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Stir-membrane solid–liquid–liquid microextraction for the determination of parabens in human breast milk samples by ultra high performance liquid chromatography-tandem mass spectrometry

Rocío Rodríguez-Gómez[a], Mercedes Roldán-Pijuán[b], Rafael Lucena[b], Soledad Cárdenas[b], Alberto Zafra-Gómez[a], Oscar Ballesteros[a], Alberto Navalón[a], Miguel Valcárcel[b,*]

[a] Research Group of Analytical Chemistry and Life Sciences, Department of Analytical Chemistry, Campus of Fuentenueva, University of Granada, E-18071 Granada, Spain
[b] Department of Analytical Chemistry, Institute of Fine Chemistry and Nanochemistry, Marie Curie Building, Campus of Rabanales, University of Córdoba, E-14071 Córdoba, Spain

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ABSTRACT

In this article, stir-membrane solid–liquid–liquid microextraction (SM-SLLME) is tailored for the analysis of solid matrices and it has been evaluated for the determination of parabens in l breast milk samples. A three-phase microextraction mode was used for the extraction of the target compounds taking advantage of their acid–base properties. The unit allows the simultaneous extraction of the target compounds from the solid sample to an organic media and the subsequent transference of the analytes to an aqueous acceptor phase. The method includes the identification and quantification of the analytes by ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). All the variables involved in the extraction procedure have been accurately studied and optimized. The analytes were detected and quantified using a triple quadrupole mass spectrometer (QqQ). The selection of two specific fragmentation transitions for each compound allowed simultaneous quantification and identification. The method has been analytically characterized on the basis of its linearity, sensitivity and precision. Limits of detection ranged from 0.1 to 0.2 ng mL^{-1} with precision better than 8%, (expressed as relative standard deviation). Relative recoveries were in the range from 91 to 106% which demonstrated the applicability of the stir-membrane solid–liquid–liquid microextraction for the proposed analytical problem. Moreover, the method has been satisfactorily applied for the determination of parabens in lyophilized breast milk samples from 10 randomly selected individuals.

1. Introduction

The alkyl esters of *p*-hydroxybenzoic acid (parabens, PBs) are a group of compounds widely used as bactericide and antimicrobial preservatives, especially against mold and yeast in cosmetic products, pharmaceuticals, and in food and beverage processing [1]. The biological activity of PBs is based on their inhibitory effects on membrane transport and mitochondrial function processes. These compounds are present, individually or in combination, in a large amount of commercial formulations. Although PBs have been considered for years to be relatively safe compounds with a low bioaccumulation potential [2], some studies suggest that they present a moderate endocrine disrupting activity and therefore they can cause adverse effects on humans and wildlife. In fact, the ability of PBs to disrupt physiologically important functions in both *in vitro* systems [3] and *in vivo* models [4–6] has been demonstrated. As well, the presence of non-metabolized PBs in breast cancer tissues [7] has focused the attention in their potential carcinogenic and toxic nature [2,6,8].

The human exposure to PBs may occur through ingestion, inhalation or dermal absorption. This exposure, estimated in 76 mg per day, involves different sources such as cosmetics and personal care products (50 mg/day), drugs (25 mg/day) or food (1 mg/day) [1]. After intake, PBs are metabolized by hydrolysis of the ester bond and by glucuronidation [9]. However, the parent compounds (free forms) can still be detected in biological samples such as urine [10], serum and seminal plasma [11] and human milk [12].

* Corresponding author. Tel.: +34 957 218 616; fax: +34 957 218 616.
E-mail addresses: qa1meobj@uco.es, qa1vacam@uco.es (M. Valcárcel).

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Since breast milk is the main route of exposure for breastfed infants, its analysis is of special scientific interest. The isolation of the target analytes from this complex biological sample is a critical aspect even when a chromatographic technique is employed due to selectivity and sensitivity issues. Selectivity aspects, including ion suppression, are also critical when mass spectrometry is employed as detection technique. Current sample preparation of breast milk typically involves a previous acid treatment and a centrifugation step [13] to release the target analytes from the sample matrix. Classic liquid–liquid extraction [14] and solid phase extraction [12] with intense cleanup procedures have been proposed for the extraction of these compounds from such samples. These classic techniques, which are usually tedious and require high volume of sample and organic solvents, are gradually being replaced by microextraction techniques, which are characterized by their simplicity, green nature and efficiency. Microextraction techniques, especially solid phase microextraction, have been proposed for the isolation of pesticides [15], polychlorinated biphenyl congeners [16] and monocyclic aromatic amines [17] from human milk samples.

Stir membrane extraction (SME) [18,19], which use a polymeric membrane as extracting phase, has been proposed as a novel technique that integrates the extraction and stirring element in the same device. The main shortcoming of SME is the limited adsorption capacity of conventional membranes which can be enhanced changing the nature of the extracting phase. The use of liquid extracting phases has brought the development of the stir-membrane liquid–liquid microextraction (SM-SLLME), both in the two-phase [20] and three-phase modes [21,22]. However, the design of the unit does not allow the processing of sample volumes lower than 10 mL which is an obvious restriction in bioanalysis. The adaptation of stir-membrane liquid phase microextraction (SM-LPME) for the extraction of volume limited biological samples, such as saliva, has also been recently published [23].

