# Determination of polycyclic aromatic hydrocarbons in chocolate using the combination of Quick Easy Cheap Effective Rugged Safe method and dispersive liquid-liquid microextraction

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**Summary**

The aim of this study was to determine the level of polycyclic aromatic hydrocarbons (PAHs) in chocolate samples using the innovative combination of the QuEChERS (Quick Easy Cheap Effective Rugged Safe) method with dispersive liquid-liquid microextraction (DLLME). The outcomes from the optimization experiment indicated that the proposed approach was a rapid and effective sample treatment resulting in acceptable recoveries, satisfactory effects in sample clean-up and good analytical performance. Analysis of real samples showed that chocolate samples were contaminated mostly with light PAHs with phenanthrene as a dominant compound, in the range of 13.5–96.6 $\mu g \cdot kg^{-1}$. Content of total PAHs was significantly lower in white chocolates (68.8 $\mu g \cdot kg^{-1}$) in comparison to samples of dark chocolate (113 $\mu g \cdot kg^{-1}$). The three heaviest PAHs were not identified in any investigated sample. The sum of 4 PAHs markers exceeded the limits established by European Union only in 2 samples of chocolate (with contents of 36.2 $\mu g \cdot kg^{-1}$ and 38.5 $\mu g \cdot kg^{-1}$, respectively).

**Keywords**

polycyclic aromatic hydrocarbons; chocolate; Quick Easy Cheap Effective Rugged Safe method; dispersive liquid-liquid microextraction

Polycyclic aromatic hydrocarbons (PAHs) are a large class of organic substances, containing in its chemical structure two or more fused aromatic rings. Among PAHs, compounds consisting of three or four rings are described as "light" PAHs, while compounds with at least five condensed rings are referred to as "heavy" PAHs [1–4]. In general, polycyclic aromatic hydrocarbons have high melting- and boiling-points, low vapour pressure, and very low water solubility, which decreases with increasing molecular mass. PAHs are soluble in organic solvents and have lipophilic properties. In the environment, PAHs arise from incomplete combustion of organic compounds. In food, PAHs are formed during its thermal processing such as drying, smoking, roasting, baking, frying or grilling. Studies in experimental animals showed that PAHs cause various toxic effects, including hematological changes, reproductive and developmental toxicity and immunotoxicity. Heavy PAHs are generally seen as more toxic and carcinogenic than light PAHs [1]. In 2002, the European Commission's Scientific Committee on Food identified 15 individual PAHs as being of major concern for human health and, in 2008, European Food Safety Authority (EFSA) concluded that four of them, namely, benzo[*a*]pyrene, benzo[*a*]anthracene, benzo[*b*]fluoranthene and chrysene are suitable indicators for the occurrence of PAHs in food [5].

Chocolate and chocolate products are important ingredients in food industry and are also regarded to be the most popular food items, especially for children. Chocolate contains cocoa solid, cocoa butter, sugar, glucose, buffering agents and aroma or flavouring substances. According to its composition, chocolates are basically classified as dark chocolate, milk chocolate or white chocolate. [6]. Among the chocolate ingredients, cocoa butter made from cocoa beans is the component considered as the main source of PAHs. Generally speaking, cocoa beans may be contaminated with PAHs mostly during drying on asphalt, on

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bitumen in the sun, or by using direct firing drying model. Storage and transport in jute or sisal bags that had been treated with batching oil may be another possibility to PAHs migration into cocoa beans [7]. However, in available literature the problem of PAHs presence in chocolate has been raised only in a few studies with particular focus on the benzo[*a*]pyrene levels, which was found to range from 0.06 $\mu g \cdot kg^{-1}$ to 0.63 $\mu g \cdot kg^{-1}$ [1, 6–9]. Therefore, it is important that the presence of these compounds is subjected to continuous monitoring with the use of simple but effective methods of sample preparation and analysis.

QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method, developed originally for pesticide residues determination in food of plant origin, has become one of the most popular sample preparation concepts in food analysis. QuEChERS involves extraction using acetonitrile, addition of sodium chloride to separate water phase and magnesium sulfate for the removal of residual water. Organic phase is then cleaned-up by dispersive solid phase extraction (SPE) employing bulk sorbents such as primary secondary amine (PSA), graphitized carbon black (GCB) and octadecylsilane-bonded silica gel (C18) [10]. Dispersive liquid–liquid microextraction (DLLME) is another procedure that has been introduced to food analysis during the last few years [11]. DLLME is based on the system of three solvents: aqueous sample, dispersive solvent and extraction solvent. The mixture of an extraction solvent (e.g. chloroform) and a dispersive solvent (water-organic miscible solvent, e.g. acetonitrile) is rapidly injected into an aqueous sample, forming a cloudy solution. After centrifugation, the analytes are pre-concentrated into the phase of extraction solvent [12–16]. However, for more complex matrices such as food samples, DLLME technique requires a pre-cleaning step [12]. Both QuEChERS and DLLME are attractive alternatives to classical sample preparation procedures because of low consumption of toxic solvents, simplicity, rapidity and high recovery rates. The combination of the QuEChERS procedure with the DLLME preconcentration technique has been applied mostly to the determination of pesticide residues in food samples [12–14, 17] or in ginseng [15], but also to analysis of bisphenols in canned seafood and vegetables [16, 18], and mycotoxins in nuts [19]. However, coupled QuEChERS-DLLME for PAHs determination in food samples has not been evaluated yet. Hence, the objective of this work was to determine PAHs levels in chocolate samples using the combination QuEChERS-DLLME.

## MATERIALS AND METHODS

### Chemicals and reagents

Hexane, chloroform and acetonitrile, HPLC-grade LiChrosolv, were purchased from Merck (Darmstadt, Germany). Magnesium sulphate anhydrous p.a. and sodium chloride p.a. were purchased from Chempur (Piekary Śląskie, Poland). PSA and C18 SPE bulk sorbent was from from Agilent Technologies (Santa Clara, California, USA). EPA 525 PAH Mix-B (containing: acenaphthylene, anthracene, benzo[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, benzo[*g,h,i*]perylene, chrysene, dibenzo[*a,h*]anthracene, fluorene, indeno[1,2,3-*cd*]pyrene, phenanthrene and pyrene), chrysene-$d_{12}$ and anthracene-$d_{10}$ were obtained from Supelco (Bellefonte, Pennsylvania, USA). Stock, intermediate and working PAHs standard solutions, chrysene-$d_{12}$ (internal standard) and anthracene-$d_{10}$ (syringe standard) at concentration 1 $\mu g \cdot ml^{-1}$ were prepared in hexane. Deionized water (18 MΩ) was produced by a Milli-Q system (Merck).

### Instrumentation

Analyses were performed using Varian 4000 gas chromatograph coupled to mass spectrometer (GC-MS) (Agilent Technologies) with a CP-8410 auto-injector (Bruker, Billerica, Massachusetts, USA) with DB-5MS column (30 m × 0.25 mm × 0.25 $\mu$m; Agilent Technologies). The injector temperature was set at 270 °C, and an injection volume was 1.0 $\mu$l. Each injection was performed in triplicate. The GC oven operated with the following temperature program: 50 °C (1.0 min) – 15 $°C \cdot min^{-1}$ – 300 °C (6.0 min). The total run time was 23.67 min with a solvent delay of 8.0 min. Helium 5.0 (Linde Group, Munich, Germany) was used as the GC carrier gas at a flow rate of 1.0 $ml \cdot min^{-1}$. The ion trap mass spectrometer operated in the internal ionization mode, scanning from m/z 45 to 500. The emission current of the ionization filament was set at 15 $\mu$A. Analyses were conducted in the selected ion monitoring mode (SIM) based on the use of one quantitative ion. Analysed compounds were identified according to three ions (one target and two qualifiers) and according to retention times (Tab. 1). The trap and the transfer line temperatures were set at 180 °C and 220 °C, respectively. Acquisition and processing of data were performed using Varian Start Workstation software (Agilent Technologies) and NIST 2.0 library (National Institute of Standards and Technology, Gaithersburg, Maryland, USA).

