Notes

Determination of Bisphenol A and 4-Nonylphenol in Human Milk Using Alkaline Digestion and Cleanup by Solid-Phase Extraction

Hiroaki Otaka,*† Akio Yasuhara,** and Masatoshi Morita**

*National Environmental Research and Training Institute, Ministry of the Environment, 3-3 Numiki, Tokorozawa, Saitama 359-0042, Japan
**National Institute for Environmental Studies, 16-9 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

A highly sensitive and selective method based on alkaline digestion for the simultaneous determination of bisphenol A (BPA) and 4-nonylphenol (NP) was developed. The method consists of digestion of the matrix with ethanolic KOH, extraction with diethyl ether under a mild alkaline condition, cleaning with successive aminopropyl (NH₂) cartridges and derivatization followed by a GC-MS analysis. The assay accuracies, expressed as recoveries, were 82 - 113% for BPA and 89 - 97% for NP. The limits of detection of BPA and NP were 0.09 ng/g and 0.50 ng/g, respectively. The procedure will be reliable for the trace analysis of BPA and NP in human milk, since alkaline digestion can diminish their documented association with protein.

(Received July 2, 2003; Accepted October 7, 2003)

Introduction

Bisphenol A (BPA, 4,4'-isopropylidenebiphenol) is a raw material for manufacturing polycarbonate, which is used in a wide array of plastic products. 4-Nonylphenol (NP) is one of the degradation products of alkylphenol polyethoxylates, which is widely used in a variety of industrial, household and commercial applications. Both compounds are of much concern because of their enormous amounts of usage and estrogenic activities.¹⁻³

BPA and NP are widely used in products to which humans are exposed. It is therefore of great importance to investigate their intake, bioavailability and metabolism in(to) humans. During the last few years, the contamination levels of these compounds in human blood have been estimated by several researchers. Sajiki et al. reported the BPA concentrations in human plasma to be 0.59 ± 0.21 ng/ml for men and 0.33 ± 0.54 ng/ml for women.¹ Inoue et al. detected trace amounts of NP from healthy human plasma.³

Exposure of fetuses and infants seems to be the most serious issue among the various problems. It is urgent to assess the exposure of highly-sensitive individuals to slight quantities of these compounds. Recent work has shown the contamination of BPA in placenta at the same level as blood,⁶ suggesting migration from a mother into the fetus.

The possible pathway for infant exposure to the compounds is the ingestion of contaminated human breast milk or infant formula. Attracting considerable attention is the discovery that trace quantities of BPA migrate from the polycarbonate of lactation bottles, or epoxy resin as a can coating material to infant formula.⁷⁻⁸ On the other hand, there is still no report on an investigation of human milk contamination. The detection of BPA from human blood and placental tissue suggests the necessity of assessing human milk. In order to recognize detailed infant exposure through human milk, it is highly important to accurately estimate the levels of the targets in this medium. Therefore, we attempted to develop a protocol to determine sub-parts-per-billion levels in human milk.

According to previous reports, the extraction of the target analytes with a reverse-phase column seems to be most suitable for liquid samples. Biles et al. extracted BPA in infant formula concentrate with a styrene/divinylbenzene polymer column.⁸ The enrichment of BPA and NP in serum using a reverse-phase cartridge was reported by several investigators.⁷⁻¹⁰ On the other hand, it was also reported that these compounds easily associate with protein.¹¹⁻¹² If the analytes exist as associates with the polyionized compound, they can pass through a reverse-phase column. The interaction of the target analytes with some components may occur in a complex matrix, such as milk. To avoid an erroneous estimation, such an interaction should be diminished in an extraction step.

Sasaki et al. adopted an alkaline digestion method based on degradation of protein and lipid with ethanolic alkali for the determination of NP and other alkylphenols in various foods.¹³ This method should be superior to general solid-phase extraction methods for the analysis of protein-abundant samples, because it can diminish the above-mentioned association.

We propose in this report a method based on alkaline digestion for the determination of BPA and NP in human milk. The proposed method involves liquid-liquid extraction (LLE) with diethyl ether under a mild alkaline condition, cleaning by successive aminopropyl (NH₂) solid-phase extraction (SPE) and derivatization followed by GC-MS analysis. To our knowledge, very few studies have been published on the simultaneous determination of these compounds in a complex matrix, such as foods or biological samples. The optimization of alkaline digestion, LLE and cleaning procedures resulted in an effective

¹ To whom correspondence should be addressed.
E-mail: hiroaki_otaka@env.go.jp
removal of the interfering matrix, and enabled the simultaneous determination of the two much-concerned analytes.

