Suzuki, 2004; Khim, Lee, & Kannan, 2001; Latorre, Lacorte, & Barcelo, 2003), sediment (Hashimoto et al., 2005; Yamashita et al., 2000), agricultural soil (Telscher, Schuller, Schmidt, & Schaffer, 2005), fish (Keith et al., 2001; Lye, Frid, Gill, Cooper, & Jones, 1999), and birds (Hu, Jin, & Wan, 2005), and these compounds have even been found in the atmosphere (Dachs, Van Ry, & Eisenreich, 1999). Guenther made the first comprehensive survey on the occurrence of NP in 60 kinds of foodstuffs in Germany, and found that NP is ubiquitous in food (Guenther et al., 2002). Shao investigated the levels of NP, OP and BPA in beverages in Beijing markets (Shao et al., 2005, Shao, Hu, Yang, An, & Tao, 2005) and conducted another study on the occurrence of NP and nonylphenol ethoxylates in drinking water and source water in the area of Changing, China (Shao, Han et al., 2005; Shao, Hu et al., 2005).

Nonylphenol (logKOW 4,48) and bisphenol A (log KOW 3,18) are lipophilic compounds. Therefore, they can easily contaminate foods of animal origin (e.g., meat, milk, cheese), which are thought to represent the most important source of human exposure to many organic pollutants. Knowledge of animal tissue concentrations is important for understanding the potential risk to animal health and performance as well as the risk to human health.

For extraction of AP and BPA from meat prior to analysis, steam distillation (Guenther et al., 2002), Soxhlet extraction with dichloromethane/methanol (Shao, Han et al., 2005; Shao, Hu et al., 2005), matrix solid phase dispersion (Zhao, van der Wielen, & Voogt de, 1999), microwave assisted extraction (Pedersen & Lindholst, 1999) and accelerated solvent extraction (ASE) (Datta, Loyo-Rosales, & Rice, 2002; Tavazzi, Benfenati, & Barcelo, 2002) were established as methods for quantifying AP and BPA. To the best of our knowledge, only one method was previously published for the simultaneous determination of NP, OP and BPA in fish liver (Tavazzi et al., 2002) using accelerated solvent extraction coupled with liquid chromatography–mass spectrometry with limits of quantification (LOQ) of 15–60 μg/kg, which is insufficient to monitor these compounds for the purpose of protecting human health.

This paper describes a sensitive method for quantification of NP, OP and BPA in meat using accelerated solvent extraction (ASE) followed by solid-phase cleanup, and LC–ESI–MS/M analysis. Method development efforts were focused on the optimization of the accelerated solvent extraction, the cleanup procedure and avoiding contamination. In the end, the developed method was successfully used to monitor the contaminant exposure originating from different meats.

2. Materials and methods

2.1. Reagents

Organic solvents such as dichloromethane, hexane, acetone and methanol from pesticide residue were purchased from Merck (Darmstadt, Germany). All of these solvents were of analytical grade. Standard BPA (>99%) and technical purity NP were both purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). OP and internal standard 4-*n*-NP of 99% purity were both from Dr. Ehrenstorfer Gmbh (Augsburg, Germany, 99%). Sep-Pak silica and amino-propyl solid phase extraction cartridges containing 500 mg materials (3 mL) were purchased from Waters (Milford, MA, USA). Ultra pure water was obtained by the Milli-Q ultrapure system (Millipore, Bedford, MA, USA). All standards were stored at −20 °C. To avoid the contamination of NP, OP and BPA, no APE detergents or plastics were allowed to be used, and all the glassware was baked for 4 h at 400 °C prior to use. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination. Neutral alumina (80–120 mesh)was from Beijing Chemical Co., baked 4 h at 600 °C and activated at 130 °C for (12 h), Celite (extrelut NT, Merck Co, Germany).

Stock solutions were prepared for all standard substances at 1000 mg/L in methanol. Spiking and calibration mixtures at various concentration levels were obtained by combining aliquots of stock solutions and by subsequent dilution with methanol. The mixtures were stored at 4 °C.

