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QuEChERS and ultra-high performance liquid chromatography–tandem mass spectrometry method for the determination of parabens and ultraviolet filters in human milk samples

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ABSTRACT

Concerns are growing about human exposure to endocrine disrupting chemicals (EDCs), especially during developmental stages. Parabens (PBs) and ultraviolet filters (UVFs) are prevalent EDCs widely used as additives in cosmetics and personal care products (PCPs). The objective of this study was to develop a method to determine four PBs and ten UVFs in human milk using QuEChERS treatment and ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS). Multivariate strategies were applied to optimize experimental parameters. Limits of quantification ranged from 0.1 to 0.2 ng mL^{-1} and inter-day variability (evaluated as relative standard deviation) from 6% to 13%. The method was validated using matrix-matched standard calibration followed by a recovery assay with spiked samples. Recovery percentages ranged from 87% to 112%. The method was satisfactorily applied to assess target compounds in human milk samples from 15 donors. This analytical procedure can provide information on newborn exposure to these EDCs.

1. Introduction

Over recent decades, humans have been exposed to numerous chemicals that can interfere with normal endocrine functioning. Exposure to these endocrine disrupting compounds (EDCs) has been correlated with uterine and ovarian diseases, breast cancer, poor semen quality, and/or testicular cancer, among other abnormalities and reproductive syndromes [1–4]. Human exposure to EDCs during pregnancy and childhood may be particularly harmful, given the specific hormonal regulation during those development periods [5]. Personal care products (PCPs) are an important source of exposure to different EDCs, which can be found in shower gels, hydrating creams, shampoos, skin protection lotions, toothpastes, perfumes, and other PCPs. This study focuses on parabens (PBs) and ultraviolet filters (UVFs).

PBs such methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB), and butylparaben (BPB) are used as antimicrobial agents for the preservation of PCPs. Evidence of the disrupting effects of some PBs has led to a modification in regulations on their utilization, with the prohibition of PBs in PCPs for children in Denmark [6] and restrictions on the use of long alkyl chain PBs (PPB and BPB) in the EU [7].

UVFs are widely used in various PCPs to protect the skin against UV radiation. Current EU regulations allow the use of 26 compounds with different chemical structures as UVFs in cosmetics [8], including benzophenone-3 (BP-3), ethylhexyl methoxycinnamate (EMC), 3-benzylidene camphor (3-BC), and octocrylene (OCR). The endocrine disrupting properties of these chemicals have been demonstrated in several *in vitro* and *in vivo* studies [9–11].

The biotransformation of EDCs depends partly on their chemical structure and exposure pathway. Most of them are transformed by organisms into β-D-glucuronide derivatives that are excreted through urine [12,13]; however, the metabolic-excretory system is not completely effective, and some EDCs can remain in human tissues [14,15].

Breastfeeding is considered the best option for avoiding disease in neonates [16], and many neonatal intensive care units coordinate with human milk banks to provide premature newborns with breast milk [17,18]. It is important to monitor the burden of EDCs (e.g., PBs and UVFs) in human milk in order to avoid or minimize EDC exposure during this critical development stage.

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Several analytical techniques have been developed to assess the EDC content in human milk samples. The most widely reported procedures are based on liquid-liquid extraction (LLE) and solid phase extraction (SPE) [19,20]. Nevertheless, recent analytical procedures have been proposed to obtain high extraction specificity and minimize the co-extraction of matrix substances. Thus, stir bar sorptive extraction (SBSE) [21], molecularly imprinted polymers (MIPs) [22], and stir-membrane solid-liquid-liquid microextraction (SM-SLLME) [23] can be remarked. In spite of their advantages, these recent procedures usually require prolonged treatment times and specific devices. These operational drawbacks are overcome by dispersive liquid-liquid microextraction (DLLME) [24], which has been used to study PBs and benzophenone-derived UVFs but not other UVFs (e.g., 3-BC or EMC). In fact, there has been scant research on the presence in human milk of many EDCs typically incorporated in PCPs, and only two studies have reported the presence of non-benzophenone UVFs, together with PBs and other cosmetic compounds [25,26]. Hence, there is a need for analytical procedures that can be rapidly and readily applied to large numbers of samples.

The QuEChERS method appears to be a good alternative option that avoids the above-mentioned drawbacks. It offers very high extraction yields for a wide range of chemicals, and the solid sorbents used in the cleaning step (polysecondary amine [PSA] or octadecylsilane [C18]) reduce the non-desired matrix burden in the final extracts. QuEChERS has been widely applied to extract pesticides, pharmaceuticals, mycotoxins, polycyclic aromatic hydrocarbons, and many other compounds in a wide variety of complex matrices [27–29]. However, it has been little used to study EDCs in humans and has never been applied for the simultaneous assessment of PBs and UVFs in human milk samples [30–32].

With this background, the main purpose of this study was to develop a method to determine four PBs and ten UVFs in human milk using QuEChERS treatment and ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC–MS/MS). The method was validated and then applied to 15 human milk samples from anonymous donors.

