Food Chemistry 114 (2009) 753–757

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

Determination of alkylphenol residues in breast and commercial milk by solid-phase extraction and gas chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 22 January 2008
Received in revised form 8 August 2008
Accepted 25 October 2008

Keywords:
Alkylphenols
GC–MS
Breast milk
Commercial cow milk
Solid-phase extraction

ABSTRACT

This study presents a feasible and sensitive method to determine alkylphenol residues (i.e., 4-t-octylphenol (4-t-OP) and the isomers of 4-nonylphenols (4-NPs)) in breast milk samples and commercial cow milk products. The matrix interference associated with the constituents in the milk was reduced by extraction with *n*-hexane and dilution with 50% methanolic solution (v/v, methanol/water), then followed by the Oasis-HLB SPE extraction. The analytes were determined by a GC–MS system in full-scan and selected ion monitoring modes simultaneously. Limit of quantitation was less than 0.05 ng/g in a 20 g (wet weight) milk sample. The 4-NPs were detected in 19 of the 20 breast milk samples at concentrations ranging from 1.7 to 11.6 ng/g, while 4-t-OP was detected in 8 samples at concentrations ranging from 0.4 to 1.1 ng/g. The 4-NPs were detected in all the testing commercial cow milk products at concentrations ranging from 2.9 to 8.8 ng/g.

1. Introduction

Humans are exposed to numerous environmental chemicals through industrial pollution, from the use of pesticides in agriculture and the consumption of food. Alkylphenols, such as 4-tert-octylphenol (4-t-OP) and the isomers of 4-nonylphenol (4-NPs), are widely used in various industries as the precursors in the production of nonionic surfactants in detergents and as emulsifying agents in many pesticide formulations. Additionally, the 4-NPs are also used to yield tris(nonylphenol)phosphite (TNPP) as the antioxidant stabilizers in plastics. They have been demonstrated to be able to mimic natural hormones by interacting with the oestrogen receptor, and inhibit ATP synthesis in mitochondria at low-level exposures (Colborn, vom Saal, & Soto, 1993; Jobling & Sumpter, 1993; Jobling, Nolan, Tyler, Brighty, & Sumpter, 1998; Tyler, Jobling, & Sumpter, 1998; Bragadin et al., 1999; Nagao, Wada, Marumo, Yoshimura, & Ono, 2001). The extensive use of alkylphenols led to their detection in many food products, such as seafood, beverages, eggs, vegetables, fruits, meats, rice and commercial milks (Guenther et al., 2002; Cheng, Liu, & Ding, 2005; Ferrara, Fabietti, Delise, Bocca, & Funari, 2001; Kannan et al., 2003; Basheer, Lee, & Tan, 2004; Casajuana & Lacorte, 2004; Fernandes, Costley, & Rose, 2003; Lu, Chen, Sung, Wang, & Mao, 2007; Shao, Han, Tu, & Huang, 2007; Shao, Hu, Yang, An, & Tao, 2005; Yang & Ding, 2005). Human body fluid such as breast milk is ideal for monitoring human exposure to alkylphenol residues, to reveal both parent and neonate exposure. Although chlorinated and brominated organic contaminants in breast milk have been reported extensively (Costopoulou, Vassiliadou, Papadopoulos, Makropoulos, & Leondiadis, 2006; Chao et al., 2004; Burke, Holden, & Shaw, 2003; Chao, Wang, Lin, & Chung, 2006; Jaraczewska et al, 2006; Johnson-Restrepo, Addink, Wong, Arcaro, & Kannan, 2007; Tsydenova et al., 2007; Gomara et al, 2007), information regarding alkylphenol residues in breast milk is scarce (Guenther et al., 2002; Otaka, Yasuhara, & Morita, 2003), especially in developing countries.

Determination of alkylphenol residues in breast milk has been based on 5-hour steam distillation extraction followed by normal-phase HPLC cleanup (Guenther et al., 2002), or overnight alkaline digestion, liquid–liquid extraction, SPE cleanup and both of them need to derivatize alkylphenols prior to GC–MS analysis (Otaka et al., 2003). These methods involved intensive sample preparation and derivatization steps. Moreover, relatively large amounts of sample (up to 80 g) were required to achieve the desired sensitivity.

