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A multiresidue method for the determination of selected endocrine disrupting chemicals in human breast milk based on a simple extraction procedure

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ABSTRACT

In recent decades, in parallel to industrial development, a large amount of new chemicals have emerged that are able to produce disorders in human endocrine system. These groups of substances, so-called endocrine disrupting chemicals (EDCs), include many families of compounds, such as parabens, benzophenone-UV filters and bisphenols. Given the demonstrated biological activity of those compounds, it is necessary to develop new analytical procedures to evaluate the exposure with the final objective of establishing, in an accurate way, relationships between EDCs concentrations and the harmful health effects observed in population. In the present work, a method based on a simplified sample treatment involving steps of precipitation, evaporation and clean-up of the extracts with C18 followed by ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) analysis for the determination of bisphenol A and its chlorinated derivatives (monochloro-, dichloro-, trichloro- and tetrachlorobisphenol A), parabens (methyl-, ethyl-, propyl- and butylparaben) and benzophenone-UV filters (benzophenone −1, −2, −3, −6, −8 and 4-hydroxybenzophenone) in human breast milk samples is proposed and validated. The limits of detections found ranged from 0.02 to 0.05 ng mL^{-1}. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery rates ranged from 91% to 110% and the precision (evaluated as relative standard deviation) was lower than 15% for all compounds, being within the acceptable limits for the selected bioanalytical method validation guide. The method was satisfactorily applied for the determination of these compounds in human breast milk samples collected from 10 randomly selected women.

1. Introduction

The overall development occurred in the last century has led man to have available a lot of manufactured products with a wide applicability that have significantly eased the life. Nevertheless, this massive development has brought an important inconvenient to the population: the exposure to a high variety of xenobiotics that could cause negative health effects. Among these compounds, endocrine disrupting chemicals (EDCs) have become in a special concern in the last years.

EDCs cover an important range of synthetic and natural substances able to alter the normal hormone function of wildlife and humans. The endocrine and reproductive effects of those compounds are believed to be due to their ability to mimic or antagonize the effects of endogenous hormones, such as estrogens and androgens, or to disrupt synthesis and metabolism of endogenous hormones and hormone receptors [1]. Beside some naturally occurring compounds (lignans, coumestans, isoflavones, mycotoxins), numerous synthetic chemicals such as are bisphenol A (BPA) and its chlorinated derivatives, benzophenone-UV filters (BPs) and parabens (PBs) have been implicated in endocrine disruption.

Since its effects, even at very low concentrations, are more detrimental and pernicious than other EDCs, BPA has received a tremendous attention from the scientific-medical community and governments [2,3]. It is the raw material used in the manufacturing of epoxy resin and polysulfones. It is also applied as antioxidant or stabilizer. However, the most important use of BPA is the production of polycarbonate plastics for a great variety of applications such as digital media (e.g., CDs, DVDs), electrical and electronic equipment, automobiles, sports safety equipment, reusable food and drink containers, medical devices and many other products [4]. Moreover, when BPA is present in treated waters, it may react with residual chlorine originally used as disinfectant, producing chlorinated BPA derivatives depending on the pH of the medium [5]. Regarding to BPs, those compounds are used as UV filters in sunscreens to protect the skin and hair from UV irradiation as they are able to absorb UV light that is harmful to the human body in the form of

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UVA (320–400 nm) and UVB (290 to 320 nm). Finally, PBs (alkyl esters of *p*-hydroxybenzoic acid) are widely used as antimicrobial preservatives, especially against mold and yeast, in cosmetic products and pharmaceuticals, and in food and beverage processing [6].

The widespread use of BPA, PBs and BPs and their potential risk to human health have prompted interest in assessing human exposure to them. It may occur through inhalation, dermal contact or ingestion [7–10] and their metabolism may differ depending upon the exposure route [7,11]. These compounds may conjugate to β-D-glucuronide and sulfate, thus reducing their bioactivity and facilitating their urinary excretion. Although free and conjugate forms can be measured in humans, only the free forms are biologically active.

Developmental exposure to EDCs is particularly important in the first stages of life because of the increased susceptibility of the brain and other organs to estrogens during this period [12]. It has been postulated that EDCs accumulate in certain human tissues and their effects might pass to the offspring via the placenta and/or breast milk [13–17]. Breastfeeding mothers exposed to EDCs may be unknowingly exposing their children to harmful levels of these compounds. In this context, it is particularly important to develop strategies for the study of this exposure through the mother after childbirth and therefore, to develop sensitive analytical methods to monitor EDCs in human milk.

Sample preparation is a critical step in complex biological matrices analysis, such as human milk. An extraction technique is usually required to purify and isolate the target compounds. Moreover, because of the low levels of EDCs in human milk, these extraction techniques must be able to concentrate the analytes. To date, BPA and its chlorinated derivatives, PBs and BPs have been extracted from human milk using liquid–liquid extraction (LLE) [18–20], ultrasound assisted extraction (UAE) [21], off-line solid-phase extraction (SPE) [22–25] and on-line SPE [21,26–28]. In the present work, a simple and cost effective sample treatment based on a precipitation of fat and proteins followed by a clean-up using a simplification of the Quick, Easy, Cheap, Effective, Rugged & Safe (QuEChERS) methodology is proposed. QuEChERS was developed by Anastassiades et al. in 2003 for the analysis of pesticides in fruits and vegetables [29]. Since then, it has become an important and widely used technique in the analysis of multiple chemical residues, including EDCs, in a great variety of matrices. Thus, it has been used for the analysis BPA and bisphenol S in canned vegetables and fruits [30], pesticides and mycotoxins in commercial milk [31] and steroid hormones or BPA and the active metabolites of methoxychlor and vinclozolin in rat testis [32]. Recently, the efficacy of this methodology has been also proven for the extraction of organochlorine pesticides in human milk [33]. However, to our knowledge, QuEChERS has not been applied for the EDCs selected in the present work in human milk samples.

