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Analysis of alkylphenol and bisphenol A in eggs and milk by matrix solid phase dispersion extraction and liquid chromatography with tandem mass spectrometry

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Abstract

A method based on matrix solid phase dispersion (MSPD) using C18 as dispersant, and a subsequent cleanup step with amino-propyl solid phase extraction cartridges and liquid chromatography electrospray ionization tandem mass spectrometry (LC–ESI–MS/MS) has been developed for the simultaneous determination of nonylphenol (NP), octylphenol (OP) and bisphenol A (BPA) in eggs and milk. Recovery studies were performed at different fortification levels. Average recoveries by MSPD varied from 79% of BPA to 98% of NP and relative standard deviations were equal or lower than 15% for egg samples. The average recoveries in milk ranged from 86 to 84% for BPA, 90 to 99% for NP and 82 to 103% for OP and relative standard deviations were equal to or lower than 8%. The limits of detection (LODs) in eggs were 0.10, 0.10 and 0.25 μg/kg for BPA, NP and OP, respectively and LODs for milk were 0.10, 0.05 and 0.10 μg/kg for BPA, NP and OP, respectively. Investigation of the levels in commercial samples indicated that NP was ubiquitous in milk and eggs at levels ranging from 4.24 to 17.60 μg/kg, and the milk samples were more heavily contaminated by NP than were the egg samples.

Keywords: Alkylphenol; Bisphenol A; Matrix solid phase dispersion; LC–ESI–MS/MS

1. Introduction

At present, pollutants and related diseases are of great concern, and bisphenol A (2,2′-bis[4-hydroxyphenyl]propane; BPA) and alkylphenols (APs) are among these pollutants. BPA is mainly used as a monomer in the preparation of polycarbonate plastic and epoxy resins, which are used in baby bottles, as protective coatings on food containers, and for composites and sealants in dentistry. It is also used as an antioxidant or stabilizer in polyvinylchloride. The most commonly used APs include octylphenol (OP) and nonylphenol (NP), which are widely used as intermediates to produce surfactants (anionic and non-ion surfactants) and as stabilizers of ethylcellulose resin, oil-soluble phenol resin and esters. BPA and APs as well as related final products can be discharged into the environment during the manufacturing process and can migrate into the environment from materials [1–5]. They also can be discharged into the environment as metabolites of alkylphenol ethoxylates, polycarbonate plastic and epoxy resins mainly by biodegradation from sewage treatment plants [6–8].

After these compounds are released into the environment and manufactured into packaging materials, food and feed may contain some of these products. The compounds enter the food chain in several different ways [9,10]. 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, sediment, agricultural soil, fish, alga and birds, and these compounds have even been found in the atmosphere [11–16]. Klaus conducted the first comprehensive survey on the occurrence of NP in 60 kinds of foodstuffs in Germany, and found that NP is ubiquitous in food [17]. Shao et al. investigated the levels of NP, OP and BPA in beverages and bottled water in Beijing markets. NP occurred at levels ranging from 36.4 to 464.6 ng/l in 13 samples, and no BPA was found [18]. An investigation was also conducted on pork, mutton, chicken, beef, duck meat and fish [19]. Among 27 samples, BPA was detectable with concentrations ranging from 0.33 to 7.08 μg/kg. OP was only found at a concentration of

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about 0.1 μg/kg, and NP occurred at levels ranging from 0.49 to 55.98 μg/kg. Among these samples, higher concentrations of NP and BPA were found in aquicolous animals (fish and duck meat). Another study reported the occurrence of NP and nonylphenol ethoxylates in drinking water and source water in the area of Chongqing, China [20].

Because of the widespread use of BPA and APs, as well as their persistence in the environment, the potential risk of human exposure is an increasing concern. Toxicological studies of laboratory animals suggest that exposure to BPA and NP is associated with morphologic, functional and behavioral anomalies related to reproduction [21–26]. Exposure of rodent fetuses to low BPA doses of 20–400 μg/kg/day produces postnatal estrogenic effects [21], bisphenol A (BPA) at 2–4 mg/l given to male or female hydra had adverse effects on both sexual and asexual reproduction [25]. Laboratory studies have shown vitellogenin production in male rainbow trout at approximately 10 μg/l and inhibition of testicular growth at concentrations greater than 30 μg/l [26]. Epidemiologic evidence on the relationship between the blood levels of BPA and polycystic ovary disease (PCOS) in Japanese women adds to the concern, although this is still in debate [27–31]. Although no clear association has been established between human exposure and BPA or NP and adverse health effects, investigation of the exposure levels of these chemicals are necessary because of their potential risk [32]. AP and BPA in food are thought to represent the most important source of human exposure to many organic pollutants [17–19,33,34]. Knowledge of concentrations of these pollutants in foods is important for understanding the potential risk to human health.

