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Biological monitoring of bisphenol A with HLPC/FLD and LC/MS/MS assays☆

Bitna Yi [a], Changsung Kim [b], Mihi Yang [a,*]

[a] Sookmyung Women's University College of Pharmacy, Seoul 140-742, Republic of Korea
[b] Korea Testing & Research Institute, Gimpo 415-873, Republic of Korea

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ABSTRACT

Biological monitoring is a necessary process for risk assessment of endocrine disrupting chemicals (EDCs), particularly, bisphenol A (BPA), in breast milk, because its human risks are not clear yet, and infants, who feed on breast milk, are highly susceptible for EDCs. Concerning biological monitoring of BPA, the HPLC/FLD has been widely used before the LC/MS/MS. However, there was no report, which simultaneously evaluated the two methods in real analyses. Therefore, we analyzed BPA with LC/MS/MS and HPLC/FLD in human breast milk and conducted comparison of two methods in analyzed BPA levels. After establishing optimal condition, e.g. linearity, recovery, reproducibility and free BPA system, we analyzed BPA levels in human breast milk samples ($N = 100$). The LOQs were similar in the two methods, i.e. 1.8 and 1.3 ng/mL for the HPLC/FLD and LC/MS/MS assays, respectively. There were strong associations between total BPA levels with the two methods ($R^2 = 0.40$, $p < 0.01$), however, only 11% of them were analyzed as similar levels with 15% CVs. In addition, the detection range of BPA was broader in the HPLC method than the LC/MS/MS method. However, the BPA levels in the HPLC/FLD analysis were lower than those in the LC/MS/MS analysis ($p < 0.01$). Thus, the differences in BPA levels between the two methods may come from mainly over-estimation with the LC/MS/MS method in low BPA samples and some of poor resolution with the HPLC/FLD in high BPA samples.

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1. Introduction

Endocrine disrupting chemicals (EDCs) have been known to interfere with endocrine systems by mimicking, blocking, and triggering actions of hormones and implicated with toxic effects, e.g. disorders in development and reproduction [1,2]. Bisphenol A [BPA, 2,2-bis (4-hydroxyphenyl) propane] is one of EDCs with a weak estrogenic activity. Many *in vitro* and *in vivo* studies have suggested a number of adverse health outcomes from BPA exposure, e.g. decreased sperm production, increased prostate gland volume, and altered development, vaginal morphology and estrous cycles [3–5]. Particularly, maternal exposure to BPA has been emphasized for second generation's health risks, e.g. genital malformations, testicular abnormalities, impairment in fertility or sexual functions [6–8]. In a case of breast milk, which is the main route of exposure to environmental chemicals for breast-fed infants, it should be monitored to know present status of maternal exposure to BPA and estimate the risk of BPA in breast-fed infants.

BPA is used to manufacture polycarbonate plastic and epoxy resins, and widely used for a variety of applications such as baby feeding bottles, food-can lining and sealants in dentistry [9]. Such an extensive use of BPA results in widespread human exposure in general population. We previously reported that the most of the subjects, who were not occupationally exposed to BPA, have detectable levels of BPA in their urine [10]. In addition, several studies have reported the occurrence of BPA in human breast milk. Sun et al. found BPA in the breast milk of all 23 healthy women at a range of 0.28–0.97 μg/L with a mean concentration of 0.61 μg/L [11]. Another study with 101 colostrum samples detected BPA at a range of 1–7 μg/L with a mean level of 3.41 μg/L [12].

Breast milk is a complex matrix that contains various substances, e.g. lipids, proteins, carbohydrates, minerals, and vitamins [13]. Therefore, breast milk samples should be processed with intensive preparation procedure. In our study, we conducted the liquid–liquid extraction in order to avoid matrix effects for analyses. In addition, a general problem with the interpretation of the results on BPA is background contamination of samples, which may interfere with quantification at low concentrations. Therefore, we replaced plastic wares with glassware throughout the entire analytic procedure in order to avoid possible BPA contamination. Moreover, blank tests, which were conducted with water instead of breast milk, were conducted at every experiment to confirm an absence of BPA contamination in the whole of our experimental

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* Corresponding author. Tel.: +82 2 2077 7179; fax: +82 2 710 9871.
E-mail address: myang@sm.ac.kr (M. Yang).