The aim of the present work was to develop a sensitive multiresidue method based on a precipitation of fat and proteins followed by a clean-up step for the simultaneous determination of free amounts of BPA and its chlorinated derivatives (monochloro-, dichloro-, trichloro- and tetrachloro-); four PBs (methyl-, ethyl-, propyl- and butylparaben) and six BPs (benzophenone-1, 2, 3, 6, 8 and 4-hidroxybenzophenone) in human milk samples. UHPLC-ESI-MS/MS has been used as detection technique. The proposed method was satisfactorily validated and applied for the determination of the free content of the above mentioned compounds in 10 human milk samples from volunteers lactating mothers who live in the province of Granada (Spain).

2. Experimental

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. PBs standards were supplied by Alfa Aesar (Massachusetts, MA, USA). Bisphenol A (BPA), tetrachlorobisphenol A (Cl_4-BPA), deuterium-labeled bisphenol A-d_{16} (BPA-d_{16}), benzophenone-UV filter standards (BPs) and deuterium-labeled benzophenone-d_{10} (BP-d_{10}) were supplied by Sigma-Aldrich (Madrid, Spain). Monochloro-, dichloro- and trichlorobisphenol A (Cl-BPA, Cl_2-BPA, Cl_3-BPA) were synthesized in our laboratory (purity > 99%) by direct chlorination of BPA [34]. Deuterium-labeled ethylparaben-d_5 (EPB-d_5) was purchased from Toronto Research Chemicals Inc (North York, Ontario, Canada). Stock standard solutions (100 μg mL^{-1}) were prepared by weighing 10 mg of each compound into a 100 mL flask. Then, acetonitrile up to the final volume was added. The solution remained stable for at least four months at 4 °C in the darkness. For calibration and validation purposes, two intermediate solutions, No. 1 and 2 (10 and 2.5 μg mL^{-1}) were prepared by diluting 1.0 and 0.25 mL respectively of the stock solution to 10 mL in acetonitrile (MeCN). Subsequently, two new intermediate solutions No. 3 and 4 (1.0 and 0.5 μg mL^{-1}) were prepared by diluting 1.0 and 0.5 mL respectively of solution No. 1 to a final volume of 10 mL in MeCN. Then, two new intermediate solutions No. 5 and 6 (0.1 and 0.05 μg mL^{-1}) were prepared by diluting 1 and 0.5 mL respectively of solution No. 3 to a final volume of 10 mL in MeCN. Finally, intermediate solution No. 7 (0.01 μg mL^{-1} was prepared by diluting 1 mL of solution No. 5 to a final volume of 10 mL in MeCN. Working standards for calibration and validation purposes were prepared by diluting 100 μL of the intermediate solutions No. 2 to 7 to a final volume of 10 mL in human breast milk. Working standards were prepared fresh from the MeCN solutions prior to the experiments.

Methanol (MeOH) and MeCN gradient grade were obtained from Merck (Darmstadt, Germany). LC-MS grade methanol and water, formic acid, ammonia (25%), zinc acetate dihydrate, phosphotungstic acid hydrate and primary-secondary amine (PSA), were also purchased from Sigma-Aldrich (Madrid, Spain). Octadecyl (C18) solid sorbent (40 μm) was supplied by J.T. Baker (Deventer, Netherlands). Glacial acetic acid (99%) was obtained from Panreac (Barcelona, Spain). The fat/proteins precipitation solution was prepared at time of use by dissolving 9.10 g of zinc acetate hydrated, 5.46 g of hydrated phosphotungstic acid and 5.8 mL of glacial acetic acid in 100 mL final volume of deionised water.

2.2. Instrumentation and software

UHPLC-MS/MS analysis was performed using a Waters Acquity UPLC™ H-Class from Waters (Manchester, UK). A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z-spray™ electrospray ionization (ESI) source was used for EDCs detection. An Acquity UPLC® BEH C18 (100 mm × 2.1 mm i.d., 1.7 μm particle size) from Waters (UK) was used as chromatographic column. A vacuum centrifugal evaporator was used to concentrate samples (LaboGene, Lynge, Denmark). MassLynx 4.1 software was used for instrument control, peak detection and integration. Statgraphics Plus version 5.0 (Manugistics Inc., Rockville, USA, 2000) was used for statistical and regression analyses.

2.3. Sample collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. Samples were anonymized, frozen at −20 °C and stored until analysis in our laboratory. The study was performed in compliance with the *Ethical Principles for Medical Research Involving Human Subjects* issued by the World Medical Association, and all volunteers signed the informed consent form.