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A validated liquid chromatographic method for the determination of polycyclic aromatic hydrocarbons in honey after homogeneous liquid–liquid extraction using hydrophilic acetonitrile and sodium chloride as mass separating agent

Anastasia Koltsakidou[a], Constantinos K. Zacharis[b,c,*], Konstantinos Fytianos[a]

[a] Environmental Pollution Control Laboratory, Chemistry Department, Aristotle University of Thessaloniki, Thessaloniki 54124, Greece
[b] Research Laboratory for the Physical and Chemical Testing of Foods, Department of Food Technology, School of Food Technology and Nutrition, Alexander Technological Educational Institute (ATEI) of Thessaloniki, 57400, Thessaloniki, Greece
[c] Analytical Development Laboratory, R&D API Operations, Pharmathen SA, Building Thermi 1, 9th klm Thessaloniki-Thermi, Thessaloniki, 57001, Greece

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ABSTRACT

In the present report, a simple and cost-effective method for the determination of twelve US EPA priority polycyclic aromatic hydrocarbons (PAHs) in honey samples after salting-assisted liquid–liquid extraction and UHPLC with fluorescence detection is proposed. The sample treatment is based on the usage of hydrophilic acetonitrile as extraction solvent and its phase separation under high salinity conditions. Due to the high sugar content of the samples the phase separation is promoted effortlessly. Several parameters affecting the extraction efficiency and method sensitivity including the concentration of the honey samples, the type and volume of the extraction solvent, the type and quantity of the inorganic salt, extraction time and centrifugation time was systematically investigated. The method was validated in-house according to the Commission Decision 2002/657/EC guidelines. The limit of detection (LOD) of the method lay between 0.02 and 0.04 ng mL^{-1} (corresponding to 0.08 and 0.16 ng g^{-1}) which are close to the quality criteria established by European Regulation (EC) 836/2011 concerning the PAHs in foodstuffs. The mean analytical bias (expressed as relative recoveries) in all spiking levels was acceptable being in the range of 54–118% while the relative standard deviation (RSD) was lower than 19%. The proposed method has been satisfactorily applied for the analysis of the selected PAHs residues in various honey samples obtained from Greek region.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are compounds that contain 2-6 fused aromatic rings and normally are produced through partial pyrolysis of organic matter and various geological processes [1]. Due to their mutagenic and carcinogenic properties these compounds have been characterized as priority pollutants by European Union and US Environmental Protection Agency [2]. The occurrence of PAHs in foodstuffs is mainly attributed to the environmental contamination or technological food processing (grilling, smoking, etc.). In honey, the source of PAHs mainly comes from the environment (e.g. air, soil, stubble burning, burned forest, placement of beehives near to industrial areas, etc.) through the bees as they transfer the nectar from flowering plants. Another possible contamination source can be through the blowing smoke into the beehives during handling by the beekeepers [3]. Since it is a food product with world-wide consumption especially among children it must be free of chemical contaminants particularly from PAHs.

In order to protect the public health, the scientific panel of Contaminants in the Food Chain (CONTAM panel) of EFSA concluded that four specific PAHs, commonly called as PAH4, (benzo[*a*]pyrene, B[*a*]P; benzo[*a*]anthracene, B[*a*]A; benzo[*b*]fluoranthene, B[*b*]F; chrysene, Chry) or eight substances (called as PAH8) including the above four and benzo[*k*]fluoranthene (B[*k*]F), benzo[*g,h,i*]perylene (B[*g,h,i*]P), dibenzo[*a,h*]anthracene (Db[*a,h*]A), indeno[*1,2,3-c,d*]pyrene (I(*1,2,3-cd*)P) are currently the most appropriate indicators of the carcinogenic potency and effect of PAHs in foodstuffs [4]. Furthermore, EFSA also deduced that the set of eight PAHs do not provide much added value compared to the group of four PAHs.

* Corresponding author. Tel./fax: +30 2310 791581.
E-mail addresses: czach@food.teithe.gr, czacharis@gmail.com (C.K. Zacharis).

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The analysis of PAHs in honey samples is mostly performed by conventional sample pretreatment techniques such as liquid-liquid extraction (LLE) [5,6] solid-phase extraction [3] or matrix solid-phase dispersion [7] in combination with separation techniques (GC or HPLC) coupled to optical (fluorescence) or mass spectrometric detectors. LLE is generally laborious and time-consuming procedure while large volumes of organic toxic solvents are usually required (e.g. CH_2Cl_2 [5]). Although that SPE is considered as the technique of choice in many laboratories, it requires additional hardware and consumables increasing the entire operational costs (typically 2–5 € per cartridge). The availability of rapid and low cost analytical schemes for the determination of persistent organic pollutants (pesticides, PAHs, etc) in honey is highly required for quality control purposes.