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process. Furthermore, we analyzed both free and total BPA. Most of BPA is metabolized into conjugated forms [14]. Generally, free BPA is thought as contamination in experimental processes.

With the development of the analytical technologies, a number of analytical methods have been developed during the last few years for determination of BPA in human bio-specimens including breast milk [11,15,16]: The analytical methods include ELISAs (enzyme-linked immunosorbent assays), single trace chromatographic separations such as HPLC/FLD (high performance liquid chromatography) with fluorescence detection, HPLC/ED with electrochemical detection, and lipid chromatography mass spectrometry (LC/MS/MS). As MS assays monitor intensity of several fragments or transitions during the chromatographic separation, they are speedy and specific. However, cost for installing and maintaining the instruments is expensive and keeps them from being widely used. On the other hand, the HPLC/FLD assays have been widely used for BPA analysis with suitable sensitivity before uses of the LC/MS/MS [17], because HPLC is more affordable to install and maintain than LC/MS/MS. In addition, there was no report, which simultaneously evaluated the two methods in real analyses rather than reviewing [14]. Therefore, we analyzed BPA with both LC/MS/MS and HPLC/FLD assays in human breast milk and evaluated efficacy if the two methods for BPA biological monitoring.

2. Materials and methods

2.1. Standards and reagents

BPA and β-glucuronidase (Helix pomatia, H1) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Internal standard, BPB, was purchased from Tokyo Kasei Chemical (Tokyo, Japan). HPLC grade acetonitrile (ACN), 2-propranol, and ethylacetate were purchased from Fisher (Fair Lawn, NJ, USA).

The enzyme solution for hydrolysis was prepared by dissolving β-glucuronidase (H-pomatia Type-1, 577,900 U/g solid) in 0.2 M sodium acetate buffer (pH 5.0).

2.2. Preparation of standards

Initial BPA stock solution was prepared by dissolving BPA in 60% ACN. Standards were generated by serial dilution of the initial stock solutions with formula milk powder. BPA levels of the spiked standards were 0.96–120 μg/L. All standard solutions were aliquoted and stored at −80 °C prior to be used.

2.3. Collection of breast milk samples

We collected 5 mL of breast milk from 100 volunteers, who lived in Seoul, Korea and delivered babies within 2 weeks. All subjects filled out informed consents to participate in this study and donated 5 mL of breast milk into capped brown glass-bottle. Collected breast milk specimens were stored at −80 °C prior to analyses. All experimental procedures were approved by the Institutional Review Boards of Inje University Paek Hospital.

2.4. Preparation of breast milk samples

To determine BPA levels in breast milk, we modified our previous method [18]. In brief, 4 mL of each milk sample was separated into 2 parts for free BPA and total (free and conjugated) BPA. In brief, 100 μl of 5 μM BPB as an internal standard, 120 μl of 2.0 M sodium acetate (pH 5.0), and 48 μl of β-glucuronidase (2784 U) were added to 2 mL of each milk sample. The mixture was incubated in water bath at 37 °C for 5 h. After incubation, we added 4 mL of 2-propranol to the mixture and extracted with a shaker (EYELA cute mixer CM-1000, Tokyo Rikakikai Co., Tokyo, Japan) for 10 minutes with maximum speed. The mixture was centrifuged (3000 *g*, 20 min) and its 3 mL of supernatant was transferred to a new glass tube. The above extraction was repeated. Total 6 mL of the transferred supernatant was evaporated in an evaporator (Savant Automatic Environmental Speedvac System AES1010, ThermoSavant, Holbrook, NY) for 2 h and the residue was dissolved in 60% ACN. The solution was centrifuged (16,000 *g*, 10 min) and the supernatant was transferred to a vial for LC/MS/MS or HPLC/FLD analyses. To determine concentrations of the free form of BPA, we followed the above procedures without enzyme.