**Experimental**

**Materials and reagents**

Bisphenol A and 4-nonylphenol (mixture of isomers) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Bisphenol A-d_{16} and 4-nonylphenol-d_{4} were obtained from Kanto Chemicals (Tokyo, Japan). Standard solutions (1000 µg/mL) of each compound were prepared in acetone. Working solutions were prepared by mixing and diluting these solutions with acetone. A surrogate solution containing BPA-d_{16} (100 ng/mL) and NP-d_{4} (70 ng/mL) was prepared in ethanol. Pyrene-d_{10} and phenanthrene-d_{10} were used as recovery standards, prepared at a concentration of 40 ng/mL in hexane. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was used as a derivatization reagent by Wako. All solutions were stored at −20°C. All solvents were of pesticide residues analytical grade, with the exception of 2-propanol of HPLC grade, purchased from Wako. NaCl and Na_2SO_4 of analytical grade were treated at 400°C before use, because serious contamination with NP was confirmed in both materials. Analytes-free water was prepared by the treatment of distilled water with an active-carbon column. N_2 gas (99.999% purity) purified by passing through an active carbon tube was used for concentrating the extracts. Isolute-NH_2 (1 g/6 mL) was purchased from Uniflex Corporation (Tokyo, Japan). No analytes could be detected from all of the reagents and materials in the individual measurements.

**Procedure**

The complete process of alkaline digestion, LLE and cleanup is shown in Fig. 1. A 2-µL aliquot of the resulting solution was injected into an HP6890 gas chromatograph equipped with an HP5973 mass spectrometer. The GC-MS conditions used for the analysis, excluding the acquisition parameters for the selected ion monitoring (SIM) mode, followed a reference. The selected ions for quantitative SIM are summarized in Table 1. Calibration standards for the derivatives of each analyte were prepared by derivatizing known amounts of the authentic standard (no solvent) with 0.1 mL BSTFA at 50°C for 30 min. The standard for the determination of BPA and that for NP were prepared separately. Briefly, after derivatization, BSTFA was removed from BPA standards with a gentle stream of N_2 to prevent contamination from the epoxy resin used in a micro syringe. The removal of BSTFA from NP standards was not performed because a pronounced decrease in the peak abundance of silylated NP was observed when the treatment was conducted.

**Results and Discussion**

**Optimization of alkaline digestion and liquid-liquid extraction procedures**

We conducted alkaline digestion by having the mixture stand overnight at room temperature, because digestion by heating (70°C for 30 min) gave an NP chromatogram with numerous interfering peaks. Various solvents were tested for the extraction of analytes from the hydrolysate. Serious emulsification was observed when dichloromethane was used. We adopted diethyl ether with which emulsification was not observed.

The analysis of BPA in fat-content matrices using alkaline digestion is a challenging task. A serious problem with a fatty sample is that much more matrix coextractants are present in the extracts that interfere with GC-MS determination when an alkaline digestion is used. Because fatty acid, a degradation product of triglyceride, is an acidic substance similar to BPA, generally-adopted LLE under an acidic condition could not be applied. The effect of the pH on the recovery of BPA from a potassium

---

**Table 1 Selected ions used for quantitative SIM**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Selected ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>207 221</td>
</tr>
<tr>
<td>NP-d_{4}</td>
<td>183 296</td>
</tr>
<tr>
<td>BPA</td>
<td>357 372</td>
</tr>
<tr>
<td>BPA-d_{16}</td>
<td>368 386</td>
</tr>
<tr>
<td>Pyrene-d_{10}</td>
<td>221 111</td>
</tr>
<tr>
<td>Phenanthrene-d_{10}</td>
<td>188 162</td>
</tr>
</tbody>
</table>

Fig. 1 Flow chart for the complete alkaline digestion, extraction and cleanup process of BPA and NP from a human milk sample. HEX, hexane; 2-PrOH, 2-propanol; DCM, dichloromethane; EA, ethyl acetate; ACE, acetone.
phosphate buffer and the amount of extracts from milk hydrolysate is shown in Fig. 2. The extraction efficiency of BPA remains constant and is maximum for pH values lower than 11, decreasing sharply for higher values. On the other hand, the amount of extracts (mainly fatty acid) from milk hydrolysate showed a sharp decrease within the pH range of 7–9. Therefore, we decided to adjust hydrolysate to pH 10–11 prior to diethyl ether extraction. This procedure permitted the extraction of a small amount of extracts (below 20 µL from a 25 g milk sample) and quantitative recoveries of the analytes.

Cleaning with solid-phase extraction

To remove the coextractants, we tested the cleaning effects of several protocols, including successive solid-phase extraction on various adsorbents and solvents. At first, by the protocol employed in this work, most of the coextractants were trapped on an NH₂ adsorbent and the analytes were satisfactorily recovered by eluting with chloroform:2-propanol (2:1).15 Thereafter, the eluate was subjected to a second cleaning with another NH₂ column. After washing with hexane:dichloromethane (1:3) in order to remove less-polar compounds, such as unsonifiable matters, the retained NP and BPA were quantitatively eluted with dichloromethane:ethyl acetate (1:1) (fraction 2) and acetone (fraction 3), respectively. Protocols using other adsorbents, such as silica gel or Florisil, or compounds, such as unsaponifiable matters, the retained NP and BPA were quantitatively eluted with dichloromethane:ethyl acetate (1:1) (fraction 2) and acetone (fraction 3), respectively. Protocols using other adsorbents, such as silica gel or Florisil, or

Linearity of the calibration curves and detection limits

The linearity of the response of the MS detector to the TMS derivative of each analyte was assessed. The detector response for silylated BPA was linear ($r^2 = 0.999$) over the range of 2.0–20 pg injected. For silylated NP, the response was also linear ($r^2 = 0.999$) over the range of 20–200 pg injected.

