ENVIRONMENTAL CHEMICAL CONTAMINANTS

Comparison of DAD and FLD Detection for Identification of Selected Bisphenols in Human Breast Milk Samples and Their Quantitative Analysis by LC-MS/MS

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Abstract

Background: Determination of bisphenols released from packaging material is undoubtedly a difficult and tricky task, requiring the chemical analyst to develop an individual approach to obtain reliable analytical information.

Objective: QuECHERS (Quick, Easy, Cheap, Effective, Rugged, and Safe)/dispersive solid-phase extraction (d-SPE) technique and high performance liquid chromatography (HPLC) coupled with modern detection techniques such as diode-array detector (DAD), fluorescence detector (FLD) or tandem mass spectrometry (MS/MS) for the determination of bisphenols such as bisphenol A (BPA), bisphenol S (BPS), bisphenol F (BPF), bisphenol B (BPB), 2-[4-[4-(4-Oxiran-2-ylmethoxy)phenyl]propan-2y]l]phenoxy] methyl]oxirane (BADGE), 3-[4-[4-(Oxiran-2-ylmethoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol (BADGE*H2O), 3-[4-[4-(2,3-Dihydroxypropoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol (BADGE*2H2O), 1-Chloro-3-[4-[4-(3-chloro-2-hydroxypropoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol (BADGE*2HCl) in human breast milk samples have been performed.

Methods: For the analysis of total analytes, prior to the extraction with acetonitrile, a deconjugation step was implemented in a tube by adding 1 mL of the enzymatic solution with the β-Glucuronidase to 5 mL of sample. The mix was homogenized and incubated for 17 h at 37 °C. Ten milliliters of acetonitrile, and a QuECChERS salt packet with 4 g anhydrous MgSO4 and 1 g NaCl were added. During the d-SPE step the extract was transferred into tube with 30 mg Z-Sep and 50 mg PSA (and also...
Many compounds such as bisphenols that are introduced into the environment by human activity can influence the health of various life forms including humans. Human exposure to bisphenols occurs mainly through ingestion of canned foods and beverages, but also through their absorption by the skin and by inhalation.

In recent years endocrine disrupting compounds have become a chemical group of special concern due to their ability to interfere with the hormonal system. One of them, bisphenol A (BPA) and its analogues, e.g., bisphenol S (BPS), bisphenol F (BPF), bisphenol B (BFB), 2-[4-[2-[(4-Oxirane-2-y1methoxy)phenyl]propan-2-yl]phenoxy]methyl]oxirane (BADGE), 3-[4-[2-[4-(Oxirane-2-y1methoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diyl (BADGE*2H2O), 3-[4-[2-[4-(2,3-Dihydroxypropoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol (BADGE*H2O), 1-Chloro-3-[4-[2-[4-(2,3-Dihydroxypropoxy)phenyl] propan-2-yl]phenoxy]propane-1,2-diol, and 1-Chloro-3-[4-[2-[4-[(Oxirane-2-y1methoxy)phenyl] propan-2-yl]phenoxy]methoxy]propane-1,2-diol (BADGE*2HCl), belong to a group of xenoestrogens, which have estrogenic effects on living organisms even though they differ chemically from the estrogenic substances produced internally by the endocrine system of organisms. However, their structural similarity to natural hormones such as estradiol and diethylstilbestrol, may “mimic” the functions of hormones, “cheating” the human organism.

BPA and its analogues are important industrial chemicals used, for example, as plasticizers in polycarbonate and epoxy resins in the plastic and paper industries. Therefore, bisphenols have attracted much concern because of their widespread occurrence. More so because bisphenols may be washed off the materials surface and transferred to food or individual elements of the environment due to the interaction with food ingredients, and also by the influence of external factors.

Human biomonitoring of all exposure to bisphenol events during a lifetime, particularly during critical life stages such as fetal development and early childhood, should be considered for a reliable assessment of human risk to mixtures of bisphenols because of the increased susceptibility of the brain and other organs to estrogens during this period.

Moreover, xenobiotics, e.g., bisphenols are characterized by lipophilicity, thereby they are capable of easily passing through biological membranes and penetrating living cells, and thus may be subject to bioaccumulation in various tissues and organs.

In biological samples, bisphenols and derivatives can exist in both the conjugated and the unconjugated form. Both glucuronides and sulfates are the most common conjugates, the first one being the predominant. Therefore, methods to determine total (both free and total concentration of bisphenols) concentration require an enzymatic deconjugation using a mixture of β-Glucuronidase and sulfatase. Due to the relatively short half-life of analytes and their properties, most bisphenols may be successfully analyzed in the urine.