2.5. Instrumentation and assay condition

The HPLC system consisted of dual Younglin SP930D pumps (Younglin, Seoul, Korea), a MIDAS COOL auto sampler (Spark Holland, Emme, The Netherlands), a Jasco FP-2020 plus Fluorescence Detector (Jasco, Great Dunmow, UK), and XTerra C18 column (5 μm, 4.6 mm × 250 mm, Waters, Wexford, Ireland). The injection volume of a prepared milk sample was 50 μl. The analyses were carried out with the gradient mode: mobile phase A, water; B, ACN; flow rate was 1.0 mL/min, ratio of A to B, 0–30 min, 70:30 to 50:50; 30–40 min, 50:50 to 0:100; 40–45 min, 0:100; 45–50 min, 0:100 to 70:30; 60–75 min, 70:30. BPA fluorescence was monitored at an excitation of 225 nm and emission of 305 nm. The peak area ratio of BPA to internal standard, BPB, was used for quantification.

The LC/MS/MS system was composed with Waters alliance 2695 XELC/MS/MS (Waters, Watford, UK), Zobax SB-C18 (5 μm, 4.6 mm × 250 mm, Agilent, USA). Separation was accomplished with a gradient mode: mobile phase A, water; mobile phase B, ACN; flow rate 0.3 mL/min, ratio of A to B, 0–3 min, 70:30; 3–4 min, 70:30 to 95:5; 4–6 min, 95:5 to 100:0; 6–25 min, 100:0; 25–30 min, 100:0 to 70:30. This method utilized ESI-LC/MS/MS operating in MRM mode. The Waters alliance 2695 Quattro Premier XE was used in negative ion ESI. The ESI settings were following: capillary voltage, 3.5 kV; cone voltage, 40 V; flow of desolvation gas (Argon gas), 800 L/h; flow of cone gas, 20 L/h; collision energy, 20 V.

2.6. Calibration curve

Calibration curves were prepared by processing various concentrations, i.e. 0.98, 4.8, 24, and 120 ng/mL of BPA working standards by diluting stock standards in formula milk.

2.7. Recovery and reproducibility

The recovery of BPA to see matrix and extraction effects was calculated with comparison between the spiked standards into milk and into water with or without extraction.

Reproducibility was evaluated among BPA-spiked standards into milk. The above 4 concentrations of BPA, 0.96–120 ng/mL, were used to test recovery and reproducibility ($N = 5$ at each BPA concentration).

2.8. Limit of detection (LOD) and limit of quantification (LOQ)

In the case of the HPLC/FLD method, LOQ was determined from the graph between BPA amounts (0.96–600 ng/mL) and coefficients of variance (CV). For the LC/MS/MS method, LOD and LOQ were calculated with signal to noise ratio 3 and 10, respectively.

2.9. Blank test

In order to avoid any BPA contamination in the analysis system, plastic wares were excluded throughout the entire analytic proce-