**Tab. 1.** Parameters of GC-MS analysis of examined compounds.

| Retention time [min] | Compound | Quantification ion | Confirmation ions |
|---|---|---|---|
| 10.11 | Acenaphthylene | 152.1 | 151.1; 153.1 |
| 11.22 | Fluorene | 166.1 | 164.1; 165.1 |
| 12.76 | Phenanthrene | 178.1 | 178.1; 179.1 |
| 12.81 | Anthracene-$d_{10}$ | 188.0 | 188.1; 177.9 |
| 12.85 | Anthracene | 178.1 | 178.1; 179.1 |
| 15.05 | Pyrene | 202.1 | 200.1; 203.1 |
| 16.98 | Benzo[*a*]anthracene | 228.1 | 226.1; 229.1 |
| 17.01 | Chrysene-$d_{12}$ | 240.1 | 228.0; 241.0 |
| 17.04 | Chrysene | 228.1 | 226.1; 229.1 |
| 18.76 | Benzo[*b*]fluoranthene | 252.1 | 250.1; 253.1 |
| 18.81 | Benzo[*k*]fluoranthene | 252.1 | 250.1; 253.1 |
| 19.39 | Benzo[*a*]pyrene | 252.1 | 250.1; 253.2 |
| 22.17 | Indeno[1,2,3-*c,d*]pyrene | 276.1 | 274.1; 277.1 |
| 22.27 | Dibenzo[*a,h*]anthracene | 278.2 | 276.0; 279.1 |
| 22.96 | Benzo[*g,h,i*]perylene | 276.0 | 274.1; 277.0 |

MS1 Minishaker (IKA, Königswinter, Germany) and MPW 350 R Centrifuge (MPW, Warsaw, Poland) were used during sample preparation. Accublock (Labnet, Edison, New Jersey, USA) with nitrogen 5.0 (Linde Group) was used to evaporate solvents and concentrate the extracts.

### Sample and standard preparation

In the optimization experiment, the samples of milk chocolate, delivered from a local market in Poland, were used for the preparation of blank and spiked samples. Recovery studies at two spiking levels (5 $\mu g \cdot kg^{-1}$ and 20 $\mu g \cdot kg^{-1}$) involved three samples being fortified with the standard solution and chrysene-$d_{12}$ solution. The spiking levels were adapted to the maximum levels set in European Union for the sum of 4 PAHs in cocoa beans and its products (30 $\mu g \cdot kg^{-1}$) [20].

Chocolate was powdered, spiked with the mixture of standards and left to stand for 15 min at room temperature prior to extraction. Then, 1 g of sample was weighed into a 50 ml propylene (PP) centrifuge tube, 5 ml of water and 10 ml of acetonitrile were added, and the mixture was shaken vigorously for 1 min. Next, 1 g NaCl and 4 g $MgSO_4$ were added, with the tube being shaken immediately after the addition of the salt. Subsequently, each sample was shaken vigorously for 1 min and centrifuged for 15 min at 8500 ×*g*. A volume of 6 ml of the supernatant was placed into a 15 ml PP tube containing 0.15 g PSA, 0.3 g $C_{18}$ sorbents and 0.9 g $MgSO_4$ (clean-up step by dispersive SPE). The tube was shaken for 2 min and centrifuged for 15 min at 10000 ×*g*. Then, 2 ml of the QuEChERS extract with the addition of 200 $\mu$l of $CHCl_3$ was rapidly injected into a 15 ml PP tube containing 6 ml $H_2O$. The tubes were gently shaken and centrifuged for 5 min at 780 ×*g*. Approximately 200 $\mu$l of the bottom chloroform layer was placed into a 1.5 ml glass vial and left to evaporate. The residues were dissolved in 225 $\mu$l of hexane, and 25 $\mu$l of the anthracene-$d_{10}$ solution (1 $\mu g \cdot ml^{-1}$) was added. A volume of 200 $\mu$l of the solution was transferred into glass inserts placed in autosampler vials and analysed by GC-MS. Blank samples and reagent blanks were prepared similarly to the fortified samples.