Human breast milk is the major or exclusive energy source for infants up to 6 months of age. Human breast milk samples are also a reflection of the internal exposure level of chemical contaminants in mothers and fetuses. Therefore, breast milk is often monitored for estimation of exposure to endocrine disrupting compounds. BPA analogues and halogenated derivatives have been as having similar or even greater toxic effects compared with those of BPA. However, there is little information on the occurrence of BPA-related compounds in human breast milk samples to date. One reason is the lack of analytical (suitably sensitive and specific) methods for the simultaneous determination of bisphenols. The reported methods to date mainly focused on the detection of BPA in human breast milk samples.

Therefore, as bisphenols can cross the placental barrier, the fetus remains exposed to these compounds. Consequently, it is important to develop more sensitive and robust analytical methods for analysis of bisphenols in biological samples. Many analytical methods for the detection of bisphenols have been developed, especially chromatographic methods. The following chromatographic methods are applied in biological samples analysis: liquid chromatography (HPLC) and ultrahigh-performance liquid chromatography (UPLC) and gas chromatography (GC) (1, 2). Nowadays chromatographic techniques coupled with modern detection techniques can find broad applications in the separation, identification, and quantification of xenobiotics (drugs and veterinary drugs, vitamins, dyes, mycotoxins, bisphenols, environmental bioindicators, allergens, and others) in different samples (1).

Analysis of bisphenols by liquid chromatography is usually carried out on octadecyl (C18) columns with mobile phases suitable for the detection method. High performance liquid chromatography coupled to mass spectrometry (HPLC-MS) or tandem mass spectrometry (HPLC-MS/MS) currently represents the most flexible and effective (with high sensitivity and selectivity) technique employed to determine chemical contaminants in many different matrices (1, 2). On-line solid phase extraction high performance liquid chromatography tandem mass spectrometry (SPE-HPLC-MS/MS) is generally preferred to analyze xenobiotics such as bisphenols, because generic and fast methods for screening purposes can be developed, and the derivatization steps needed for a GC-MS analysis can be omitted (3). MS with atmospheric pressure ionization (API) interfaces and triple quadrupole analyzers operating in the multiple reaction monitoring (MRM) mode are most often applied for detection of bisphenols. On-line SPE-HPLC-MS/MS has been applied for the determination of BPA in human breast milk samples (4, 5). Niu et al. analyzed bisphenols in human breast milk samples by UPLC-MS/MS with application of the reversed system (RP) (6). Also, the RP system was applied for LC-MS/MS determination of BPA in human breast milk samples (7).

150 mg MgSO4 for LC-MS/MS analysis. MeOH–water (20:80, v/v) were added to the dry residue and the extract was reconstituted in 150 μL. 25-fold analytes pre-concentration is achieved. Next bisphenols were identified by HPLC-DAD-FLD and quantified by LC-MS/MS equipment.

**Conclusions:** During the bisphenols HPLC-DAD-FLD analysis, from 6 min a reinforcement of 15 was used, which allowed analytes to be identified at 750 pg/mL. Application of LC-MS/MS allowed quantification of bisphenols in the range from 2.12 to 116.22 ng/mL in a total 27 human breast milk samples.

**Highlights:** First QuEChERS/d-SPE coupled with HPLC-DAD-FLD or LC-MS/MS method for the quantification of bisphenols and its analogues in breast milk Faster and cheaper alternative to traditional extraction methods The method was applied for the first biomonitoring of bisphenols and its analogues in breast milk.
However, a major drawback of the LC–MS/MS methodology is the charge competition observed in electrospray ionization, leading to questionable results especially where complex biological matrices are concerned (8, 9).

More importantly, for non-specialized, basic laboratories in developing countries, the price of GC–MS and LC–MS instruments is still prohibitive. Expensive instrumentation, time-consuming sample pre-treatment and expert personnel for operation limit the practical application of these methods (10).

Apart from these methods, HPLC-FLD has great potential for simultaneous determination of multiple bisphenols due to its simple operation and low cost. A more simple technique, LC-FLD has found interesting applications related to the determination of mixtures of bisphenols and/or diglycidyl ethers in food, and environmental and biological samples (11–13). Xiong et al. described simultaneous determination of selected bisphenols in human milk samples with application of HPLC-FLD (14).

Sample preparation is a critical step in complex biological matrices analysis, such as in human breast milk samples, because it has a multifarious role related to target analyte extraction, pre-concentration and clean-up from co-existing compounds. In the literature, there are few reports on the application of the QuEChERS approach for the analysis of bisphenols in human breast milk samples to date. However, to our knowledge, only Niu et al. (6), Tuzimski et al. (15), and Deceuninck et al. (16) have studied human breast milk levels of BPF and/or BPs. The study described by Tuzimski et al. (15) optimized the QuEChERS-based sample preparation procedure for selected bisphenols analysis in human breast milk samples by HPLC-DAD and liquid chromatography coupled with triple-quadrupole mass spectrometry (LC-QqQ-MS). In the experiments described (15) during the d-SPE step zirconium-based sorbents (50 mg Z-Sep or 45 mg Z-Sep and 5 mg Z-Sep Plus) were applied (15).