As part of an extensive effort to evaluate the impact of alkylphenol residues in dietary components that are consumed by infants and consumers in Taiwan, this study developed a simple, reliable and sensitive method to routinely determine trace levels of alkylphenol residues in breast milk samples and commercial milk products by direct GC–MS analysis. Milk samples were pretreated with liquid–liquid extraction, the extract was diluted and the analytes were then extracted using an HLB SPE cartridge. This work

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doi:10.1016/j.foodchem.2008.10.059

represents a preliminary study of alkylphenol residues in breast milk samples and commercial milk products sold in Taiwan, to support children's health and food safety programs.

2. Experimental

2.1. Chemicals and reagents

Unless stated otherwise, all chemicals and solvents were purchased in high purity grade from Aldrich (Milwaukee, WI, USA), Tedia (Fairfield, OH, USA) and Merck (Darmstadt, Germany) and used without further purification. 4-Nonylphenols (4-NPs, tech. grade), 4-*tert*-octylphenol (4-t-OP, purity >97%), and cumylphenol (purity >99%, used as a surrogate) were purchased from Aldrich. Internal standard [$^2H_{14}$]-p- terphenyl was obtained from ChemServices (West Chester, PA, USA). Stock solutions of each analyte (1000 μg/ml) were prepared in methanol. Mixtures of the analytes for the preparation of working standards and for sample fortification were also prepared in methanol. All stock solutions and mixtures were stored in the dark at −4 °C.

2.2. Sample collection and pretreatment

Twenty breast milk samples were collected from healthy mothers in the central Taiwan suburban area between December 2000 and November 2001, as described elsewhere (Chao et al., 2004; Chao et al., 2006). Participants collected their milk samples in chemical-free glass-bottles with Teflon seals and froze them at home. After 40–60 ml of samples was collected, the bottles were immediately transferred to the laboratory at National Health Research Institutes (NHRI) and kept frozen at −20 °C until analyses were conducted. Seven commercial cow milk products, including four whole milk samples, two low-fat milk samples and one no-fat milk, were purchased from nationwide wholesale markets. They were stored unopened until analysis at 4 °C.

Before analyses, the stored frozen breast milk or cold commercial cow milk was brought to room temperature and homogenised by vertical mixing for 5 min. Twenty gram of a milk sample was spiked with the surrogate cumylphenol at the final concentration of 5.0 ng/g. We modified the sample pretreatment procedures from our previous work dealing with the analysis of bisphenol A and phytoestrogens in infant formula powders (Kuo & Ding, 2004). First, the sample was extracted three times with *n*-hexane (10 ml each) by liquid–liquid extraction (Otaka et al., 2003). The organic extracts were combined, the volume of the extract was reduced to 2 ml with rotary evaporation, and this extractable fraction was considered to be "fat extract". Since *n*-hexane only can extract analytes from lipids but not protein, the quantitative results of alkylphenols in this study were called "*n*-hexane extractable residue levels". The extract was redissolved in 50 ml of 50% methanolic solution (v/v, methanol/water) (for details, see Section 3), and then filtered through a 0.45 μm membrane filter (Gelman Scientific, Ann Arbor, MI, USA) (Kuo & Ding, 2004). The extract was diluted by another 100 ml of 50% methanolic solution (v/v, methanol/water) and was passed through the filter to make the "fat extract" as clean as possible to improve flow rate during SPE, and reduce the viscosity. The total 150 ml of methanolic solution was then applied to an HLB SPE cartridge (3-ml, 60 mg, surface area 810 m^2/g, Waters, Milford, MA, USA) for extraction. The HLB SPE cartridge was employed herein because it is much more efficient than other hydrophobic SPE cartridges (i.e., RP-C_{18} and PS-DVB) and has been used extensively in the analysis of moderate polar residues (Waters Crop, 2001). Before HLB SPE extraction, each cartridge was conditioned with 3 ml of methyl-*t*-butyl ether (MTBE), 3 ml of methanol and 3 ml of deionized water on an SPE manifold (VacMaster, IT Sorbent Technology, Cambridge, UK) to eliminate the contamination. The methanolic extract was passed through the SPE cartridge at a flow rate of about 3–4 ml/min via a siphon tube with the aid of a vacuum. When the extraction was completed, the cartridge was dried under vacuum for 5 min. The analytes were eluted from the cartridge with 3 ml MTBE. The extract was then passed through an alumina/anhydrous sodium sulphate column (10 cm × 0.5 cm i.d.) for cleanup. The eluate was evaporated to dryness under a gentle stream of nitrogen. The residue was then redissolved in 100 μl of dichloromethane containing 5.0 μg/ml of [$^2H_{14}$]-p-terphenyl as an internal standard, and made ready for GC–MS analysis.