2.2. Sample collection and preparation

All samples including pork, fish, rabbit, duck meat and chicken were purchased from supermarkets in Beijing. For fish, rabbit, duck and chicken, one whole was purchased, the internal organs and skin were removed, the edible muscle tissue were homogenized. For pork, thin meat were stochastically purchased and homogenized. They were stored unopened at 4 °C until analysis.

Homogenized samples weighing 10 g were placed into a mortar, and 0.1 ml of 4-*n*-NP with a 200 μg/L concentration level was added to the samples as internal standard, and the samples were kept at a stable state for 30 min. Eight grams of celite were added and the samples were ground into powder using a pestle. The mixtures were packed into 33-ml stainless steel ASE cells capped with two filter disks, and 5 g activated alumina was placed on the bottom of each filter disk. The filter disks and cells were extracted with acetone three times to remove any possible contamination. The extraction was performed on an ASE

300 apparatus (ASE 300, Dionex, Sunnyvale, CA, USA). The extraction conditions were: 3 min static mode, 1500 p.s.i pressure, 60% flush volume, cell temperature 100 °C and 5 min, 120 s N_2 purge, static cycle 3 times. The crude extracts were concentrated with a rotary evaporator to near dryness and used for further cleanup by solid phase extraction.

The residues were redissolved with 20 ml dichloromethane/hexane (50:50), and passed dropwise through an amino-propyl SPE cartridge preeluted with 10 ml methanol–acetone (V/V, 50:50) and preconditioned with 5 ml hexane. Then 5 mL dichloromethane/hexane (V/V, 50:50) was used to wash the interference, and the vacuum was reduced to dry the cartridge. Finally, the analytes were eluted with 10 ml methanol–acetone (V/V, 50:50). The eluents were dried under a gentle nitrogen stream, and reconstituted with 0.5 ml mobile phase.

2.3. LC–MS/MS analysis

Identification and quantification of analytes were carried out using an Alliance 2695 (Waters, USA) liquid chromatograph equipped with a Quattro Ultima Pt (Micromass, UK) tandem mass spectrometer according to procedures previously developed by our lab (Shao, Han et al., 2005; Shao, Hu et al., 2005). A symmetry C-18 column (150 mm × 2.1 mm ID, 3.5 μm) was used for LC separation. The column oven temperature was 40 °C, the flow rate was 0.2 mL/min, and the injection volume was 10 μL. Methanol and water with 0.1% ammonia were used as mobile phases. The methanol was linearly increased from 10% to 55% within 10 min, then increased to 85% in 10 min and held for 7.5 min, finally brought back to 10% and held for 15 min until the next injection. The mass spectrometer was operated in negative mode electrospray ionization in multiple-reaction monitoring (MRM) mode. The capillary voltage was maintained at 3.5 kV. The cone voltage was 70 V. The multiplier voltage was 650 V. The nebulizing, desolvation and cone gas were supplied with nitrogen. The nebulizing gas was adjusted to the maximum, and the flow of the desolvation gas and cone gas were set to 550 L/h and 80 L/h, respectively. The source temperature and desolvation gas temperature were held at 100 °C and 300 °C. The RF lens 1 and RF lens 2 were set at 50 and 0.5. The ion energy 1 and ion energy 2 were both 0.5. The entrance and exit were zero. The collision gradient was 3.2. During tandem mass spectrometric analysis, UHP argon was used as the collision gas and the pressure of the collision chamber was kept at 2.8×10^{-3} mbar.

3. Results and discussion

3.1. Sample preparation

Temperature, solvent, and pressure are closely related with the extraction efficiency. First, dichloromethane, acetone, methanol and methanol–dichloromethane (V/V, 15:85) were used as solvents to evaluate the extraction efficiency of spiked samples (5 μg/kg). The recoveries for the three analytes ranged from 78% to 91%, 24% to 55%, 24% to 69%, and 71% to 83%, respectively. Therefore, dichloromethane was used as the extraction solvent. Then the extraction temperature was optimized between 80 and 120 °C. The results indicated that when the temperature was up to 100 °C, there was no obvious increase in extraction efficiency. As for the pressure, when 1500 psi was used, high efficiency was obtained; therefore, no further optimization was necessary.