Optimization and application of QuEChERS/d-SPE coupled with the HPLC-DAD (and LC-QqQ-MS) method for the simultaneous determination of bisphenols (A, S, F, B, BADGE and derivatives) in milk samples from a can and human breast milk samples was described by Tuzimski and Szubartowski (17). In the described study, six different salts including Primary and Secondary Amines (PSA), Z-Sep, Z-Sep Plus, C18, EMR-Lipid, Chitin and their mixtures during d-SPE step, were evaluated for the removal of matrix interferences and the recovery of bisphenols (17).

In another paper, the presence of BPA and its analogues such as BPF and BPS in a total of 120 human breast milk samples was described by Duval et al. (18).

The purpose of this study was to develop a sensitive enough method for the simultaneous determination of selected bisphenols (BPA, BPS, BPF, BPB, BADGE and derivatives) in human breast milk samples. In the method developed, samples were prepared using the QuEChERS extraction technique and analyzed with HPLC–DAD-FLD. The economic benefits and wide use of HPLC–DAD-FLD were combined with the environmentally-friendly QuEChERS/d-SPE extraction technique for sample pre-treatment. Also, to our knowledge, comparison of DAD and FLD detection for analysis of selected bisphenols in human breast milk samples is described here for the first time. This method could be applied for screening analysis of selected bisphenols in large numbers of human breast milk samples. The application of LC-MS/MS allowed quantitative analysis of bisphenols in nanograms per milliliter in 27 samples.

Materials and Methods

Chemicals and Reagents

Analyte standards

Standards for the bisphenols under investigation, such as bisphenol S (BPS, No. 1), bisphenol F (BPF, No. 2), bisphenol A (BPA, No. 3), [3-[4-[2-[(3R,4R)-4-(Oxiran-2-ylmethoxy)phenyl]propan-2-yl]phenoxy]propane-1,2-diol (BADGE-H2O, No. 4), bisphenol B (BPB, No. 5), 1-Chloro-3-[4-[2-[4-[3-(chloro-2-hydroxypropoxy)-phenyl] prop-2-yl]phenoxy]propan-2-ol (BADGE-2HCl, No. 6), 2-[4-[2-[4-[3-(Oxiran-2-ylmethoxy)phenyl]propan-2-yl]phenoxy]methyl]oxirane (BADGE, No. 7), 3-[4-[2-[4-[2-(3-Dihydroxypropoxy)phenyl]prop-2-yl]phenoxy]propane-1,2-diol (BADGE-2H2O, No. 8) were obtained from Sigma–Aldrich (Bellefonte, PA, USA). The standard purity indicated by the manufacturers for all of the reference standards of bisphenols was ≥98.0%.

Solvents and mobile-phase solutions

LC-MS/MS grade methanol (MeOH), acetonitrile (MeCN), and ammonium acetate were obtained from E. Merck (Darmstadt, Germany). LC-MS/MS grade water was purchased from Sigma-Aldrich (St. Louis, MO, USA). Also, the Deionized water (0.07–0.09 mS cm−1) was obtained by means of the Hydrolab System (Gdansk, Poland) in our laboratory.

The polypropylene material used for sample analysis and reagents (including QuEChERS) were checked previously for BPA contamination and its analogues. All glassware was cleaned with methanol prior to the analysis. Moreover, quality control blanks were periodically prepared and analyzed. All solvents were checked for the presence of the target analytes before use.

Individual stock standard solutions were prepared in methanol and were stored in stock capped glass tubes at 3 ± 2°C in the dark. A bisphenols standard mixture containing all the analytes was prepared by combining suitable aliquots of each individual standard stock solution and diluting them with methanol and was stored at 3 ± 2°C for up to 2 weeks. This mixture was used for calibration preparation, as well as for fortification of the breast milk samples.

Enzymatic standard and solutions

β-Glucuronidase from Helix pomatia type H1 was obtained from Sigma Aldrich (St. Louis, MO, USA). The enzymatic solution was prepared weekly by dissolving the β-Glucuronidase purified powder in 1M ammonium acetate (acetic acid was added to pH = 5) to obtain a solution of 3500 U/mL.

QuEChERS salts and sorbents

Sodium chloride (NaCl) and anhydrous magnesium sulfate (MgSO4) were obtained from POCH (Gliwice, Poland). Single-packed sorbents, such as primary secondary amine (PSA) and Z-Sep, which were obtained from Sigma–Aldrich (Bellefonte, PA, USA) were used to prepare their sets of mixtures during the d-SPE stage.