The recovery experiments were performed using the spiked commercial whole milk samples. Standard solutions of 4-t-OP (1.0 μg/ml) and the 4-NPs (total concentration: 1.0 μg/ml) in 100 μl of methanol were carefully added into a 20 g milk sample by a glass syringe. The samples were mixed by tumbling for 30 min. The spiked samples (final concentration: 5.0 ng/g) were then stored in tightly closed brown glass vials at room temperature for at least 12 h prior to analysis. The spiked samples were subjected to the sample pretreatment and GC–MS analytical procedures, and then the recovery was calculated by comparing the concentrations obtained with the known amounts of the spiked analytes.

Due to the ubiquity of alkylphenol, to avoid the contamination of alkylphenol, no alkylphenol polyethoxylates detergents or plastics were allowed to be used, and all glassware was cleaned and rinsed subsequently with hot tap water, deionized water, methanol and acetone before drying, and then heated overnight at 250 °C prior to use. In addition, procedural blanks were analysed for each batch of samples as a check for contamination from solvents and glassware used in the analysis.

2.3. GC–MS analysis

Analyses were performed on a Finnigan Focus gas chromatograph coupled directly to a Focus DSQ quadrupole mass spectrometry (Finnigan, CA, USA) operated in full-scan and selected ion monitoring (SIM) modes simultaneously. Samples (1 μl) were injected with the injection temperature at 280 °C in the splitless mode. A DB-5MS capillary column (15 m × 0.25 mm i.d., 0.1 μm film, J&W, CA, USA) was used. The GC temperature program was as follows: 70 °C for 2 min, a temperature ramp of 30 °C/min up to 130 °C, a temperature ramp of 8.5 °C/min up to 300 °C, and then holding this temperature for 5 min. The temperature of transfer line was set at 275 °C; the ion source temperature was 200 °C. Quantitation of the analytes was performed in the SIM mode. The selected ions were those at m/z 107 and 121 for 4-t-OP; m/z 107, 121, 135 and 149 for the 4-NPs; m/z 197 and 212 for cumylphenol (as a surrogate); and m/z 244 for [$^2H_{14}$]-p- terphenyl (as an internal standard). The dwell time was 100 ms/ion/scan and the solvent delay was 5 min. The detector was tuned using perfluorotributylamine (PFTBA) and the autotune program. The electron energy was 70 eV.

3. Results and discussion

Since the use of dichloromethane as the solvent for liquid–liquid extraction cause serious emulsification (Otaka et al., 2003; Kuo & Ding, 2004), *n*-hexane was adopted to extract analytes (with lipid) from the milk samples. Casajuana et al. noted that the effective and reproducible SPE extraction from milk samples depends on disrupting the milk fat globule membranes by adding relatively large amounts of alcoholic solutions to dilute the "fat extract" prior to SPE extraction (Casajuana & Lacorte, 2004). In this study, 50%

(v/v) water: methanol and water: ethanol solutions were firstly evaluated as the "dilute solution" in the SPE extraction. When methanolic solution was used, the recoveries of 4-t-OP and the 4-NPs were 81% (with RSD 4%, $n = 3$) and 93% (with RSD 6%), respectively. However, for ethanol solution, the recoveries were 53% (with RSD 6%) and 84% (with RSD 8%), respectively. Therefore, the milk sample was firstly extracted by *n*-hexane liquid–liquid extraction, and the "fat extract" was diluted with 50% of methanolic solution to digest the milk's emulsion before HLB SPE extraction.