For extraction of APs and BPA from eggs and milk, different extraction methods including liquid–liquid extraction [9], steam distillation [17], solid phase extraction [34] and accelerated solvent extraction (ASE) [35] have been developed for extraction of AP and BPA. To the best of our knowledge, no method has been published for the simultaneous determination of NP, OP and BPA in eggs and milk using the matrix solid phase dispersion method.

This paper describes a comprehensively analytical method for the simultaneous determination of NP, OP and BPA residues in eggs and milk. The matrix solid-phase dispersion (MSPD) method was used to extract NP, OP and BPA from eggs and milk followed by solid-phase cleanup and LC–ESI–MS/MS analysis. Method development efforts were focused on the optimization of the matrix solid-phase dispersion, the cleanup procedure and avoidance of contamination. In the end, the developed method was successfully used to monitor the contaminant exposure from milk and eggs.

2. Experimental

2.1. Standards and reagents

Organic solvents such as dichloromethane, hexane, acetone and methanol free pesticide residue were purchased from Merck (Darmstadt, Germany). All organic 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%). The solid-phase material used for MSPD was Discovery DSC-18 (Supelco Co., USA), which is a polymerically bonded, trifunctional C18 silica with 50 μm particle size, 70 Å pore diameter, 480 m^2/g specific surface area and 0.9 cm^3/g pore volume. Sep-Pak silica and amino-propyl solid phase extraction cartridges containing 500 mg materials (3 ml) were purchased from Waters (Milford, MA, USA) for cleanup. Ultra pure water was obtained using the Milli-Q ultrapure system (Millipore, Bedford, MA, USA). All standards were stored at −20 °C.

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 mobile phase. The mixtures were stored at 4 °C.

2.2. Sample collection and preparation

All samples were purchased from supermarkets in Beijing. They were stored unopened until analysis at 4 °C.

One gram of homogenized milk and 25 ng 4-*n*-NP or 0.5 gram eggs and 10 ng 4-*n*-NP were placed into a glass beaker and gently blended with 1 g of C18 powder for 5 min using a pestle. Then the mixtures were blown with a gentle nitrogen stream until free flowing powder was obtained. This mixture was introduced into a 6-ml solid phase extraction column fitted with a front frit, covered with a frit at the top, and tapped gently to remove the air pockets inside the materials. The analytes were eluted with 10 ml methanol that was allowed to elute dropwise by applying a slight vacuum. The eluent was collected and evaporated to dryness under a stream of nitrogen at 35 °C. The extracts were 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 (50:50, v/v) and preconditioned with 5 ml hexane. Then 5 ml dichloromethane/hexane (50:50, v/v) 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 (50:50, v/v). The eluents were dried under a gentle nitrogen stream, and reconstituted with 0.5 ml mobile phase for egg samples and 0.2 ml for milk samples.

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 [19]. A Symmetry C18 column (150 mm × 2.1 mm I.D., 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 containing 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, and finally brought back to 10% and held for 15 min before 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 was set to 550 l/h. The source temperature and desolvation gas temperature were held at 100 and 300 °C. The RF lens 1 and RF lens 2 were set at 50 and 0.5 V. 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 3.0×10^{-3} mbar.

3. Results and discussion

3.1. Sample preparation

Different parameters that affect MSPD extraction such as dispersant agent, cleanup and eluent solvent were studied. Nonpolar solid phase C18 and graphite carbon black (GCB) were tested using 10 ng spiking standard for matrix dispersion. High recoveries (>85%) for all analytes were obtained using C18. Although the GCB achieved better recoveries for NP and OP (>80%), the recovery of BPA was relatively low, which may be attributed to the stronger adsorbance to relative polar compounds BPA, which made it was difficult to elute from the cartridge. Therefore, in this study C18 powder was used as the MSPD material. In addition, conventional liquid–liquid extraction (LLE) method described in previous paper [9] was used to compare these two methods. Compared with MSPD method, although the precision is below 5%, the recoveries in milk and egg are no more than 80% for milk and egg samples, especially for BPA, the recoveries are as low as no more than 65% for eggs. Moreover, LLE method takes more hazard organic solvent than MSPD such as dichloromethane and the sample extracts contain more lipids especially for egg sample.