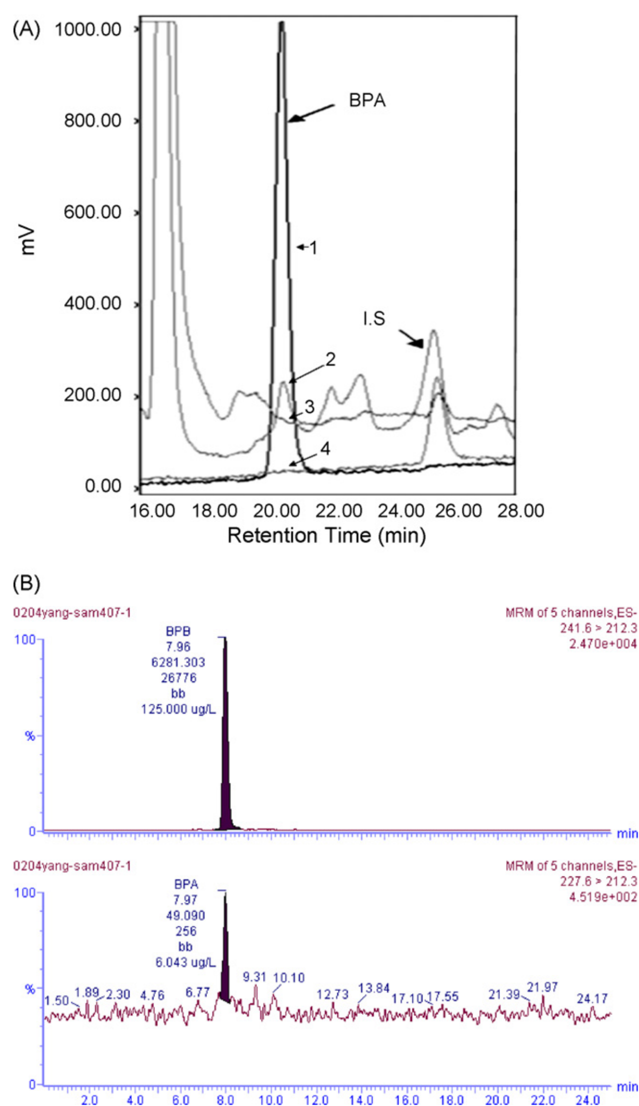

Fig. 1. Profiles of BPA in human breast milk with the HPLC/FLD (A) and LC/MS/MS (B) methods: A, 1, BPA standard (100 μg/L) in 60% ACN; 2, 24 μg/L of BPA-spiked breast milk; 3, a BPA-non-detected breast milk sample; 4, blank (IS, Internal Standard, 62.3 μg/L of BPB). B, upper, 125 μg/L of BPB(IS); lower, 6.043 μg/L of BPA.

dure, and replaced with glass wares. At every lot of experiment, we conducted blank tests ($N = 2$), which were prepared with water instead of breast milk, to confirm an absence of BPA contamination in the whole experimental processes.

2.10. Statistical analysis

A regression analysis and a pair analysis were used to study the similarity and difference in BPA levels between the two methods, respectively. All statistical analyses including histograms, and skewness were conducted with JMP Version 4 (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

BPA profiles of the two assays were shown in Fig. 1: Two-fold short retention time of BPA reflects high-throughput characteristics in the LC/MS/MS method than the HPLC/FLD method. The HPLC/FLD assay, which we have performed for several years, also had proper characteristics for analyses of BPA.

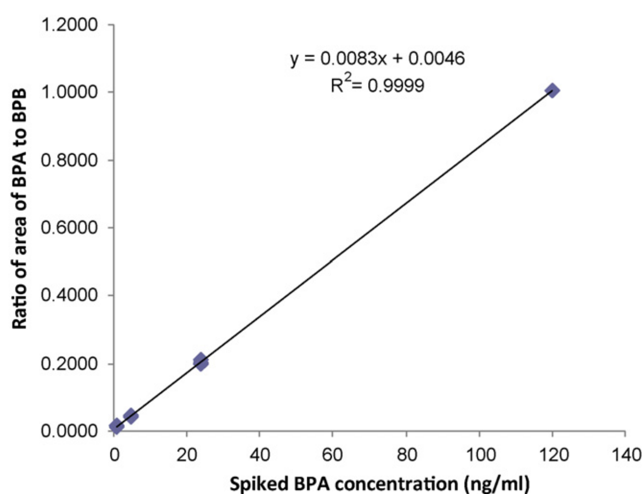

Fig. 2. A calibration curve between BPA-spiked concentrations (0.96–120 μg/L) into milk and area ratio of BPA to BPB in the HPLC/FLD method ($N = 5$ at each concentration).