A promising approach called as salting-out assisted liquid–liquid extraction (SALLE) has been introduced by Matkovich and Christian aiming on the extraction of metal chelates using a binary homogenous system of acetone and water [8,9]. This technique is based on the usage of a low dielectric constant organic solvent such as acetonitrile, methanol, acetone etc, which is soluble with water in any portion (homogeneous liquid-liquid extraction, HLLE). The contact area between the two "phases" is practically infinitive compared to the LLE or dispersive liquid–liquid microextraction (DLLME) facilitating fast mass transfer. Furthermore, no vigorous shaking of the sample or even the application of ultrasound is required [10,11]. The phase separation is based on the (i) temperature, (ii) pH of perfluorinated surfactants, (iii) formation of ion-pair and (iv) addition of an electrolyte (salting-out). A detailed investigation of salting-out phenomenon in water-acetonitrile homogenous system has been recently carried out by Valente et al. [12]. The main advantages of this technique include simplicity in operation, low cost, reduction the extraction time, usage of non-halogenated or aromatic solvents compared to the conventional LLE [13] or DLLME [14] and have attracted much attention by many researchers. It has been successfully applied for the determination of various analytes in biological matrixes [15], foods [16] and environmental samples [17]. Recent literature findings revealed that there are only three methods using the above sample preparation technique in honey analysis aiming to the determination of sulfonamides [18], fluoroquinolones [19] and sulfanilamide [20].

The aim of this study was therefore to develop and validate a simple analytical tool for the determination of twelve PAHs in honey using the SALLE approach. To the best of our knowledge, it is the first SALLE method for the determination of PAHs in honey. The proposed analytical scheme was validated according to Commission Decision 2002/657/EC guidelines [21] and successfully applied to the quantification of the selected pollutants in commercially available honey samples of different floral origins.

2. Experimental

2.1. Reagents and solutions

All reagents used throughout this study were of analytical grade or highest. A certified mixture of 13 PAHs (EPA 525 PAH Mix A) containing 500 μg mL^{-1} each in dichloromethane was provided by Supelco Analytical. Acetonitrile (ACN), acetone, ethanol and isopropanol were of HPLC grade and supplied by Panreac (Barcelona, Spain) while methanol was purchased by Fluka. Salts including $MgSO_4$, Na_2SO_4, $(NH_4)_2SO_4$, $ZnSO_4$ were provided by (Merck, Darmstadt, Germany) and NaCl from Chem-Lab NV (Zedelgem, Belgium). Ultrapure water (18 MΩ cm resistivity) was used throughout this study (Millipore Direct-Q UV, Millipore S.A.S., Molsheim, France).

A standard PAHs stock mixture (50 mg L^{-1}) was prepared in ACN stored at −18 °C and protected from the light. Working standard solutions were prepared daily by appropriate dilutions of aliquots obtained by the stock solutions in ACN. It should be mentioned that during SALLE experiments, the volume fraction of ACN in the spiked solutions was less than 0.5% v/v in all cases. To avoid carry over effects all glassware used in this study was previously washed with water and acetone.

2.2. Instrumentation and chromatographic conditions

All separations were performed on an Acquity UPLC binary solvent system (Waters) equipped with a fluorescence and/or PDA detector. The analytical column was a reversed phase Acquity UPLC C18 BEH (100 × 2.1 mm, 1.7 μm) and was protected by guard column (VanGuard 5 × 2.1 mm, 1.7 μm, Waters). The instrument control and the data acquisition were carried out via the Empower 2 Pro software. Samples were filtrated through syringe filters (0.22 μm PVDF, Millex® HV, Millipore) prior to the analysis. Glass tight syringes with volumes of 100 μL and 1000 μL (Hamilton, Nevada, USA) were employed for measurements of the volumes of the extraction organic solvents and for the DLLME processes. An EBA 20 (Hettich, Germany) centrifuge and an ultrapure water (18 MΩ cm resistivity) purification system were used throughout this study (Direct-Q 3UV, Millipore S.A.S., Molsheim, France).

The analytes of interest were separated by a binary gradient elution program using water (A) and ACN (B). The initial ratio was 50% v/v of B and linearly increased to 60% in 3 min and then to 70% in 6 min. The next 1 min was kept constant and increased to 90% in 12 min and finally to 100% in 13 min. At time of 13.1 min the gradient elution is changed to the initial ratio and stated for a period of 5 min to maintain a stable and reproducible separation. The flow rate was set to 0.35 mL min^{-1} while the injection volume was 10 μL. The column was thermostated to 30 °C. The analytes were detected spectrofluorimetrically using a two-channel program as described in Table 1. Between injections the autosampler was sequentially washed with 1500 μL water and 500 μL ACN to remove any sample residuals.

2.3. Sampling and SALLE protocol

All honey samples were collected from local markets including different botanical origins. They were kept in their original containers in dark place at ambient temperature as in everyday use. Prior to sampling each specimen was mechanically homogenized by stirring for 3 min [22]. PAH-free honey samples were used during the optimization of the extraction parameters and validation of the method. A pooled sample ($n = 5$) was prepared and homogenized by mixing 50 ± 0.1 g of five honeys of different floral origins. A 250 g L^{-1} honey solution was prepared in water, spiked with the analytes and used throughout this study. Aqueous solutions (250 g L^{-1}) of individual, commercially available honey samples were spiked with PAHs and left to "equilibrate" for at least for 15 min prior to the extraction.

An aliquot of 5 mL of the pooled or individual honey aqueous samples (blank or spiked) was transferred in a 10 mL screw cap glass tube with conical bottom. A volume of 1700 μL ACN was added in the extraction vessel and the mixture was vortexed for 30 s. Afterwards an amount of 1 g of NaCl was added in the aqueous-acetonitrile homogeneous phase and the mixture was manually shaken for 5 min in order to dissolve the solid salt. Then the mixture was centrifuged at 4000 rpm for 5 min to enhance the phase separation. A portion of ca 500 μL of the resulted PAH-riched acetonitrile phase (upper phase) was retrieved using a 1 mL glass microsyringe.