A series of standard solutions in hexane and in a chocolate matrix were prepared by dilution of the standard mixture solution (1 $\mu g \cdot ml^{-1}$): 0.1, 1, 5, 10, 40, 70 and 100 $ng \cdot ml^{-1}$. Each standard solution contained 100 $\mu$l of anthracene-$d_{10}$ solution (1 $\mu g \cdot ml^{-1}$) and was prepared in triplicate. Calibration curves were constructed by plotting integrated peak areas, divided by the area of anthracene-$d_{10}$, against concentrations of compounds. GC-MS chromatogram of standard solution in hexane is presented in Fig. 1.

## RESULTS AND DISCUSSION

### Optimization of the method

To evaluate the usefulness of developed QuEChERS-DLLME combination, the percent recoveries for each compound were calculated by substracting values in an unspiked blank sample from values in a spiked sample. The obtained re-

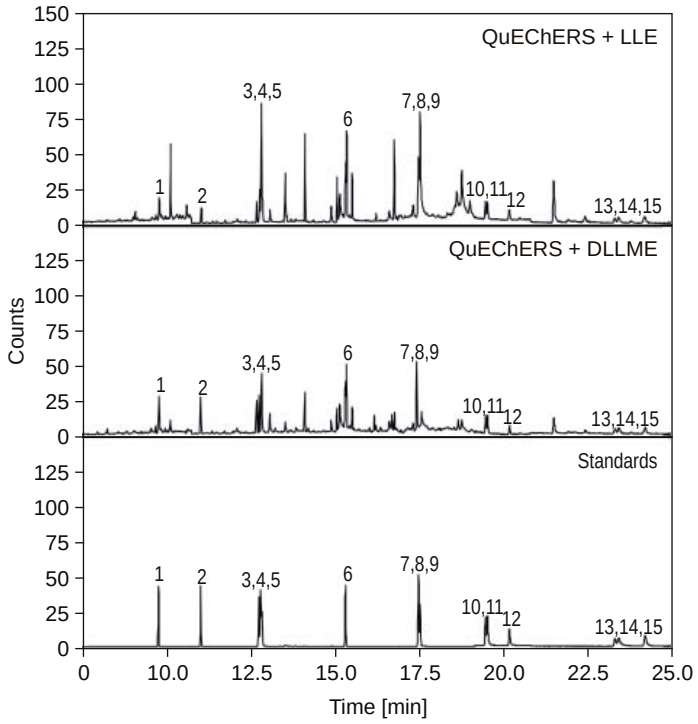


**Fig. 1.** GC-MS chromatograms of sample extracts prepared by QuEChERS+LLE, QuEChERS+DLLME and standards.

QuEChERS + LLE – combination of the QuEChERS method with the preconcentration by liquid-liquid extraction, QuEChERS + DLLME – combination of the QuEChERS method with dispersive liquid-liquid microextraction.
1 – acenaphthylene; 2 – fluorene; 3 – phenanthrene; 4 – anthracene-$d_{10}$; 5 – anthracene; 6 – pyrene; 7 – benzo[*a*]anthracene; 8 – chrysene-$d_{12}$; 9 – chrysene; 10 – benzo[*b*]fluoranthene; 11 – benzo[*k*]fluoranthene; 12 – benzo[*a*]pyrene; 13 – indeno[1,2,3-*c,d*]pyrene; 14 – dibenzo[*a,h*]anthracene; 15 – benzo[*g,h,i*]perylene.

coveries were 50–120% [21] for all analytes at both spiking levels (Tab. 2). Repeatability of recovery values, expressed as relative standard deviation (*RSD*), was lower than 9.3% for all compounds. These results were compared to our previously developed method based on the QuEChERS concept with the application of PSA + florisil + $C_{18}$ sorbents and acetonitrile-hexane exchange in the presence of NaCl solution. This approach (described in brief as QuEChERS + LLE) was successfully used for the determination of PAHs in cocoa samples [22]. However, in case of chocolate samples it was found that the recoveries achieved by QuEChERS + LLE were too low for the two lightest compounds (acenaphthylene and fluorene). The presence of co-extracts in GC-MS chromatograms was also more prominent for QuEChERS + LLE than for the proposed QuEChERS + DLLME combination (Fig. 1). Therefore, acetonitrile-chloroform replacement in the presence of water entailed better recovery and clean-up than acetonitrile-hexane substitution with the addition of NaCl solution.