Human Breast Milk Sample Collection

Human breast milk samples were obtained from 27 patients from the Department of Obstetrics and Pathology of Pregnancy, Medical University of Lublin, Poland. Sample collection was conducted from May to June in 2019. After collecting their breasts with abundant clean water, samples were collected by the mothers in a glass container using a BPA-free breast pump. All samples were collected in the glass bottles and immediately, on
a regular basis, analyzed or frozen immediately at –8°C until analysis. This study was approved by the ethics committee of the Medical University of Lublin, Poland (No. KE-0254/271/2018).

**Sample Preparation of Human Breast Milk Samples**

Concerning the analysis of unconjugated analytes, after the thawing and shaking the sample (5 mL human breast milk) was transferred into a polypropylene centrifuge tube (50 mL, checked as free of BPA and its analogues).

For the analysis of total bisphenols, prior to the extraction with acetonitrile, a deconjugation step was implemented in a tube by adding 5 mL of sample and 1 mL of the enzymatic solution with β-Glucuronidase (3500 U/mL). The mix was homogenized and incubated for 17 h at 37°C.

Next, 10 mL of acetonitrile was added and the tube was shaken vigorously for 1 min. Then QuEChERS salts such as 4 g anhydrous MgSO4 and 1 g NaCl were added and the tube shaken for 1 min. After shaking and centrifugation (6000 rpm, 3480 rcf, 10 min), the total acetonitrile layer (~6–8 mL) was isolated in a polypropylene tube and stored in fridge (45 min at –8°C), followed by evaporating to dryness, and reconstituting in 1.2 mL acetonitrile.

After the start of the preparation sample (d-SPE step), the solid residue was discarded and the extract was transferred into a 15 mL polypropylene tube with 50 mg primary secondary amine (PSA) and 30 mg Z-Sep (and also 150 mg MgSO4 for LC-MS/MS analysis). The tube was then shaken for 1 min, stored in fridge (10 min), and centrifuged for 5 min (6000 rpm, 3480 rcf). Next the tube was stored in fridge (15 h), and then centrifuged twice for 5 min (6000 rpm, 3480 rcf). The acetonitrile supernatant (~800 µL) was obtained with a pipette and evaporated to dryness. A MeOH–water (20:80, v/v) mixture was added to the dry residue and the extract was reconstituted in the same mixture (150 µL) and analyzed by HPLC-DAD-FLD or LC-MS/MS. The flow-chart of the procedure is presented (Figure 3).

**HPLC-DAD-FLD**

**DAD detection**

An Agilent Technologies 1200 HPLC system with a quaternary pump was used for the LC analysis. Analytes were separated using a Scherzo SM-C18 150 × 4.6 mm column, with a 3-µm particle size (Imtakt, Portland, OR, USA). The column was thermostatted at 22°C. A mobile phase was used consisting of 50 mM HCOOH in water (component A) and 50 mM HCOOH in acetonitrile (component B). Gradient elution at 0.4 mL/min mobile phase flow was applied. The gradient program was as follows: 0–10 min from 40% eluent B to 100% B; 10–16 min isocratic 100% B. Final samples were injected onto the column using a Rheodyne manual injector with a 20 µL loop.

Detection was carried out simultaneously at five different wavelengths (260, 265, 270, 275, and 280 nm). Identification of analytes was accomplished on the basis of their retention times and by comparison between the UV spectra of the reference compounds in the chromatograph and the UV spectra of the detected peaks of the human breast milk samples.

**FLD detection**

Detection was carried out simultaneously at four different excitation wavelengths (225, 230, 235, and 240 nm). The emission wavelength was set at 300 nm. Identification of analytes was accomplished on the basis of their retention times and by comparison between the UV spectra of the reference compounds in the chromatograph and the UV spectra of the detected peaks of the human breast milk samples.

### Table 1: Validation Data Including Retention Time, Optimal Wavelength, Calibration Curve and Range, LOD, and LOQ for Bisphenols in Human Breast Milk Samples Obtained Using the Proposed QuEChERS-HPLC-DAD-FLD Procedure and the QuEChERS-HPLC-DAD Procedure in a Published Paper (17)