The detection limit and the quantitation limit of each analyte was calculated from the blank standard deviation by applying the methodology proposed in previous report. The results are summarized in Table 2.

Application of the proposed method to human milk

The proposed method was applied to analyze BPA and NP from human milk. The recovery of each compound was calculated by a comparison with the recovery standard (pyrene-d₁₀ with BPA and BPA-d₁₆, and phenanthrene-d₁₀ with NP and NP-d₄). The overall recoveries were 82–113% for BPA and 89–97% for NP, as shown in Table 3. The accuracy of the data obtained from replicated measurements of three different breast milk samples spiked with the same concentration of standard solution are presented in Table 4. The assay accuracies were 6.4% for BPA and 3.3% for NP.

Table 2 Detection limit and quantitation limit of each compound in the proposed procedure

<table>
<thead>
<tr>
<th>Compound</th>
<th>Procedure blank (ng/g, n = 5)$^a$</th>
<th>Detection limit$^b$ (%)</th>
<th>Quantitation limit$^b$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>0.33 ± 0.058</td>
<td>0.50 ± 0.09</td>
<td>0.91 ± 0.21</td>
</tr>
<tr>
<td>BPA</td>
<td>0.033 ± 0.018</td>
<td>0.09 ± 0.03</td>
<td>0.21 ± 0.01</td>
</tr>
</tbody>
</table>

Table 3 Recovery of each compound from a spiked human milk sample

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (ng/g sample)</th>
<th>Number of replicate</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>10</td>
<td>3</td>
<td>97 ± 13</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>3</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>NP-d₄</td>
<td>1.4</td>
<td>12</td>
<td>86 ± 11</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>3</td>
<td>92 ± 3</td>
</tr>
<tr>
<td>BPA</td>
<td>1.0</td>
<td>3</td>
<td>113 ± 11</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3</td>
<td>82 ± 5</td>
</tr>
<tr>
<td>BPA-d₁₆</td>
<td>2.0</td>
<td>12</td>
<td>100 ± 23</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>3</td>
<td>84 ± 6</td>
</tr>
</tbody>
</table>

Table 4 Assay precision and accuracy for BPA and NP in spiked human milk samples (n = 3)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Expected</th>
<th>Observed</th>
<th>Precision % C.V.</th>
<th>Accuracy % Bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>NP</td>
<td>9.2</td>
<td>9.5</td>
<td>3.7</td>
<td>3.3</td>
</tr>
<tr>
<td>BPA</td>
<td>0.94</td>
<td>0.88</td>
<td>2.3</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Fig. 2 Effect of the pH on the recovery of analytes from the buffer (10 mM-potassium phosphate + 15%-ethanol) and the amount of extracts from milk (50 g) hydrolysate. ● ●, amount of extracts; ▲ ▲, recovery of BPA; ■ ■, recovery of NP.

Table 5 gives the results for the determination of compounds in three human milk samples provided by different volunteers by the proposed method. BPA was significantly detected in two samples (0.65 and 0.70 ng/g). Representative chromatograms of a human milk sample are shown in Fig. 3. No interfering peak was observed, demonstrating that sufficient removal of fatty acid residues was achieved.

The detected BPA levels were very consistent with those of human plasma reported by Sajiki et al. If an infant of 5 kg body weight takes 200 g of the milk per day, the BPA intake level should be around 25 ng/kg/day, which is much lower than the no-observed effect level estimated by Tyl et al. 1665

---

1. S. Tyl, et al.
2. S. Sajiki, et al.
4. N. Sajiki, et al.
5. N. Tyl, et al.
mg/kg/day). However, determining more samples is required to understand the general contamination level of human milk. In addition, the quantitation of analytes in breast milk and blood provided by one individual will provide helpful information about the migration of compounds from blood into breast milk.

It has been reported that phenolic compounds mostly exist as glucuronides in biological samples.18 In this study, the very low concentration of BPA and NP may be caused by their metabolism. Not only unconjugated targets, but also conjugated metabolites should be measured for an exact risk assessment to humans. It is our expectant subject to establish an analytical method for metabolites by using a β-glucuronidase treatment.

In conclusion, the proposed alkaline digestion method was successfully applied to the determination of BPA and NP in human milk. Moreover, it can be used as a reference for evaluating other developed simple methods.

### References