3.1. Linearity

The linearity between the responses and BPA concentrations were examined with calibration curves. As per results, the HPLC/FLD method showed a good linearity from 0.96 to 120 μg/L of BPA (Fig. 2). The LC/MS/MS method also had a proper linearity within the above range ($r^2 = 0.996$).

3.2. Recovery and reproducibility

The recovery of BPA in the HPLC assay was in the range 65–82% (Table 1). Reproducibility of the method was measured with CVs and the CVs were below 15%. These levels were similar to those in the LC/MS/MS (recovery range, 68–82%; CVs < 15%).

3.3. LOD and LOQ

After establishing the proper recovery and reproducibility within 0.96–120 μg/L of BPA (Table 1) and calibration curves (Fig. 2), we obtained following LODs and LOQs. In the case of the HPLC/FLD method, LOQ was 1.8 μg/L at the 10% CV and the LOD was calculated as: LOD = LOQ/3, 0.6 μg/L. For the LC/MS/MS method, LOD and LOQ were 0.39 and 1.3 μg/L, respectively.

Table 1
Recovery and reproducibility.

A. The HPLC/FLD assays[a]

BPA concentration (μg/L)		Recovery[b] (%)	Coefficient of variance (%)	
Spiked	Detected		Intra-day	Inter-day
0.96	1.25 ± 0.14	65.58 ± 28.05	11.73	13.22
4.8	4.69 ± 0.12	82.63 ± 2.26	2.51	9.87
24	23.92 ± 0.69	78.11 ± 5.89	2.87	3.12
120	120.69 ± 0.13	82.39 ± 2.97	0.11	1.34

B. The LC/MS/MS assays

BPA concentration (μg/L)		Coefficient of variance (%)	
Spiked	Detected	Intra-day	Inter-day
0.96	0.87 ± 0.12	13.73	14.58
4.8	4.56 ± 0.27	5.89	8.95
24	22.59 ± 0.50	2.23	9.26
120	123.81 ± 1.57	1.27	7.30

[a] $N = 5$ for each concentration.
[b] $N = 5$ for each spiked concentration into milk and into 60% ACN.

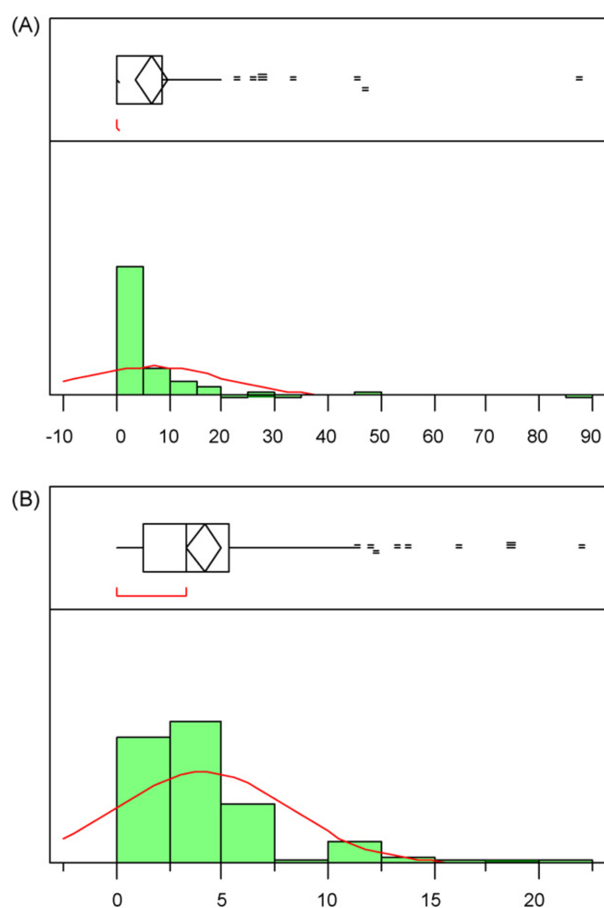

Fig. 3. Histograms of conjugated BPA levels with the HPLC/FD (A) and the LC/MS/MS methods (B): A, skewness = 3.63; B, skewness = 1.97; Upper parts show an outlier box plot with the square in the box showing the interquartile range.

