Talanta 188 (2018) 299–307

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Phenanthrene metabolites determination in human breast and cow milk by combining elution time-emission fluorescence data with multiway calibration

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ARTICLE INFO

Keywords:
Phenanthrene metabolites
Elution time-emission fluorescence matrices
Multiway calibration
Human breast milk
Cow milk

ABSTRACT

Phenanthrene is the most released polycyclic aromatic hydrocarbon into the environment by anthropogenic action. Because of the absorption and biotransformation pathways, this compound is metabolized and the most abundant metabolites are hydroxylated derivatives, such as 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene, which are excreted through biological fluids, included mammals milk. For the resolution and quantitation of co-eluted analytes, elution time-emission fluorescence matrices were analysed with different second-order calibration algorithms: n-way and unfolded partial least squares, both coupled with residual bilinearization (N-PLS/RBL and U-PLS/RBL), and multivariate curve resolution-alternative least squares (MCR-ALS). Once optimized the chromatographic parameters, in isocratic mode, the elution time was of 5.5 min. The second-order data were obtained exciting at 250 nm, with an emission range from 330 to 430 nm, each 1 nm, and elution time from 0 to 5.5 min each 5.4 s. The ranges for the second-order multivariate methods in validation samples were from 1.0 to 9.0 ng mL^{-1} for 1-, 2-, 3- and 4-hydroxyphenanthrene, and from 5.0 to 45.0 ng mL^{-1} for 9-hydroxyphenanthrene. Root mean square errors of prediction between 0.45 and 1.82 ng mL^{-1} (relative errors of prediction 7–22%) were obtained. The optimized procedures were applied in the analysis of human breast milk and in whole and semi-skimmed commercial cow milk. N-PLS/RBL and U-PLS/RBL algorithms show satisfactory results for the five metabolites with recoveries ranging between 82% and 115%.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants generated by the incomplete combustion of organic materials. Moreover, although they are not chemically synthesized for industrial purposes, they are used as intermediaries in pharmaceuticals, agricultural products and other chemical industries [1]. Human exposure to combustion products, containing mixtures of PAHs, has long been associated with cancer occurrence [2]. PAHs are lipid soluble and would be absorbed from the gastrointestinal tract of mammals, transferred through lungs and distributed in tissues and/or accumulated in adipose tissues. The metabolism, via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation, yield hydroxylated metabolites that are excreted in feces, urine or milk [3]. The metabolites of PAHs are used as biomarkers to assess exposure to PAHs [4]. Until now, 1-hydroxypyrene (1OHPy) and 3-hydroxybenzo[a] pyrene (3OHBaP) have been proposed to be the most representative biomarkers of global exposure to PAHs [5–7]. However, in a recent study, it is recommended that total body burden of PAHs should not be assessed exclusively by 1OHPy biomonitoring. The study is concluding that, as detected in biological fluids, the hydroxy-PAHs derivatives are also representative of contamination of biological matrices [8].

The US Environmental Protection Agency [9] has established a list of priority pollutants including 16 PAHs, being phenanthrene (Ph) one of them. The hydroxymetabolites of phenanthrene (OHPhs), appear to be particularly useful for PAHs biomonitoring and, specifically, 1-, 2-, 3-, 4- and 9-hydroxyphenanthrene (1-OHPh, 2-OHPh, 3-OHPh, 4-OHPh and 9-OHPh). These OHPhs (Fig. 1) have been proposed as the main markers of exposure to phenanthrene [10,11]. Of the phenanthrene metabolites, 1- and (2 + 3)-OHPhs appear to be particularly useful for biomonitoring studies and, in urine samples, represent more than 50% of the total OHPAHs measured [12]. Recently, a HPLC method for the simultaneous analysis of 2-hydroxyfluorene (2-OHF), various hydroxyphenanthrene metabolites, (1 + 9)-, (2 + 3)-, and 4-OHPh, 1-

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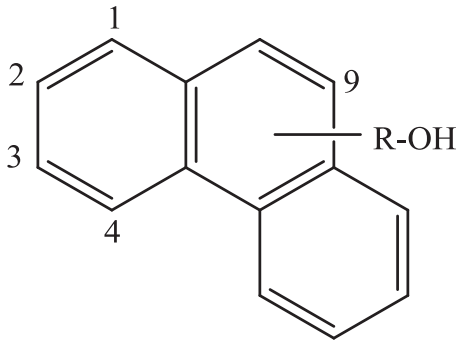
https://doi.org/10.1016/j.talanta.2018.05.096
Received 4 April 2018; Received in revised form 24 May 2018; Accepted 28 May 2018
Available online 30 May 2018
0039-9140/



1-Hydroxyphenanthrene (1-OHPh)

2-Hydroxyphenanthrene (2-OHPh)

3-Hydroxyphenanthrene (3-OHPh)

4-Hydroxyphenanthrene (4-OHPh)

9-Hydroxyphenanthrene (9-OHPh)

Fig. 1. Chemical structures for hydroxyphenanthrene (OHPh) metabolites.

hydroxypyrene (1-OHPy) and 3-hydroxybenzo[a]pyrene (3-OHB[a]Py) in human urine, has been proposed [13].

Although the principal elimination routes of OHPAHs are through biological fluids, their transference to mammals' milk is also taken place. Because of this, the determination of the above-mentioned metabolites in cow and breast milk has a great significance, due to their correlation with environmental or indoor contamination.