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

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 [36]. In our previous paper analyzing the alkylphenol and bisphenol A in animal tissues, the amino-propyl SPE cartridge was proven to be the preferable cartridge for purifying the crude extracts; thus, further steps were not necessary to optimize the purification [19].

Table 1
Recoveries and RSD of spiked egg

Compound	Added (ng)	Recovery (%) ($n=5$)	RSD (%)
BPA	1	79.15	7.42
	10	86.84	7.41
	50	82.60	2.86
NP	1	81.45	14.44
	10	82.46	6.57
	50	98.05	3.23
OP	1	84.59	8.31
	10	85.05	11.50
	50	96.37	3.20

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 the standard area to the internal standard area (4-*n*-nonylphenol) against analyte concentrations ranging from 1.0 to 500.0 μg l^{-1} containing 50 μg l^{-1} internal standard, i.e., 10.0–5000 pg with 10 μl injection. Good linearity was obtained for all analytes, with correlation coefficients of $r > 0.99$. The analyte recovery of this procedure was evaluated by spiking different levels of standard analyte and internal standard to samples at three levels in replicates of five. Fig. 1 shows the chromatogram of the milk sample spiked at 50 ng/g. The results are listed in Tables 1 and 2. The average recovery of each compound in eggs ranged from 79 to 87% for BPA, 81 to 98% for NP and 84 to 96% for OP. The average recovery of each compound in milk ranged from 86 to 84% for BPA, 90 to 99% for NP and 82 to 103% for OP. The reproducibility of this method was represented by the percent relative standard deviation (RSD) at each fortification level for each compound, and these values are also summarized in Tables 1 and 2. The precisions of this method for milk are all below 8% (Table 2), and most of those for eggs are lower than 10% except for NP at 1 μg/kg of spiking level and OP at 10 μg/kg of spiking level (Table 1). For each analyte, the within- and between-day reproducibilities were

Table 2
Recoveries and RSD of spiked milk

Compound	Added (ng)	Recovery (%) ($n=5$)	RSD (%)
BPA	1	93.03	5.29
	10	93.90	3.15
	50	85.73	4.10
NP	1	89.99	7.78
	10	98.58	3.27
	50	94.94	3.11
OP	1	99.96	1.36
	10	102.45	3.66
	50	82.31	6.50