| No. | Analyte          | Calibration curve | LOD, ng/mL | LOQ, ng/mL | Calibration curve | LOD, ng/mL | LOQ, ng/mL |
|-----|------------------|-------------------|------------|------------|-------------------|------------|------------|
| 1   | BPS              | ~9.1              | 0.0063     | 0.9717     | 250–2000          | 0.4926     | 2.5367     |
| 2   | BPF              | ~10.35             | 0.0036     | 0.986     | 250–2000          | 0.4926     | 2.5367     |
| 3   | BPA              | ~11.75             | 0.0036     | 0.986     | 1500–2000         | 0.4926     | 2.5367     |
| 4   | BADGE+H2O        | ~13.15             | 0.0036     | 0.986     | 1500–2000         | 0.4926     | 2.5367     |
| 5   | BADGE*H2O        | ~14.5              | 0.0036     | 0.986     | 1500–2000         | 0.4926     | 2.5367     |
| 6   | BADGE*2H2O       | ~15.9              | 0.0036     | 0.986     | 1500–2000         | 0.4926     | 2.5367     |
| 7   | BPA              | ~8                 | 0.75–60    | 1.4853     | 250–2000          | 0.4926     | 2.5367     |
| 8   | BADGE*H2O        | ~11.5              | 0.75–60    | 1.4853     | 250–2000          | 0.4926     | 2.5367     |
| 9   | BADGE*2H2O       | ~13.5              | 0.75–60    | 1.4853     | 250–2000          | 0.4926     | 2.5367     |
| 10  | BADGE*H2O        | ~15.5              | 0.75–60    | 1.4853     | 250–2000          | 0.4926     | 2.5367     |

For this concentration range, BADGE*2H2O was not detected using a DAD detector.

For this concentration range, BPA was not detected using FLD detector.
chromatograph and the UV spectra of the detected peaks of the human breast milk samples.

Quantitative analysis in this regard was carried out on the basis of a seven single-point calibration, in which response factors were calculated as amount-to-area ratios of the analytes in the calibration sample and used in the analyte-concentration calculation in fortified samples.

Method Optimization and Validation

HPLC method validation

The standard calibration curves of the analytes were constructed by plotting analyte concentration against peak area. Bisphenols standards prepared as solutions in methanol were prepared at seven concentrations in the range from 0.75 to 60 ng/mL for HPLC-DAD-FLD (Table 1) for systems in the conjugated form (with incubation with β-Glucuronidase) and injected in triplicate under the same chromatographic conditions. The calibration curves of bisphenols under investigation showed satisfactory linearity and correlation between concentration and peak area over the studied range with the determination coefficient, \( R^2 \), >0.9947. Method sensitivity was evaluated based on limits of detection (LOD) and quantification (LOQ). LODs were calculated using following formula (Equation 1):

\[
\text{LOD} = 3.3 \frac{\text{SD}}{S}
\]  

(Equation 1)

and the LOQs for all analytes were calculated using following formula (Equation 2):

\[
\text{LOQ} = 10 \frac{\text{SD}}{S}
\]  

(Equation 2)

where: SD is the standard deviation of y-intercept of regression lines (calculated using the LINES function in MS Excel 2010), and S is the slope of the calibration plot. Retention times \( t_{R} \) and full calibration data including LODs and LOQs are presented in Table 1.

LC-MS/MS analysis

An Agilent 6540 UHD Accurate-Mass Q-TOF mass spectrometer (Agilent Technologies, USA) equipped with a 1260 Infinity LC system (Agilent Technologies, Germany) was used. The LC-MS/MS system was controlled by MassHunter software. LC separation was carried out on an Agilent ZORBAX Eclipse XDB-C18 (50 x 1.8 mm). Eluent A was an aqueous ammonium acetate (5 mM/L; pH = 4.0) and eluent B was an acetonitrile-ammonium acetate buffer (5 mM/L; pH 7.9) (9:1, v/v). The linear gradient was used with a flow rate of 0.4 mL/min and the following elution profile: 0–13 min, from 20 to 95% B; 13-13.1 min, from 95–100% B; 13.1–14 min isocratic with 100% B; 14.1–25 min, isocratic with initial conditions. The column oven was set to 40°C and the injection volume was 5 µL. The JetStream source was operated in negative ESI mode using the following source parameters: drying gas: (temp. 300°C, flow rate: 10 L/min), nebulizer: - 40 psi; ion spray voltage - 4000 V; sheath gas flow: (temp.300°C, flow rate 12 L/min).

Recovery and precision studies for LC-MS/MS analysis

An attempt was made to determine recoveries using MS detection. When it was possible to obtain 15 mL of milk from one patient, the human breast milk sample was divided into three parts, each of 5 mL. One 5 mL sample was blank, the other 5 mL sample was enriched with a mixture of bisphenols standards, the third 5 mL sample was analyzed, and analytes were identified in this sample.

Human breast milk samples were spiked with the bisphenols under investigation at following concentration level: 50 ng/mL for LC-MS/MS analysis. Samples were fortified with the appropriate volume of the working standard mixture. Recovery
studies were performed on the basis of three replicates from the spiking procedure (n = 3) at each concentration level. Relative standard deviations expressed as a percentage (RSD, %) were calculated for all of the analytes. LOQs were set as the minimum spiking level (ng/mL) that can be quantified with acceptable accuracy and precision.