Several techniques are available for the analysis of OHPhs in milk samples, mainly liquid chromatographic methods with fluorescence detection [7,14,15]. Moreover, gas chromatographic-mass detection methods, with previous chemical derivation, have been also proposed to detect and analyse Ph and OHPhs [16–18]. In one of these methods [16], 1-, 2-, 3-, 4-, and 9-OHPh were analysed in milk of lactating goats, among other matrices. The proposed method includes enzymatic hydrolysis, liquid-liquid extraction, and Envi-Chrom P SPE column purification. Capillary electrophoresis has been also used to analyse some OHPAHs in milk, including 3OHPh and 9OHPh [19].

The main disadvantages of the reported methodologies are the relatively long chromatographic elution times, and the incomplete chromatographic separation between phenanthrene metabolites, in most cases. Grova et al. [16] achieved a complete resolution of the five metabolites by GC-MS but, after the pretreatment of the samples for the protein precipitation and analytes isolation, it is necessary a derivatization of the extracts previous to the chromatographic analysis. As indicated, studies about the determination of OHPhs in milk samples from mammals are scarce, but studies in breast milk are even more limited, being focused on analysing the most abundant PAHs according to the United States Environmental Protection Agency (US EPA) list [20–24]. Although food consumption is the major route of human exposition, no exhaustive information on infants exposure via breastfeeding is available actually [25]. In addition, in some regions where the high concentration of atmospheric PAHs was assessed, mothers are also exposed via inhalation of polluted air [25]. However, procedures for the analysis of PAH metabolites in breast milk have not been described to date.

According to the revised literature, HPLC proposed methods allow the analysis of only one or two metabolites of Ph. Methods for the simultaneous determination of the five OHPhs, in only one chromatographic run and using fluorimetric detection, have not been reported. Taking into account that these analytes show overlapped chromatographic signals and practically identical fluorescence profiles, the use of multivariate calibration approaches opens a possibility for the quantification of all of them. In this sense, the combination of chromatographic data with spectroscopic techniques, such as HPLC with UV–vis diode-array detection (DAD) or fast-scanning luminescence detection (FSLD), is able to yield spectral-time second-order data, and second-order multivariate calibration can be performed when full selectivity in the chromatographic separation is not achieved. Applications of second-order calibration to chromatographic data, with diode array (DAD) and/or fast scanning fluorescence detection (FSLD), have been recently revised [26]. Data about the use of FSLD for generating second-order-chromatographic data are very scarce. Different second-order multivariate calibration algorithms, applied to LC data with fluorimetric detection, have been compared for the analysis of four fluoroquinolones in urine [27]. Multivariate curve resolution-alternating least squares (MCR-ALS) was applied to resolve the co-elution of fluorene, pyrene and benzo[b]fluoroanthene with some interferences in smoked paprika samples [28]. MCR-ALS has been also proposed for the quantification of sex hormones in water and sediments [29], and for the determination of marker pteridines in urine samples [30].

The aim of the present paper is focussed in two ways: first, to investigate the combination between three-way multivariate calibration approaches and HPLC data in combination with a fast-scanning spectrofluorometer as detector, for the simultaneous determination of hydroxyphenanthrene metabolites; second, application of the optimized methodology for the analysis of 1-OHPh, 2-OHPh, 3-OHPh, 4-OHPh and 9-OHPh in breast and cow milk samples. The second-order data, based on elution time-emission measurements, were processed with several multi-way algorithms, such as N-PLS/RBL, U-PLS/RBL and MCR-ALS.

2. Material and methods

2.1. Chemicals and reagents

2-, 3-, and 9-hydroxyphenanthrene (2-, 3-, 9-OHPh) pure solids and individual solutions of 1-, and 4-hydroxyphenanthrene (1-OHPh, 4-OHPh), at 10 $\mu g\,mL^{-1}$ in acetonitrile, were obtained from LGC-Dr Ehrenstorfer GmbH (LGC Standards, SLU, Barcelona). Individual stock standard solutions of solid analytes were prepared in acetonitrile at concentrations of 50 $\mu g\,mL^{-1}$ and stored at −4 °C in darkness. Working solutions were prepared by dilution of the appropriate aliquots with acetonitrile. Acetonitrile (ACN), HPLC-grade, was purchased from Panreac (Barcelona, Spain). β-glucuronidase (30 U/mL)/aryl sulphatase (60 U/mL), from Helix pomatia aqueous solution, was provided by Merck (Barcelona, Spain).

Ultrapure water provided by a Milli-Q purification system (Millipore S.A.S., Molsheim, France) was used. Solvents and samples, used to perform the chromatographic technique, were filtered through 0.22 μm hydrophobic fluoropore filters (PTFE) before each injection.

2.2. Instrumentation and software

The chromatographic studies were carried out on an Agilent 1100 High Performance Liquid Chromatograph equipped with degasser, quaternary pump, column oven, autosampler Agilent 1290 infinity thermostated at 5 °C, fast scanning fluorescence detector (FSLD) and the ChemStation software to control de instrument, data acquisition and data analysis. The analytical column used was a Tracel Excel ODS-B column (2.1 × 100 mm; 3 μm, Teknokroma Analítica, Spain). The column temperature was set at 20 °C. The mobile phase consisted in a mixture of acetonitrile and water (45:55, v/v) in isocratic mode. The flow rate was 0.4 $mL\,min^{-1}$ and the injection volume was 5 μL.

Data matrices were collected in the chromatographic system with a FSLD, in multiemission mode, using a wavelength emission range from 330 to 430 nm, each 1 nm, and elution times from 0 to 5.5 min, every