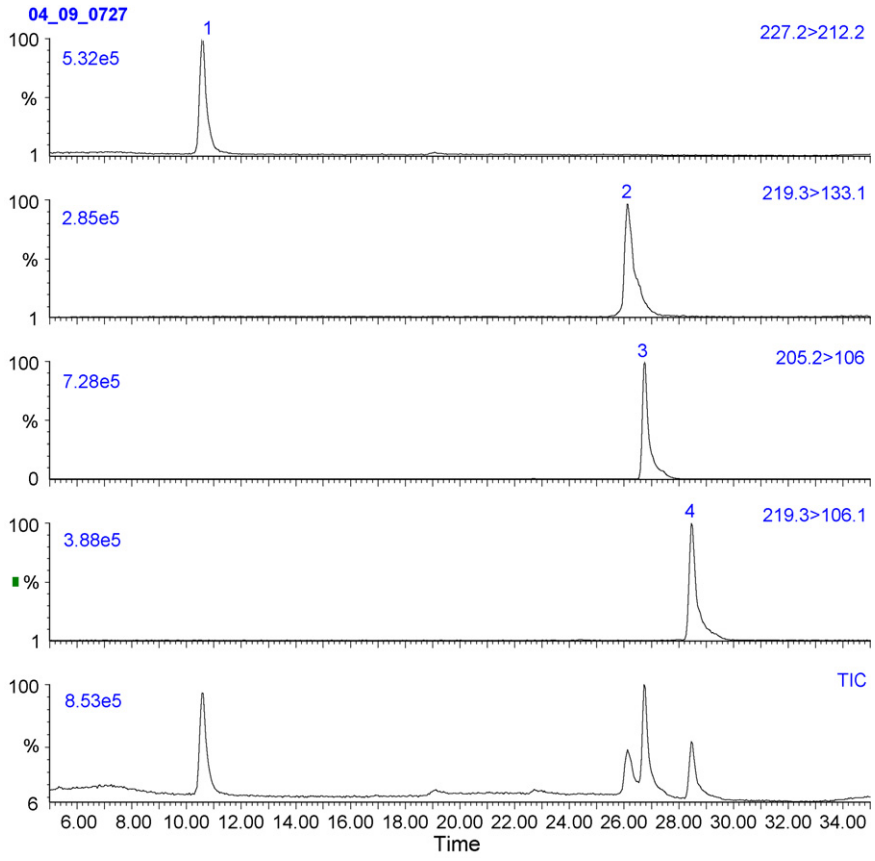

Fig. 1. Chromatogram of spiked milk sample (50 ng/g).

determined by testing six replicates independently, with samples extracted at a level of 10 μg/kg. The within-day reproducibility ranged from 3.2 to 8.9% and the between-day reproducibility ranged from 8.2 to 16.3%. The within-day and between-day precisions of milk samples are also below 10%. The large variations for the precisions of eggs may attribute to small quality of sample using (1 g) and adhesivity of eggs, which can adhere to the vessels such as glass beaker, pestle, and cause sample loss. In eggs, the limits of detection (LODs), defined as the concentration that yields an S/N equal to three, were 0.10, 0.10 and 0.25 μg/kg for BPA, NP and OP, respectively. The LODs for milk were 0.10, 0.05 and 0.10 μg/kg for BPA, NP and OP, respectively.

3.3. Application to real samples

Samples of 10 kinds of eggs and 10 kinds of milk commercially available from the market were assayed. Table 3 lists the concentration of each compound detected in the samples. Among the milk samples, BPA was detectable in only one sample, at a level of 0.49 μg/kg. OP was found in three of the 10 samples at about 0.10 μg/kg, and NP was found in seven samples with levels ranging from 4.24 to 17.60 μg/kg. As for eggs, NP occurred at levels ranging from 1.24 to 2.94 μg/kg in eight of the 10 samples. OP was found in one sample, with a level of 0.41 μg/kg, and three samples contained 0.35–10.45 μg/kg of BPA. The results indicated that the milk samples were more heavily contaminated by NP than the egg samples, which can be attributed to the migration of NP from plastic containers. Nevertheless, it seems that the egg samples contained higher levels of BPA than the milk samples.

Table 3
The concentration of BPA, NP and OP in eggs and milk

Sample no.[a]	BPA (μg/kg)	NP (μg/kg)	OP (μg/kg)
1	ND[b]	ND	ND
2	ND	1.60	ND
3	0.63	ND	ND
4	ND	2.94	0.41
5	ND	0.57	ND
6	ND	2.39	ND
7	10.45	2.80	ND
8	ND	1.24	ND
9	0.35	1.57	ND
10	ND	1.81	ND
11	ND	15.93	0.10
12	ND	5.38	ND
13	0.49	11.29	ND
14	ND	5.50	ND
15	ND	ND	ND
16	ND	ND	ND
17	ND	17.60	0.10
18	ND	7.68	ND
19	ND	4.24	0.10
20	ND	ND	ND

[a] 1–10 are eggs and 11–20 are milk.
[b] ND: not detected.

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 [37]. 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 also been derived [38]. As for intake of BPA, according to the EU's risk assessment report on bisphenol A, a temporary tolerable daily intake of BPA (10 μg/kg) from food was set in 2002 [39,40]. According to the National Health and Nutrition Examination Survey in 2003–2004, the maximum consumption of eggs and milk in the Beijing population is 200 g eggs and 500 ml milk 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 much lower than the temporary tolerable daily intake of BPA (10 μg/kg bw). Even though the residual levels of NP, BPA and OP are lower than the temporary tolerable daily intake, further comprehensive risk assessment is needed to safeguard human health.

4. Conclusion

A comprehensive extraction and clean-up method has been developed for fractionating NP, OP and BPA from eggs and milk and followed by sensitive LC–ESI–MS/MS. The method was successfully applied to commercial samples. Although higher levels of NP and BPA were found in eggs and milk, the maximum intake of resident is much lower than temporary tolerable daily intake.

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