All of the concentrations of the alkylphenol residues are reported herein on a wet-weight basis. Quantitation of the 4-NPs was based on the sum of the peak areas for all of the isomers, which was calculated from the five-point calibration curve, as indicated by the response factors, covering the range from 0.1 to 10 μg/ml, each divided by the fixed concentration of the internal standard (Cheng et al., 2005; Yang & Ding, 2005). The precision of the calibration curves, as indicated by the relative standard deviation (RSD) of the response factors, was 4.7% and 6.2% for 4-t-OP and the 4-NPs, respectively; the correlation coefficients exceeded 0.997. The curves covered a range equivalent to the concentrations of the analytes in 20 g milk sample after the extract was concentrated to 100 μl (approx. 200-fold concentration). The instrumental detection limits, defined as the lowest amount (S/N $\geqslant$ 3) that can be detected by the instrument, were 0.01 and 0.3 pg for 4-t-OP and the 4-NPs, respectively, as summarised in Table 1. The limit of detection (LOD), defined as the concentration that yielded an S/N ratio of higher than or equal to 3, and the limit of quantitation (LOQ), defined as the concentration that yielded an S/N ratio of higher than or equal to 10, were determined by the sample pretreatments of the spiked commercial or breast milk samples. The LOQ values were 0.03 ng/g for 4-t-OP and 1.0 ng/g for the 4-NPs in 20 g (wet weight) commercial whole milk; and 0.2 ng/g for 4-t-OP and 1.2 ng/g for the 4-NPs in 20 g (wet weight) breast milk (Table 1).

Tables 2 and 3 present the versatility of the developed method. Table 2 lists the recovery rates of the spiked samples and the concentrations of alkylphenol residues detected in commercial cow milk products with various lipid contents. For commercial cow milk products, the spiked recoveries of 4-t-OP ranged from 77% to 85% (average 81 ± 4%, $n = 3$), the spiked recoveries for the 4-NPs were 86 to 93% (average 89 ± 4%, $n = 3$), and the RSD ranged from 3% to 9%. Recoveries of surrogate (cumylphenol) were 85% to 95% (average 89 ± 4%, $n = 10$) and RSD ranging from 3% to 6%. The residues of the 4-NPs were detected in all the testing samples ($n = 7$) at concentrations ranging from 2.9 to 8.8 ng/g (wet weight), and relatively higher concentration of the 4-NP residues was found in whole milk samples than those in low- or no-fat milk samples.

Table 3 shows that the 4-NPs was detected in 19 of total the 20 breast milk samples at concentrations ranging from 1.7 to 11.6 ng/g (average 4.4 ± 2.3 ng/g, $n = 19$). Recoveries of the surrogate ranged from 81% to 94% (average 87 ± 4%, $n = 20$) and RSD ranging from 5 to 7%. Since the limiting amounts of breast milk samples were collected, no matrix-spiked experiment was performed in this study. Triplicate analyses of breast milk samples were conducted to evaluate the analytical precision. The RSD of the concentrations of the analytes in these samples were less than 10%, indicating the good repeatability of the method. Fig. 1 displays the typical SIM chromatograms of 4-t-OP and the 4-NPs detected in (a) the commercial cow milk sample-5, (b) the breast milk sample-3 and (c) the breast milk sample-10 used as matrix blank. No interference peak was observed, indicating that the developed sample pretreatment procedure sufficiently removed the fatty acid residues from the extract. The peaks were identified and quantitated using the retention times with the sum of their selected ions (as lists in Section 2.3) of the SIM trace and the response factors related to the internal standard ([$^2H_{14}$]-p-terphenyl), respectively.

Table 1
IDL, LOD and LOQ.

Compound	IDL (pg)	LOD (ng/g) Commercial milk	LOQ (ng/g)	
			Commercial milk	Breast Milk
4-t-OP	0.01	0.01	0.03	0.2
4-NPs	0.3	0.3	1.0	1.2

Table 2
Concentrations of alkylphenol residues found in commercial milk samples and their spiked recoveries.