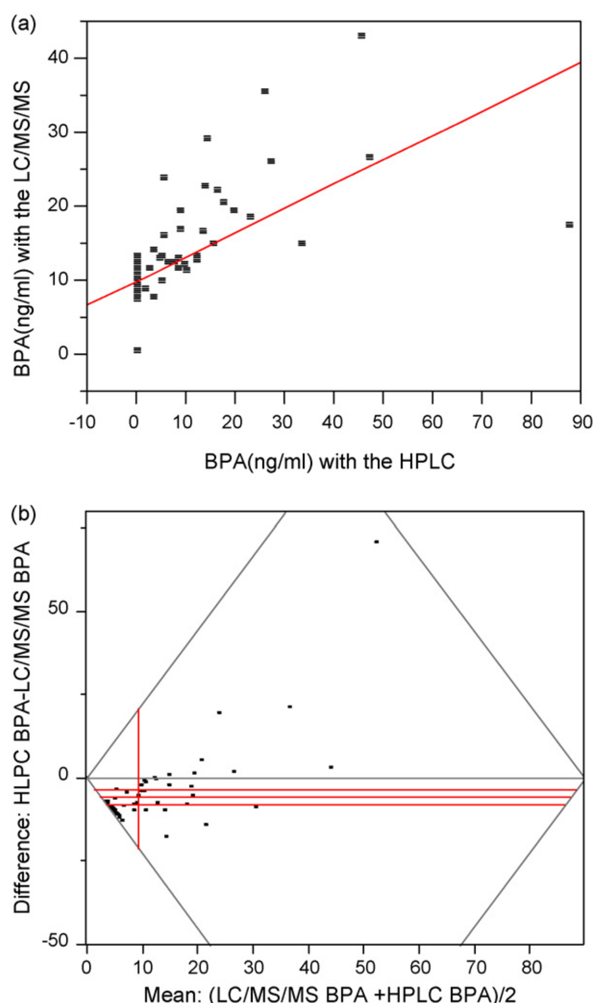

Fig. 4. Similarity and difference between the two methods: (A) Association between total BPA levels (μg/L) with two different methods: $R^2 = 0.40$, $p < 0.01$. (B) Difference between total BPA levels (μg/L) with two different methods: mean difference, −5.49 μg/L; correlation, 0.63; $p < 0.01$.

3.4. Blank tests

We established BPA free condition in the whole analysis system and started to analyze BPA in the breast milk samples. Fig. 1A shows BPA profiles with a blank test in the HPLC/FLD assay.

3.5. Comparison of BPA levels between the HPLC/FLD and the LC/MS/MS methods

Each breast milk sample was analyzed with the two methods. As per results, the frequencies of detection and median levels were higher in the LC/MS/MS method than those in the HPLC/FLD method (Table 2). In addition, we compared between distributions of conjugated formed BPA, which we chose the exposure biomarker for BPA, in the two methods (Fig. 3): The BPA levels in the LC/MS/MS method were somewhat broad compared to those in the HPLC/FLD method. That is, the LC/MS/MS method obtained various values for the samples, which were not detectable (N.D.) in the HPLC method. Thus, the detection rate of total BPA in all samples was higher in the LC/MS/MS method than the HPLC/FLD analysis. In the case of free forms of BPA, they were not detected in any breast sample with the HPLC/FLD analysis, but detected in all of breast samples with the LC/MS/MS analysis. Considering similar LODs Between the two methods, we suspect that the above difference may result from false positives in the LC/MS/MS method.