The optimization of QuEChERS-DLLME procedure involved also determination of the volumes of the extraction solvent, water and evaluation of the benefits from salt addition. The volume of QuEChERS extract was established at 2 ml, as it was assumed that smaller volume would result in a decrease in the detection limit. To study the effect of the extraction solvent volume, 2 ml of QuEChERS extracts containing different volumes of $CHCl_3$ (50, 100, and 200 $\mu$l) was submitted to the DLLME procedure. In order to study the effect of water volume, experiments were carried out in the range of 4–6 ml of water, with the intervals of 0.5 ml. In case of the use of 50 $\mu$l chloroform, the recovery was low, below 30% (data not shown). Extraction efficiency was slightly better for the samples with the addition of 100 $\mu$l $CHCl_3$, and increased significantly with an increase in the volume of $CHCl_3$ from 100 $\mu$l to 200 $\mu$l (Tab. 2). Regarding water volume optimization, the cloudy solution was not formed when 4 ml, 4.5 ml and 5 ml of water were used. The volume of 5.5 ml of water was the minimum at which the extraction

solvent was settled down. Thus, 6 ml of water was selected as the optimum volume that ensured an appropriate formation of ternary component solvent system. The influence of ionic strength was a further parameter assessed in the experiment. According to certain authors [23, 24], the addition of salt (salting-out effect) improves extraction efficiency by decreasing the solubility of analytes in the aqueous phase and by reducing the solubility of organic solvents in water. In this experiment, 0%, 5%, and 10% (w/w) NaCl aqueous concentrations were tested. However, the presence of NaCl resulted in lack of formation of a cloudy solution. Hence, it was decided that the addition of salt in the DLLME procedure is not used.

The proposed analytical method was validated including determination of method specificity (matrix effects), linearity, limit of detection, limit of quantification, repeatability, accuracy and uncertainty, according to the criteria set by the European Union [21]. The matrix effects in chocolate sample extracts were assessed by means of statistical comparison between the slopes of the calibration curves prepared in hexane, and in the extract of chocolate samples (Tab. 3). The t-test ($p = 0.05$) showed insignificant differences between the results for most of the analytes, with the exception of pyrene and chrysene. For these two compounds, the type of the matrix effect (ME), suppression or enhancement, was estimated based on the percentage calculation (the percentage of the difference between the slope values of the matrix-matched calibration curve and the solvent one. The calculated ME values were 107% and 93%, respectively. So, it can be concluded that despite significantly different values of calibrations slopes, no matrix effects were found for pyrene and chrysene.

Linearity, expressed as the correlation coefficient of the calibration slope, was calculated for solvent and matrix-matched calibration. The values were higher than 0.99 for all compounds (Tab. 3). Limit of detection (*LOD*) and limit of quantification (*LOQ*) were calculated from the signal-to-noise ratio (S/N) measured at the lowest calibration level, using the GC-MS software. The S/N values of 3 and 10 were used for the calculation of *LOD* and *LOQ*, respectively. *LOQ* were lower than 0.83 $\mu g \cdot kg^{-1}$. Repeatability ($RSD_r$), calculated from data on spiked samples, was below 5.5% with the value $HORRAT_r < 2$ for each of PAHs (Tab. 3). Estimation of reproducibility ($RSD_R$) included preparation and analysis of spiked samples by different laboratory technicians, using different series of reagents and calibration curves. A new gas chromatography column (DB-5MS) was also employed in the study. $RSD_R$ was lower than 8.2% with $HORRAT_R$ value also below 2 for all compounds. The accuracy of the method was determined with the recovery using spiked samples, because no certified reference material of chocolate with PAHs was available. All results were within acceptable limits and ranged