Sample	Lipid content (%)	Analytes		
		4-t-OP	4-NPs	cumyphenol (Surrogate, %)
Whole milk				
Sample-1	3.8			
Concentration (ng/g, $n = 3$)		n.d.	8.8[a] (8)[b]	94[c] (3)[d]
Spiked recovery (%, $n = 3$)		85[e] (4)[d]	93 (6)	95 (6)
Sample-2	3.8			
Concentration (ng/g, $n = 3$)		n.d.	3.8 (9)	87 (4)
Spiked recovery (%, $n = 3$)		77 (8)	86 (8)	85 (3)
Sample-3 (ng/g)	3.2	n.d.	4.4	83
Sample-4 (ng/g)	3.8	n.d.	5.7	86
Low-fat milk				
Sample-5	1.5			
Concentration (ng/g, $n = 3$)		0.1 (9)	5.0 (8)	93 (4)
Spiked recovery (%, $n = 3$)		82 (3)	88 (7)	90 (4)
Sample-6 (ng/g)	1.5	n.d.	3.6	88
No-fat milk				
Sample-7 (ng/g)	<0.5	n.d.	2.9	91

n.d. not detected at LOD, as listed in Table 1.
[a] Mean concentration (ng/g, $n = 3$) of alkylphenol residues found in the samples.
[b] Relative standard deviation (%RSD) of detected concentration is given in parentheses ($n = 3$).
[c] Mean surrogate recovery (%, $n = 3$) found in the samples.
[d] Relative standard deviation (%RSD) of recovery is given in parentheses ($n = 3$).
[e] Mean spiked recovery (%, $n = 3$) at final concentration of 5 ng/g for each analyte.

Table 3
Concentrations (ng/g) of alkylphenol residues found in breast milk samples.

Sample	Analytes		
	4-t-OP	4-NPs	cumyphenol (Surrogate, %)
1 ($n = 3$)	0.2[a] (10)[b]	3.5[a] (7)[b]	85[c] (7)[d]
2	n.d.	11.6	89
3	n.d.	3.5	91
4	n.d.	4.2	87
5	n.d.	1.7	90
6	n.d.	2.8	85
7	0.4	6.5	86
8	0.4	5.6	81
9	n.d.	3.4	82
10 ($n = 3$)	n.d.	n.d.	82 (5)
11	1.0	2.3	90
12	n.d.	2.9	86
13	n.d.	6.1	90
14	0.4	4.4	87
15	n.d.	4.1	85
16	0.5	6.9	87
17	n.d.	4.2	91
18	1.1	4.1	94
19	0.5	2.0	93
20 ($n = 3$)	n.d.	4.0 (10)	86 (5)

n.d. not detected at LOD, as listed in Table 1.
[a] Mean concentration (ng/g, $n = 3$) of alkylphenol residues found in the samples.
[b] Relative standard deviation (%RSD) of detected concentration is given in parentheses ($n = 3$).
[c] Mean surrogate recovery (%, $n = 3$) found in the samples.
[d] Relative standard deviation (%RSD) of recovery is given in parentheses ($n = 3$).