**Results and Discussion**

**Chromatographic Conditions**

Chromatographic conditions applied during the experiments were preliminarily elaborated by the authors previously (15, 17). Chromatographic separation using a Scherzo SM-C18 column provided satisfactory results for a wide range of bisphenols. The applied gradient elution program allowed appropriate separation of the analytes under investigation in a single chromatographic run, as presented in Figures 1 and 2, and Table 1. Gradient elution at 0.4 mL/min mobile phase flow was applied. The gradient program was as follows: 0–10 min from 40% eluent B to 100% B; 10–16 min isocratic 100% B. Detector FLD: from 6 min a reinforcement of 15 was used.

In a previous study (17), HPLC-DAD was applied in the experiments allowing determination and quantitation of the analytes at the nanogram per milliliter level of the milk samples. Instrumental LODs and LOQs were from 53 to 306 ng/mL and from 159 to 926 ng/mL; and from 142 to 693 ng/mL and from 430 to 2102 ng/mL for unconjugated and conjugated analytes with /-Glucuronidase, respectively (17).

The purpose of this study was to compare two types of detection, such as DAD and FLD, of selected bisphenols. The applied gradient elution program allowed appropriate separation of the analytes under investigation in a single chromatographic run, as presented in Figures 1 and 2, and Table 1.
Figure 2. Continued.
Optimization of Excitation Wavelength and Emission Wavelength

The excitation and emission wavelengths were two factors decisive among the instrument parameters for sensitivity, so they were optimized in single factor experiments. Experiments to address the relationship between peak height, and excitation and emission wavelengths were designed. A standard mixture of the selected bisphenols was analyzed using different excitation and emission wavelengths. After the first step of experiments, the excitation and emission wavelengths were chosen. Wavelengths of 235 nm was chosen as optimal for excitation, and 300 nm for emission (when peak heights for the bisphenols were greatest).

At these wavelengths: 235 nm for excitation and 300 nm for emission, the range of peak heights for the analyzed bisphenols was at a maximum and the best sensitivity was obtained. A comparison of six chromatograms at different concentration standard mixtures of the bisphenols with application FLD detection (0.75, 1.5, 3, 6, 12, and 60 ng/mL) as presented in Figure 2.

The FLD detector allows the amplification of analyte signals (in the range from 0 to 18). During the bisphenol analysis, from the sixth minute of analysis, a reinforcement of 15 was applied, which allowed analytes to be identified at 750 pg/mL.

Sample Preparations and Optimization of the QuEChERS-Based Procedure

The flowchart of the procedure is presented (Figure 3). In a published studies (15, 17), we applied the QuEChERS approach for bisphenol residue extraction from human breast milk samples. To obtain satisfactory extraction efficiency and sample purification should be adjusted individual stages of procedure. Therefore, during the QuEChERS/d-SPE extraction technique, the following factors should be optimized such as solvent type and volume matched to the sample amount and its kind; salts type and amount, buffer additives, extract volume for clean-up and d-SPE sorbent type and amount.

In published experiments (17) we evaluated, for this purpose, various sorbents like PSA, Z-Sep, Z-Sep Plus and C18. Also we tested EMRL-Lipid and Chitin which were recommended to remove lipids. As reported (17), during optimization of the dispersive-solid phase (d-SPE) clean-up step, negligible matrix interference was observed for most of the analytes due to application of 50 mg PSA and 30 mg Z-Sep. As described also earlier, the main function of PSA is removal of co-extracted constituents such as fatty acids, ionic lipids, and sugars, whereas Z-Sep Plus and Z-Sep are used to adsorb the majority of fatty non-polar interferences (17).

The introduction of pre-concentration steps before chromatographic analysis led to an increase in sensitivity of the whole analytical procedure. During the elaborated procedure, a 25-fold analytes pre-concentration is achieved (Figure 3). Compared to the published procedure in the previous paper (17), the d-SPE stage has been slightly modified. For even better purification of samples from interferences, after the first centrifugation with salts such as Z-Sep and PSA (and also 150 mg MgSO4 for LC-MS/MS analysis), the samples were stored for 15 h in a refrigerator (4 ± 2 °C) and then centrifuged twice (6000 rpm, 3480 rcf, twice for 5 min). The described extraction procedure was assessed in terms of recovery and repeatability, and method LODs and LOQs were established.

Application of the Procedure to the Identifications of Bisphenols in Human Breast Milk Samples

The HPLC-DAD-FLD method was applied to the identification of bisphenols in human breast milk samples. Application of the HPLC-DAD-FLD conditions described in Materials and Methods allowed proper separation and identification of the selected bisphenols. The FLD detector allows the amplification of analyte signals (in the range from 0 to 18). During the bisphenol
Figure 4. Example chromatogram of human breast milk samples showing detected bisphenol residues.

Figure 5. Example correlation between UV spectra of analytes and library bisphenols standards in human breast milk sample.