In the present work, SM-SLLME is tailored to the analysis of solid samples and it has been evaluated for the determination of PBs in lyophilized human milk samples. The unit allows the extraction of the target compounds from the solid sample to an organic media and the subsequent transference of the analytes to an aqueous acceptor phase which is compatible with ultra high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS). The new proposal gives a selectivity and sensitivity enhancement which is critical when biological samples are studied, even if a UHPLC-MS/MS system is employed. In addition, the extraction technique seems to be versatile enough to face up the isolation and preconcentration of hydrophobic ionizable compounds from different solid matrices. The method was validated and satisfactorily applied to determine the free PBs content in samples collected from 10 volunteers.

2. Experimental

2.1. Chemicals and materials

All reagents were of analytical grade or better. Methylparaben (MPB), ethylparaben (EPB), propylparaben (PPB), butylparaben (BPB) and ethylparaben $^{13}C_6$-ring labeled (EPB-$^{13}C_6$) were supplied by Sigma-Aldrich (Madrid, Spain). Stock standard solutions of each compound were prepared in acetonitrile (Panreac, Barcelona, Spain) at a concentration of 100 mg L^{-1} and stored at 4 °C in the dark. Working solutions were prepared by a rigorous dilution of the stocks in human milk samples prior to their lyophilization. Potassium hydroxide, hydrochloric acid, acetonitrile, hexane and dichloromethane, employed in the sample treatment, were purchased from Panreac (Barcelona, Spain). LC-MS grade acetonitrile and water, used as components of the chromatographic mobile phase were also purchased from Sigma-Aldrich. Polytetrafluoroethylene (PTFE) tape (75 μm, 0.5 μm of pore size) from Miarco (Valencia, Spain) and Eppendorf tube (2 mL in volume) were employed in the construction of the extraction device. Sample agitation for the extraction procedure was carried out in an eight-position digital agitator-vibrator purchased from J.P. Selecta (Barcelona, Spain).

2.2. Chromatographic analysis

Two chromatographic systems, including different detectors, were employed in the development of the present research. The optimization of the extraction procedure was carried out on a Waters-Acquity™ Ultra Performance LC system (Waters, Manchester, UK) using an Acquity UPLC® BEH C_{18} column (1.7 μm particle size, 2.1 mm × 100 mm) maintained at 45 °C. The mobile phase consisted of 60% of Milli-Q ultrapure (Millipore Corp, Madrid, Spain) water and 40% acetonitrile under isocratic conditions. The flow rate was maintained at 0.5 mL min^{-1}. The injection volume was 1 μL with partial loop with needle overfill mode. The separated analytes were detected using a PDA eλ (extended wavelength) Detector (Waters) at 254 nm. System control was achieved with Empower software.

The extraction performance is calculated in relative terms (extraction recovery and enrichment factors) and the obtained values are independent of the instrument employed. For this reason, UPLC-MS/MS was finally used as instrumental technique in order to improve the sensitivity and selectivity of the UV detection. In this sense, method validation and sample analysis were performed on a Waters Acquity UPLC™ H–Class (Waters, Manchester, UK), consisting of an ACQUITY UPLC™ binary solvent manager and an ACQUITY UPLC™ sample manager. A Xevo TQS tandem quadrupole mass spectrometer (Waters) equipped with an orthogonal Z–spray™ electrospray ionization (ESI) source was used for PBs detection. An Acquity UPLC® BEH C_{18} column (1.7 μm particle size, 2.1 mm × 100 mm) was used. The compounds were separated using a gradient mobile phase consisting of LC–MS grade water (solvent A) and LC–MS grade acetonitrile (solvent B). Gradient conditions were: 0.0–2.0 min, 40% B; 2.0–5.0 min, 40–90% B; 5.0–5.1 min, 90–100% B; 5.1–8.0 min, 100% B and back to 40% in 0.1 min. Flow rate was 0.5 mL min^{-1}. Total run time was 10.0 min. The injection volume was 10 μL and the column temperature was maintained at 40 °C.

For mass spectrometric analysis, the tandem mass spectrometer was operated in the selected reaction monitoring (SRM) mode and Q1 and Q3 quadrupoles were set at unit mass resolution. ESI was performed in the negative ion mode. The mass spectrometric conditions were optimized for each compound by continuous infusion of standard solutions (1 mg L^{-1}). The ion source temperature was maintained at 150 °C. Instrument parameters were as follows: capillary voltage, 0.60 kV; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow, 150 L h^{-1}; desolvation gas flow, 500 L h^{-1}; collision gas flow, 0.15 mL min^{-1}, and nebulizer gas flow, 7.0 bar. Nitrogen (99.995%) was used as cone and desolvation gas, and argon (99.999%) was used as a collision gas. Dwell time was set at 25 ms. Optimized parameters for each compound are also listed together with the mass transitions in Table 1.

2.3. Samples collection and storage

Human milk samples were obtained from healthy lactating women living in Granada, Spain. It is important to point out that none of them follow a medical treatment. Samples were collected using a breast pump in a 100 mL PTFE flask and immediately cold