For analysis of samples of animal origin, lipids may cause the main interference in the analysis of some contaminants in biological materials. HPLC columns are highly sensitive to trace amounts of lipidic material, which affect the active surface of the stationary phase and degrade the resolution power of the column. Thus, the presence of lipids in the extracts must be avoided or reduced as much as possible in order to extend the column lifetime and to improve detection and quantification limits (Hess, de Boer, Cofino, Leonards, & Wells, 1995). Therefore, in this paper, celite was mixed with the samples. In addition, activated neutral alumina was placed on the bottom of cells to remove part of the fat. The sample extracts using activated neutral alumina were cleaner than those without it. In a previous paper, sodium sulfate was mixed with the samples before extraction, which may lead to hardening of the mixture.

For the crude extracts, further cleanup was needed. In this study, silica and amino-propyl solid phase extraction cartridges were compared to purify the crude extracts. Ten milliliters of dichloromethane–hexane (15:85) solution containing 0.2 g animal fat and 1.0 mg/L analytes was used to evaluate the cleanup effect. The results indicated that the recoveries for analytes were all more than 91% when silica and amino-propyl solid phase extraction cartridges were used. However, when silica cartridges were used, more fat was detected after cleanup.

Because of the ubiquity of alkylphenol and bisphenol A, to avoid the contamination of NP, OP and BPA, no APE detergents or plastics were allowed to be used, and all of the glassware was baked for 4 h at 400 °C prior to use. The stainless steel ASE cells and filter disks were prewashed with ultra pure water, dichloromethane/hexane and methanol–acetone (V/V, 50:50) solution before use. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination.

3.2. Method validation

The calibration curves for detection of the target compounds were obtained by performing a linear regression analysis on standard solution using the ratio of standard area to internal standard area (4-*n*-nonylphenol) against analyte concentrations ranging from 1.0 μg L^{-1} to 500.0 μg L^{-1} containing 20 μg L^{-1} internal standard, i.e. 10.0–5000 pg with 10 μL injection. Good linearity was