Table 2. Validation data (LC-MS/MS) including retention time, range and calibration curve, $R^2$, and method LOQs for selected bisphenols in human breast milk samples obtained after the proposed QuEChERS-HPLC-DAD-FLD procedure. Recovery rates and SDs for selected bisphenols from human breast milk samples were obtained using LC-MS/MS

| No. | Analyte | $t_R \pm SD$, min | Range, ng/mL | Calibration curve | $R^2$ | LOQ, ng/mL | Fortified level, ng/mL | Recovery, % | SD, % |
|-----|---------|-------------------|--------------|------------------|-------|------------|------------------------|-------------|-------|
| 1   | BPS     | 5.938 ± 0.029     | 66–330       | $y = 78954x + 876853$ | 0.9995 | 53         | 50                     | 113.9       | 7.2   |
| 2   | BPF     | 7.974 ± 0.004     | 33–330       | $y = 242x + 11131$ | 0.9996 | 25         | 50                     | 41.5        | 6.3   |
| 3   | BPA     | 9.399 ± 0.046     | 66–330       | $y = 171.92x + 17358$ | 0.9989 | 35         | 50                     | 42.4        | 5.4   |
| 4   | BPB     | 10.393 ± 0.028    | 33–330       | $y = 257x + 3006$  | 0.9999 | 32         | 50                     | 90.7        | 5.3   |

*For other bisphenols, the average recoveries were less than 40%.
application, from 6 min a reinforcement of 15 was used, which allowed analytes to be identified at 750 pg/mL. An example of a chromatogram of a biological sample showing detected bisphenol residues is presented in Figure 4. Suitable matches between UV spectra of the residues identified in biological samples and library standards were observed (Figure 5).

Application LC-MS/MS After QuEChERS/d-SPE Procedure to the Quantitative Analysis of Bisphenols in Human Breast Milk Samples

Although HPLC-DAD-FLD provides valuable information for the separation and quantification of compounds present in biological samples, the use of conventional approaches based on
absorption/emission spectra and retention time is often limited when samples contain very similar compounds. Modern high-performance chromatographic techniques combined with other detectors are applied for the structural profiling and elucidation of active compounds. These techniques (such as MS) are often necessary for structure identification.

For quantitative determination of bisphenols in human breast milk samples, the LC-MS/MS conditions were optimized. Validation data (LC-MS/MS) including retention time, optimal wavelength, calibration curve and range, $R^2$, and method LODs and LOQs for bisphenols in breast milk samples obtained after the proposed QuEChERS-HPLC-DAD-FLD procedure are presented in Table 2.

Quantitative analysis in this regard was carried out on the basis of a seven-point calibration, in which response factors were calculated as amount-to-area ratios of the analytes in the
Figure 7. Chromatograms showing the determination of bisphenols in breast milk samples, which were enriched with a mixture of bisphenols standards, and analyzed by LC-MS/MS. Chromatographic conditions in the description of Figure 6.
calibration sample and used in the analyte-concentration calculation in fortified samples.

Sufficient sensitivity MS detection allows was achieved applying these conditions, with LOQs ranging from 25 to 53 ng/mL (Table 2). Recovery studies conducted at a spiking level of 50 ng/mL (Table 2) proved that the elaborated QuEChERS/d-SPE extraction procedures, and applications of MS/MS detection, support the possibility of BPS, BPF, BPA, and BPB residue determination of bisphenols in human breast milk samples.

Chromatographic separation using an Agilent ZORBAX Eclipse XDB-C18 (50 × 4.6 mm; 1.8 μm) column provided satisfactory results for a selected of bisphenols. The applied gradient elution program allowed appropriate separation of the selected analytes under investigation in a single chromatographic run, as presented in Figure 6 and Table 2. The mobile phase consisted of aqueous ammonium acetate (component A, 5 mM/L; pH = 4.0) and an acetonitrile–ammonium acetate buffer (component B, 5 mM/L; pH 7.9) (9:1, v/v). The linear gradient was used with a flow rate of 0.4 mL/min and the following elution profile: 0—13 min, from 20 to 95% B; 13—13.1 min, from 95—100% B; 13.1—14 min isocratic with 100% B; 14:1—25 min, isocratic with initial conditions.

When it was possible to obtain 15 mL of milk from one patient, the human breast milk sample was divided into three parts, each with 5 mL. One 5 mL sample was blank, the other 5 mL sample was enriched with a mixture of bisphenols standards (Figure 7), the third 5 mL sample was analyzed, and analytes were identified in this sample.

Twenty-seven samples were analyzed utilizing extraction and chromatographic conditions described in Materials and Methods of the paper. Successful purification of extracts utilizing PSA, Z-Sep and 150 mg MgSO4 as well as presence of enrichment steps in the extraction procedure, allowed determination of bisphenols at a low nanogram per milliliter level.

The Agilent 6540 UHD accurate-mass quadrupole time-of-flight (Q-TOF) mass spectrometer is an accurate mass MS/MS instrument based upon quadrupole and time-of-flight technology.

