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On-line SPE chromatography with spectrophotometric diode array detection as a simple and advantageous choice for the selective trace analysis of benzo(a)anthracene degradation products from microalgae

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ABSTRACT

In this work, a methodology based on on-line solid phase extraction (SPE) chromatography with spectrophotometric diode array detection was optimized and validated for the trace analysis of benzo(a)anthracene dihydrodiol degradation products from microalgae cultures 5,6-dihydrodiol, 8,9-dihydrodiol and 10,11-dihydrodiol. The two on-line methods for the constituents of the culture, an SPE/on-line SPE chromatographic method for liquid medium and a matrix solid phase dispersion (MSPD)/on-line SPE chromatographic method for biomass presented good linearity in the ranges of 0.5–47 ng mL^{-1} and 2–80 ng mg^{-1} of samples, respectively, with correlation coefficients $r > 0.99$. The percent relative standard deviation (RSD%) values were ≤4.9%. For the liquid medium and biomass methods, the global recoveries were between 84% and 90% and between 67% and 78%, and the limit of detection LODs were ≤0.3 ng mL^{-1} and ≤0.8 ng mg^{-1} respectively. The methodology was applied to exposure bioassays, and for the first time the three metabolites were detected and quantified individually by their appearance in the biomass and when they were excreted into the liquid medium. The metabolite formed in the greatest amount was 10,11-dihydrodiol, and the maximum production of all metabolites was at 6 h of exposure. This work contributes to the study of the degradation route of BaA, which has not been elucidated for microalgae until now.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental contaminants characterized by a structure made up of carbon and hydrogen atoms forming two or more fused aromatic rings. Low molecular weight PAHs having two or three aromatic rings, such as naphthalene, phenanthrene and anthracene, are more easily degraded and rapidly volatilized than higher molecular weight PAHs, which are more stable and toxic, persisting in the environment due to their low volatility. All PAHs are non-polar compounds with a highly lipophilic nature, but high molecular weight PAHs are more stable and toxic than low molecular weight PAHs [1,2]. Although there are various physicochemical methods used to remove PAHs from our environment, they have many limitations. However, biological methods using degrading microorganisms have a high potential for bioremediation [3]. Research about the biodegradation of PAHs by microbes is a high-interest branch of environmental research fields, and research emphasis has recently changed from finding PAHs-degrading microorganisms to metabolic pathways of microbes and genetic regulation and construction of high-efficiency engineered microorganisms [4]. Among the microorganisms capable of degrading PAHs are bacteria, fungi and algae [5,6]. However, the metabolic pathways used by these organisms are only well documented for the low molecular weight PAHs. Additionally, for most of the PAHs having four or more aromatic rings, the degradation pathways are not fully defined, especially in the case of microalgae, and therefore certain products resulting from their biodegradation are not fully known. Bacterial degradation pathways have been reported for PAHs such as pyrene (PYR), with four rings, and benzo(a)pyrene (BaP), with five rings. These studies demonstrated the production of *cis*-dihydrodiol metabolites at the first stage of biodegradation [7–10]. In the case of fungi, some metabolites of the benzo(a)anthracene (BaA) were found to be of the dihydrodiol type: the *trans*–3,4-dihydrodiol, *trans*–8,9-dihydrodiol, *trans*–10,11-dihydrodiol [11].

Although, some studies have demonstrated the ability of microalgae to degrade high-molecular weight compounds [12], the degradation

Abbreviations: PAH, Polycyclic aromatic hydrocarbon; BaA, benzo(a)anthracene; PYR, pyrene; BaP, benzo(a)pyrene; SPE, solid phase extraction; MSPD, matrix solid phase dispersion; HPLC, high-performance liquid chromatography; UV-DAD, ultraviolet diode array detector; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantification; CF, concentration factor; USEPA, United States Environmental Protection Agency; AOAC, Association of Official Analytical Chemist

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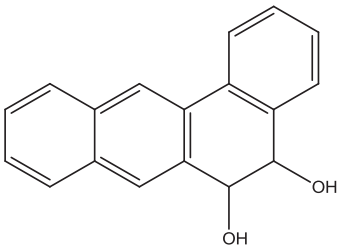
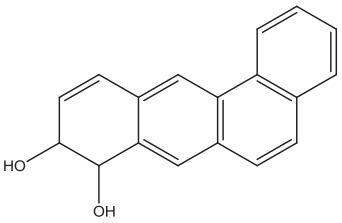
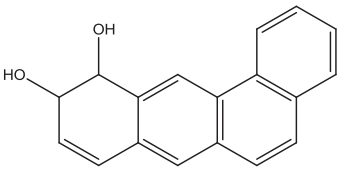
products of heavy PAHs have been documented principally for BaP, which also produces dihydrodiols (9,10-dihydrodiol, 4,5-dihydrodiol, 11,2-dihydrodiol, and 7,8-dihydrodiol) [13–15]. A more recent work addresses the microalgal degradation of a mixture of PAHs, where dihydrodiol production was also observed [16]. Certainly, the analysis of microalgae metabolites is not an easy task as their isolation must be performed with very selective analytical and/or extraction techniques because the aqueous cultures and biomass represent very complex biological matrices from the analytical point of view. Additionally, a trace enrichment step is almost mandatory because metabolites are formed in much lower quantities than their parent compounds.