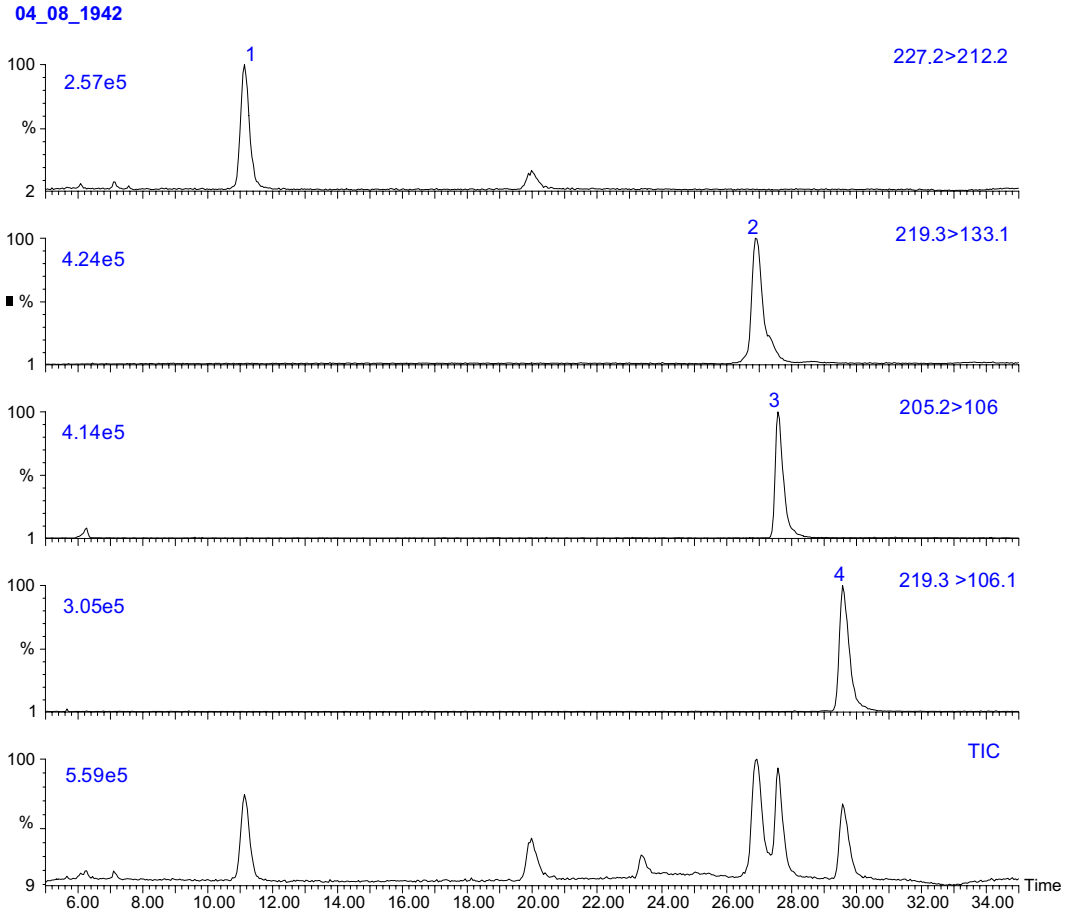

Fig. 1. LC–MS/MS chromatogram of the sample spiked at 5.0 μg/L.

obtained for all analytes, with correlation coefficients of $r > 0.99$. The analyte recovery of this procedure was evaluated by spiking 1, 10 and 50 ng of each standard analyte and 20 ng internal standard to 10 g tissue sample at three levels in replicates of five. Fig. 1 shows the chromatogram of the sample spiked at 5.0 μg/L. The results are listed in Table 1. The average recoveries of the compounds ranged from 91.5% to 99.9% for BPA, 89.0% to 93.3% for NP and 97.8% to 101.3% for OP. The reproducibility of this method was represented by percent relative standard deviation (RSD) at each fortification level for each compound, and these values are also summarized in Table 1. The results show that the method's precision was within 15%, which is very satisfactory. For each analyte, the within- and between-day reproducibilities were determined by testing six replicates independently and extracting samples of each control concentration on five different days. One control concentration (1 μg/kg) was used. The within-day reproducibility ranged from 4.2% to 11.2% and the between-day reproducibility ranged from 6.3% to 14.9%. The limits of detection, defined as the concentration that yields an S/N equal to three, were 0.30, 0.05 and 0.10 μg/kg for BPA, NP and OP, respectively. The limits of quantification (LOQ), defined as the concentration that yields an S/N equal to ten, were 1.00, 0.20 and 0.40 μg/kg for BPA, NP and OP, respectively, which were far less than previous methods (Guenther et al., 2002; Shao, Han et al., 2005; Shao, Hu et al., 2005; Tavazzi et al., 2002).

Table 1
Recoveries and RSD of spiked samples

Compound	Added (ng)	Recovery (%) ($n = 5$)	RSD (%)
BPA	1	96.92	6.23
	10	99.91	2.87
	50	91.58	7.91
NP	1	93.33	14.69
	10	88.59	8.61
	50	89.03	9.86
OP	1	97.79	7.48
	10	98.11	5.78
	50	101.33	2.70

3.3. Application to real samples

Twenty-seven kinds of samples including six pork, six mutton, three chicken, three beef, two duck's meat, and seven fish, commercially available from the market were tested. Table 2 lists the concentration of each compound detected in the samples. Among the samples, BPA was

Table 2
The concentration of each compound detected in real samples from Beijing market