Concentrations of identified bisphenols residues ranged from 2.12 to 116.22 ng/mL in human breast milk samples (Table 3). Residues of BPS were detected in all 27 samples but below the LOQ. Residues of BPF and BPA were detected in few samples but also below LOQs of bisphenols. Residues of BPB were detected in all 27 samples. Content of BPB in 5 mL of human breast milk was in the range of 10.6 to 581.1 ng (2.12–116.22 ng/mL, \( n = 27 \)).

**Table 3.** BPS, BPF, BPA, and BPB residues identified in human breast milk samples conducted from May to June 2019 from 27 patients at the Department of Obstetrics and Pathology of Pregnancy, and Department of Gynaecological Oncology and Gynaecology, Medical University of Lublin, Poland by LC-MS/MS

| Breast milk sample | BPS  | BPF  | BPA  | BPB  |
|--------------------|------|------|------|------|
| 1                  | <LOQ<sup>a</sup> | —<sup>b</sup> | —    | 22.7 |
| 2                  | <LOQ  | —    | —    | 10.6 |
| 3                  | <LOQ  | —    | —    | 31.5 |
| 4                  | <LOQ  | —    | —    | 35.1 |
| 5                  | <LOQ  | —    | <LOQ | 14.5 |
| 6                  | <LOQ  | <LOQ | <LOQ | 17.2 |
| 7                  | <LOQ  | —    | —    | 65.1 |
| 8                  | <LOQ  | —    | —    | 26.3 |
| 9                  | <LOQ  | —    | —    | 38.1 |
| 10                 | <LOQ  | —    | —    | 33.1 |
| 11                 | <LOQ  | —    | —    | 32.1 |
| 12                 | <LOQ  | <LOQ | —    | 27.1 |
| 13                 | <LOQ  | —    | —    | 69.2 |
| 14                 | <LOQ  | —    | —    | 197.1|
| 15                 | <LOQ  | —    | —    | 20.1 |
| 16                 | <LOQ  | —    | —    | 25.1 |
| 17                 | <LOQ  | —    | —    | 50.2 |
| 18                 | <LOQ  | —    | —    | 55.2 |
| 19                 | <LOQ  | —    | —    | 48.2 |
| 20                 | <LOQ  | —    | —    | 58.2 |
| 21                 | <LOQ  | —    | —    | 36.1 |
| 22                 | <LOQ  | —    | —    | 247.3|
| 23                 | <LOQ  | —    | —    | 430.1|
| 24                 | <LOQ  | —    | —    | 39.1 |
| 25                 | <LOQ  | —    | —    | 581.1|
| 26                 | <LOQ  | —    | —    | 176.1|
| 27                 | <LOQ  | —    | —    | 142.1|

<sup>a</sup> <LOQ — Bisphenols were identified in the 5 mL of sample but below the LOQ (without their quantification).

<sup>b</sup> — Bisphenols were not detected in the samples.

### Conclusions

Since human breast milk provides a dosimeter of prenatal exposure, it is considered as a biomarker of previous bisphenol maternal exposure and a target biological matrix for priority exposure assessment. HPLC-DAD-FLD and LC-MS/MS methods using QuEChERS for preconcentration was established for the simultaneous determination of selected bisphenols in human breast milk samples for the first time. Good quality results,
including recovery, precision, linearity, and LOD and LOQ were achieved. Finally, human breast milk samples collected from patients were analyzed to demonstrate the applicability of the method. Among bisphenols, BPS, BPF, BPA and BPB were quantified in samples. Proper extract purification, during d-SPE step was achieved using a mixture of 50 mg PSA, 30 mg Z-Sep and 150 mg MgSO4. Successful purification of extracts utilizing PSA and Z-Sep and 150 mg MgSO4, as well as presence of enrichment steps in the extraction procedure, allowed determination of bisphenols at low nanogram per milliliter levels. The method developed is suitable for rapid determination of trace amounts of bisphenols in human breast milk samples in routine analysis and could be used as a screening method with identification of analytes at 750 pg/mL in laboratories and by testing agencies.

To our knowledge, this is the first biomonitoring study of bisphenols in human breast milk samples in Poland with comparison of different detections techniques, such as DAD detection with much more sensitive FLD and MS/MS detection techniques. Identification and quantification of bisphenol residues in natural samples, even at a lower level then validated LOQs, confirms the usefulness of the elaborated analytical procedure.

Conflict of Interest

Tomasz Tuzimski, Szymon Szubartowski, Renata Gadzała-Kopciuch, Andrzej Miturski, Monika Wójtowicz-Marzec, Wojciech Kwaśniewski, Tomasz Gorczyca, Bogusław Buszewski declare that they have no conflict of interest. Ethical approval: this article does not contain any studies with human participants or animals performed by any of the authors. Informed consent: not applicable.

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