After a regression analysis, we found a strong association between total BPA levels with two different methods (Fig. 4A). In detail, the detection range of BPA was broader in the HPLC method than the LC/MS/MS method due to some outliers. However, the BPA levels under 10 ng/mL were somewhat higher in the LC/MS/MS methods than the HPLC methods. After the further matched pair analysis, we found that the BPA levels in the HPLC/FLD were lower than those in the LC/MS/MS (Fig. 4B). Thus, as mentioned above, low levels of BPA may be overestimated in the LC/MS/MS, however, some outliers, which showed higher levels of BPA in the HPLC method, reflect the drawback of the HPLC/FLD, i.e. poor resolution for other compounds, which have similar characteristics with BPA in the LC and fluorescence.

Table 2
Comparison of BPA levels in breast milk between the HPLC/FD and the LC/MS/MS methods.

Method	Form of BPA	Frequency of detection (%)	Range (μg/L)	Median (μg/L)
HPLC/FD	Total	80	N.D.–87.7	N.D.
	Free	0	N.D.	N.D.
	Conjugated	80	N.D.–87.7	N.D.
LC/MS/MS	Total	100	0.65–42.6	10.4
	Free	100	0.65–29.9	6.6
	Conjugated	100	0–22.1	3.3

N.D., non-detectable.

When we simply estimate the environmental exposure to BPA among the infants, who feed 0.5 L of breast milk per day and had 3.24 kg of body weight (b.w.) with the bio-monitored BPA levels in breast milk, 3.3 ng/mL (median of conjugated BPA with the LC/MS/MS: Table 2), the exposure level of BPA is 0.51 μg/kg b.w./day. This level is approximately 20,000-fold lower than the BPA-TDI, 50 μg/kg b.w./day [19], therefore, current maternal exposure to BPA in Korea seems to be safe for the breast milk feeding infants.

4. Conclusion

Biological monitoring is a necessary process for surveillance as well as risk assessment of environmental toxicants. Particularly, end points of BPA on human health are not clearly understood, yet. Thus, continuous biological monitoring of BPA is a unique method for protection of unknown health risks. Our group has followed the HPLC/FLD method for BPA biological monitoring and developed quite reliable method. However, the HPLC/FLD method has several drawbacks, e.g. long running time and overestimation or false positives. On the other hand, the LC/MS/MS method becomes popular to overcome the drawbacks of the HPLC method, i.e. short running time and accuracy of identification. In this study, we performed the comparison between the two methods. At first, the analyzed BPA levels with the HPLC/FLD method are confirmed with the LC/MS/MS method. However, there is some possibility of false positives in both methods: Among the samples which were N.D. with the HLPC/FLD assay, the LC/MS/MS assay may have some false positives, because the LC/MS/MS- analyzed BPA levels were various and mostly bigger than LOQ of the HPLC/FLD assays (Fig. 4). In the case of the HPLC/FLD, it also may have false positives in some samples, which were N.D. with the LC/MS/MS and provide some higher levels than the LC/MS/MS does. Only 11% of the total BPA levels showed the similar values, which had lower 15% CV. Therefore, we should carefully consider the merits and drawbacks of the two methods.

In conclusion, we have analyzed BPA with the HPLC/FLD method in various bio-species for biological monitoring. In this study, we compared its efficacy in breast milk samples to that of the LC/MS/MS method. Even though there was a strong positive association between BPA levels, which were analyzed with the two methods, the BPA levels in the HPLC/FLD were lower than those in the LC/MS/MS. Thus, to avoid error in biological monitoring of BPA, we recommend severe guidelines for identification of BPA in the LC/MS/MS method and confirmation of BPA identification with the LC/MS/MS method, particularly in high levels of BPA, which were obtained with the HPLC method.

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