Sample	BPA (μg/kg)	NP (μg/kg)	OP (μg/kg)
Pork	ND	4.52	ND
Pork	7.08	16.87	ND
Pork	0.89	1.74	ND
Pork	ND	ND	ND
Pork	0.91	1.53	ND
Pork	ND	ND	ND
Mutton	ND	ND	ND
Mutton	ND	0.49	ND
Mutton	0.54	ND	ND
Mutton	ND	2.51	ND
Mutton	ND	3.57	ND
Mutton	ND	6.01	ND
Chicken	0.73	ND	ND
Chicken	ND	3.23	ND
Chicken	ND	ND	ND
Beef	ND	3.17	ND
Beef	0.33	5.35	ND
Beef	ND	ND	ND
Duck	0.49	15.97	ND
Duck	0.85	4.36	ND
Fish	0.33	1.89	ND
Fish	0.27	5.02	0.08
Fish	ND	17.79	0.11
Fish	0.62	55.98	ND
Fish	0.95	9.13	ND
Fish	ND	11.24	0.13
Fish	1.01	14.86	ND

detectable in 13 of 27 samples with concentrations ranging from 0.33 to 7.08 μg/kg, which is much lower than results of canned meat and fat-containing food from Japan (Yoshida, Horie, Hoshino, & Nakazawa, 2001) at the levels ranging from 17 to 602 μg/kg and Austria (Braunrath, Podlipna, Padlesak, & Cichna-Markl, 2005) at the levels ranging from 4.8 to 17.6 μg/kg. The investigated results from Japan (Yoshida et al., 2001) and Austria (Braunrath et al., 2005) also indicated that 100% samples are found containing BPA, which means that the high levels of BPA in the canned products are mainly from the migraion from resin coat. OP was only found in three samples at a concentration of about 0.1 μg/kg, and NP was found in 21 samples. Of these samples, NP occurred at levels ranging from 0.49 to 55.98 μg/kg, which was higher than that found in a German investigation (Guenther et al., 2002) with average concentration 14.4 μg/kg in butter, 10.2 μg/kg in lard, about 13.0 μg/kg in liver sausage and 3.8 μg/kg in chicken meat. Among these samples, higher concentrations of NP and BPA were found in aquicolous animals (fish and duck meat), which may be attributed to the contamination of the aquatic environment.

As for human health implications, epidemiological studies are not available, and toxicological ones are limited. Therefore, the human health implications associated with these results are difficult to predict. An oral subchronic toxicity of 90 days for rats indicated that 4-NP did not cause any effects at 50 mg/kg bw/day (Cunny, Mayes, Rosica, Trutter, & Van Miller, 1997). Results from standard developmental toxicity assays in rodents resulted in the establishment of a no observable adverse effect level (NOAEL) of 640 mg/kg for fetal effects in rats and 1000 mg/kg in mice. A "provisional" level of 50 mg/kg bw has been derived (CSTEE, 2002). As for intake of BPA, according to the EU's risk assessment report on bisphenol A, based on the now available, extensive database on repeated-dose toxicity, reproductive and developmental toxicity of BPA in rodents and on the comparison of toxicokinetics in primates, including humans, and rodents, the scientific panel established a full TDI of 0.05 mg BPA/kg bw, derived by applying a 100-fold uncertainty factor to the overall NOAEL of 5 mg/kg bw/day (ESFA, 2007).

According to the National Health and Nutrition Examination Survey in 2002–2003, the maximum meat consumption in the Beijing population is about 200 g per person per day, and the maximum intake of NP and BPA was estimated to be 11.2 and 1.4 μg/person/day, or 0.19 and 0.02 μg/kg bw, which is lower than the temporary tolerable daily intake of BPA (50 μg/kg bw). Even though the residual levels of NP, BPA and OP were lower than the temporary tolerable daily intake, we should note that the intake values exclude other exposure ways such as air, water, vegetable, fruit, eggs, milk, etc. Therefore, further investigation is necessary to comprehensively evaluate the risk of BPA, NP and OP on human safety.

4. Conclusion

A method based on accelerated solvent extraction and subsequent cleanup with amino-propyl SPE cartridges and LC–ESI–MS/MS has been developed for the simultaneous determination of nonylphenol (NP), octylphenol (OP) and bisphenol A (BPA) in meat. This method was successfully applied to investigate the exposure levels of alkylphenols and bisphenol A in different kinds of meat available from Beijing markets. The maximum intakes of NP and BPA were estimated to be 11.2 and 1.4 μg/person/day, or 0.19 and 0.02 μg/kg bw.

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