For studies of the biodegradation by microalgae, it is important to consider the literature reports, which indicate that the dihydrodiol-type products should be the first focusses of the search, since these compounds seem to be formed at the early stages of microalgae degradation. Unfortunately, the detection and quantification of this type of compounds is very difficult because when they come from the same parent PAH, they are isomers of each other, which can often lead to partial and unresolved chromatographic peaks having the same chromatographic behavior. Their identification by mass spectrometry can also be difficult, because the dihydrodiol metabolites from the same parental PAH each have the same mass and generally share the same mass spectrum. For these reasons, it was necessary to be very careful in selecting the strategy concerning the extraction, analytical separation, and detection of these compounds.

Solid-phase extraction (SPE) is considered one of the most important techniques for sample cleanup and preconcentration, and according to the principles of Green Analytical Chemistry (GAC) is considered a green sample preparation and extraction technique [17]. SPE is an important methodology to avoid the use of large amounts of organic solvents in preconcentration and extraction steps [18]. This is one of the key goals to be achieved in greening analytical methods [19]. From a green perspective, on-line SPE is the preferred format in SPE extraction and when it is coupled to a liquid chromatograph, the main advantages are higher throughput and limited manual processing, as well as low cost [20]. On-line systems are beneficial in the domain of method development when the amount of sample is limited or when very high sensitivity is required. Applications for liquid samples involving an on-line coupling of extraction and chromatography are diverse [21]. Solid-phase-based extraction is one of the most promising on-line solid phase techniques because of its versatility, easy coupling and environmentally friendly characteristics [22]. For this reason, the use of on-line SPE chromatography as a means of metabolite concentration was considered a good option in this work to achieve the enrichment of traces. This was because the concentration factors that can be achieved are very large and thus allow the detection and quantification of the metabolites by using a spectrophotometric ultraviolet (UV) detector, which is much less sensitive than a fluorescence detector. SPE-LC systems are widely used, particularly in bioanalysis and they are a common feature in miniaturized or nano-LC systems [23,24]. Generally, on-line preconcentration techniques involve the use of SPE cartridges, or short columns or pre-columns, coupled to the analytical column via a switching valve [25].

The mono-hydroxy PYR and BaP metabolites have been analyzed by on-line or column-switching methodologies in biological fluids [26–28]. To the best of our knowledge, on-line SPE and HPLC analysis of dihydrodiol metabolites has been reported only for the microalgae dihydrodiol-BaP products [14]. Now, the aim of this work was to develop an analytical methodology based on on-line SPE chromatography with spectrophotometric diode array detection for the individual determination of three benzo(a)anthracene- dihydrodiol metabolites formed by the microalgae Selenastrum capricornutum in exposed cultures: 5,6-dihydrodiol, 8,9-dihydrodiol and 10,11-dihydrodiol BaA (Table 1). BaA is a PAH with genotoxic and carcinogenic properties [29] and is included in the priority list of the United States Environmental Protection Agency (USEPA) [30]. The use of the diode array detector allowed the identification and the quantification of all three metabolites even with two of these isomers, 8,9- and 10,11-dihydrodiol, being eluted with poor resolution between them. This bioanalytical method was successfully applied to the monitoring of the aforementioned dihydrodiol metabolites in cultures of the freshwater microalgae *Selenastrum capricornutum* exposed to BaA at different incubation times. The results were of interest because the three metabolites were detected individually in their appearance in biomass and when they were excreted to liquid media. On-line SPE chromatography with spectrophotometric diode array detection was a simple and advantageous choice for the selective trace analysis of these benzo(a) anthracene degradation products from microalgae. This has not been

Table 1
Physicochemical properties of metabolites from benzo(a)anthracene.

	Metabolites from benzo (a)anthracene		
Compound	**5,6**-dihydrodiol	**8,9**-dihydrodiol	**10,11**-dihydrodiol
Structure			
Molecular Formula	$C_{18}H_{14}O_2$	$C_{18}H_{14}O_2$	$C_{18}H_{14}O_2$
Weight (g/mol)	262.31	262.31	262.31
Physical state	solid	solid	solid
Melting point (°C)[a]	178.26	178.04	178.04
Boiling Point (°C)[a]	455.78	455.3	455.3
Solubility in water (mg L^{-1})[a]	2.654	1.866	1.866
log K_{ow}[a]	3.25	3.42	3.42
Vapor pressure (mmHg)[a]	3.78×10^{-11}	3.94×10^{-11}	3.94×10^{-11}

[a] Calculated values from the software *Estimation Program Interface*® developed by the Unites States Environmental Protection Agency (US-EPA), version 4.11 (http://www.epa.gov/opptintr/exposure/pubs/episuite.htm).