2. Materials and methods

2.1. Chemicals and reagents

All reagents were analytical grade unless otherwise specified. Water (18.2 MΩ cm) was purified using a Milli-Q system from Millipore (Bedford, MA). Ethylhexyl dimethyl p-amino benzoate (EDP), 3-benzylidene camphor (3-BC), 4-methylbenzylidene camphor (MBC), ethylhexyl p-methoxycinnamate (EMC), octocrylene (OCR), benzophenone-1 (BP-1), benzophenone-3 (BP-3), benzophenone-6 (BP-6), benzophenone-8 (BP-8), 4-hydroxybenzophenone (4-OH-BP), methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB), and butylparaben (BPB) were supplied by Sigma-Aldrich (Madrid, Spain). The purity of these standards was ≥99%. Isopropyl p-amino benzoate (IsPP), isobutyl cinnamate (IsBC), ethyl 2-cyano-3,3-diphenylacrylate (ECDA), labeled deuterium benzophenone (BP-d_{10}), and ethylparaben ring $^{13}C_6$ labeled (EPB-$^{13}C_6$) were also purchased from Sigma-Aldrich (Madrid, Spain). The structural formula of these compounds is shown in supplementary material (Fig. S1). Stock standard solutions of compounds (100 mg L^{-1}) were prepared in acetonitrile and stored at 4 °C in the dark. The solutions were stable for at least four months. Working standards were prepared by dilution with acetonitrile immediately before use.

β-glucuronidase/sulfatase (*Helix pomatia*, H1) was purchased from Sigma-Aldrich and prepared daily by dissolving 10 mg of β-glucuronidase/sulfatase ($3{\cdot}10^6$ U g solid^{-1}) in 1.5 mL of 1 M ammonium acetate/acetic acid buffer solution (pH 5.0). Methanol, ethanol, acetone, and acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Sodium chloride, magnesium sulfate, polysecondary amine sorbent (PSA), and octadecylsilane sorbent (C18) were purchased from Sigma-Aldrich (Madrid, Spain). Ammonium acetate, LC–MS grade acetonitrile and water, ammonia (25%), and formic acid were also purchased from Sigma-Aldrich.

2.2. Instruments and software

UHPLC–MS/MS analysis was performed with an Agilent Series 1290 LC system (Agilent Technologies, Santa Clara, CA) and an API 4000 (triple quadrupole) mass spectrometer (AB SCIEX). Statgraphics Plus version 5.0 (Manugistics Inc., Rockville, MD) was used for statistical analyses.

2.3. Sample collection and storage

Human milk samples were collected from 15 donors attending the Human Milk Biobank of Granada (Granada University Hospital Complex, Spain). All volunteers signed the informed consent form. The study was approved by the Institutional Ethical Committee of the hospital. Samples were coded and stored at −86 °C until chemical analysis.

2.4. Enzymatic treatment

A previously published procedure was used with minor modifications [24]. Briefly, 1 mL of milk sample was placed in a glass centrifuge tube and spiked with 10 μL of surrogate standard solution (IsPP, IsBC, ECDA, EPB-$^{13}C_6$, and BP-d_{10}; 4000 ng mL^{-1}), giving a concentration of 40 ng mL^{-1} milk for each surrogate. Then, 15 μL of the enzyme solution was added, and the sample was incubated at 37 °C for 24 h (temperature and time conditions are reported elsewhere [33,34]).

2.5. Sample treatment

Two and a half milliliters of acetonitrile were poured into each of the enzymatic treated samples, followed by 30 s of mechanical agitation (vortex) and the addition of a mixture of 150 mg NaCl and 150 mg $MgSO_4$ to promote salt-assisted liquid-liquid extraction (SALLE). After manual shaking for 60 s and centrifugation at 4000 rpm for 5 min, the extract was transferred to a conical glass tube.

A clean-up step was then performed using dispersive solid-phase extraction (d-SPE). A mixture of 250 mg PSA, 50 mg C18, and 25 mg $MgSO_4$ was added to the extract followed by manual agitation for 60 s and centrifugation at 4000 rpm for 10 min, drying the supernatant under nitrogen stream. The residue was dissolved in 100 μL of acetonitrile-water (70:30 v/v), vortexed for 30 s, and centrifuged at 13000 rpm for 5 min. The sample was then ready for injection into the LC system.

2.6. Chromatography and mass spectrometry conditions

The instrumental conditions were previously reported [35]. Chromatographic separation of compounds was carried out using a Gemini C18 column (100 mm × 2 mm i.d., 3 μm particle) from Phenomenex (Torrance, CA, USA). Injection volume was 10 μL, and column temperature was maintained at 20 °C. Two different conditions were applied according to the analytes selected. UVFs were separated using an acidified mobile phase consisting of 0.1% formic acid solution in acetonitrile:water 10:90 (solvent A) and 0.1% formic acid solution in acetonitrile: water 90:10 (solvent B). The gradient program was: 0.0–1.0 min, 30% B; 1.0–10.0 min, 30–70% B; 10.0–11.0 min, 70% B; back to 30% in 0.1 min; 11.1-12.5 min, 30% B. Flow rate was 0.700 mL min^{-1}. PBs were separated using a gradient mobile phase consisting of 0.025% (v/v) aqueous ammonia

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