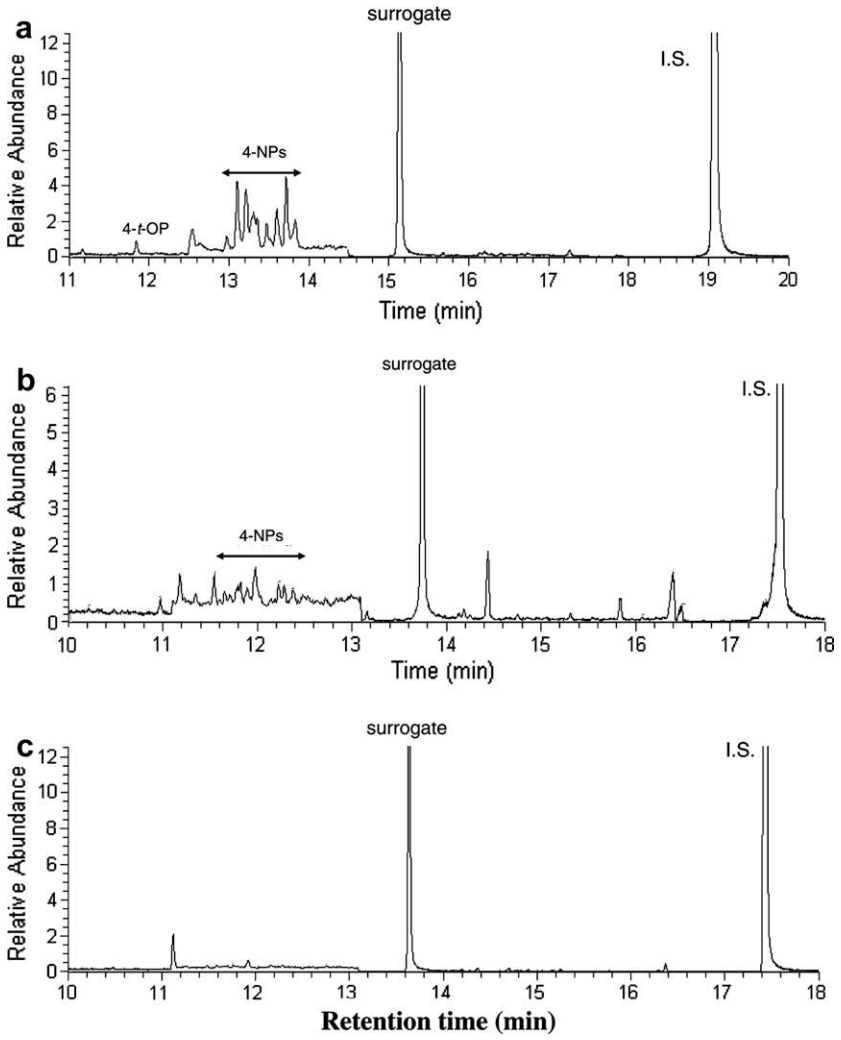

Fig. 1. SIM chromatograms of 4-t-OP and the 4-NP isomers detected in (a) the commercial cow milk sample-5, (b) the breast milk sample-3 and (c) the breast milk sample-10 used as matrix blank (neither 4-t-OP nor 4-NPs was detected).

The mean level of the 4-NP residues in breast milk samples in Taiwan exceeded those in samples from Germany (average 0.3 ng/g) and Japan (average 1.0 ng/g) (Guenther et al., 2002; Otaka et al., 2003). However, if an infant with a body weight of 5 kg took 700 g of the breast milk per day (Chao et al., 2004), then the 4-NPs intake should be around 610 ng/kg b.w./day, which is lower than the no-observed adverse effect level (NOAEL) estimated by Nagao et al (10 mg/kg b.w./day in the next generation on reproductive capacity) (Nagao et al., 2001). Taken together, our results reveal that this developed method is appropriate for analysing the levels of alkylphenol residues in commercial and breast milk samples.

4. Conclusion

We have developed and optimised a reliable, sensitive and convenient GC–MS method, coupled with liquid–liquid extraction and SPE extraction for the determination of traces of alkylphenol residues in commercial cow milk and breast milk samples. The lower LOQ of the developed method (less than 1.2 ng/g in 20 g (wet weight)) is sufficient to determine toxicologically relevant concentrations. Our preliminary results indicate that the 4-NPs are present in breast milk samples and commercial cow milk products sold in Taiwan. Although the results suggest that the levels of alkylphenol residues in milk samples are unlikely to be of concern, the oestrogenic activity of the 4-NPs at low levels has been discussed (Cooney, 2000). Moreover, since milk is the main nourishment of babies and is consumed by many adults, we recommend that the contents of these residues in breast milk and commercial milk products should be monitored and reported routinely to satisfy health and food safety concerns of infant and consumers.

Acknowledgement

This study was supported by a grant from the National Science Council of Taiwan under contract No. 96-2113-M-008-001.

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