**Tab. 2.** Recoveries and relative standard deviations of method variants.

| | QuEChERS + LLE [18] | | QuEChERS + DLLME | | | | | |
|---|---|---|---|---|---|---|---|---|
| Spiking level [$\mu g \cdot kg^{-1}$] | 20 | | 20 | | 20 | | 5 | |
| $CHCl_3$ [$\mu l$] | – | | 100 | | 200 | | 200 | |
| Compounds | *R* [%] | *RSD* [%] | *R* [%] | *RSD* [%] | *R* [%] | *RSD* [%] | *R* [%] | *RSD* [%] |
| Acenaphthylene | 33.0* | 12.0 | 118 | 4.7 | 109 | 5.5 | 93.6 | 6.8 |
| Fluorene | 46.6* | 13.6 | 75.7 | 4.7 | 112 | 4.5 | 77.2 | 9.3 |
| Phenanthrene | 51.4 | 8.8 | 55.2 | 7.2 | 101 | 0.6 | 73.5 | 5.5 |
| Anthracene | 68.2 | 4.0 | 50.4 | 8.0 | 96.7 | 0.8 | 88.8 | 7.7 |
| Pyrene | 111 | 7.0 | 54.6 | 2.0 | 83.7 | 0.3 | 83.5 | 5.7 |
| Benzo[*a*]anthracene | 105 | 0.7 | 56.8 | 2.7 | 87.8 | 2.1 | 76.6 | 6.6 |
| Chrysene | 101 | 3.1 | 75.8 | 0.6 | 112 | 1.3 | 93.5 | 7.7 |
| Benzo[*b*]fluoranthene | 105 | 4.9 | 56.2 | 5.7 | 92.2 | 4.1 | 84.7 | 5.7 |
| Benzo[*k*]fluoranthene | 89.2 | 4.3 | 50.5 | 1.3 | 77.8 | 4.7 | 75.9 | 8.1 |
| Benzo[*a*]pyrene | 87.1 | 8.2 | 45.1* | 8.2 | 76.8 | 2.0 | 65.0 | 5.4 |
| Indeno[1,2,3-*c,d*]pyrene | 60.2 | 2.3 | 35.4* | 6.8 | 78.4 | 2.2 | 52.8 | 9.3 |
| Dibenzo[*a,h*]anthracene | 85.5 | 5.4 | 51.3 | 4.9 | 78.8 | 1.9 | 70.7 | 4.6 |
| Benzo[*g,h,i*]perylene | 72.8 | 9.5 | 42.5* | 3.0 | 69.0 | 3.4 | 64.8 | 4.5 |

QuEChERS + LLE – combination of the QuEChERS method with the preconcentration by liquid-liquid extraction, QuEChERS + DLLME – combination of the QuEChERS method with dispersive liquid-liquid microextraction, *R* – recovery, *RSD* – relative standard deviation. (*) – values beyond acceptable limits (50–120%).

**Tab. 3.** Parameters of the validation study of examined compounds.

| Compound | Solvent calibration slope | $r$ | Matrix matched calibration slope | $r$ | Recovery [%] | $RSD_r$ [%] | $HORRAT_r$ | $RSD_R$ [%] | $HORRAT_R$ | $rU_c$ [%] | $LOD$ [$\mu g \cdot kg^{-1}$] | $LOQ$ [$\mu g \cdot kg^{-1}$] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acenaphthylene | 0.0088 | 0.9989 | 0.0086 | 0.9987 | 109 | 5.5 | 0.4 | 8.2 | 0.5 | 13.7 | 0.09 | 0.31 |
| Fluorene | 0.0060 | 0.9995 | 0.0060 | 0.9983 | 112 | 4.5 | 0.3 | 6.0 | 0.4 | 11.7 | 0.11 | 0.36 |
| Phenanthrene | 0.0093 | 0.9937 | 0.0093 | 0.9954 | 101 | 0.6 | 0.1 | 0.7 | 0.4 | 11.5 | 0.08 | 0.25 |
| Anthracene | 0.0090 | 0.9992 | 0.0085 | 0.9966 | 96.7 | 0.8 | 0.1 | 0.9 | 0.6 | 12.7 | 0.08 | 0.28 |
| Pyrene | 0.0103 | 0.9994 | 0.0111 | 0.9984 | 83.7 | 0.3 | 0.1 | 0.4 | 0.1 | 11.2 | 0.09 | 0.31 |
| Benzo[*a*]anthracene | 0.0110 | 0.9986 | 0.0113 | 0.9990 | 87.8 | 2.1 | 0.2 | 2.4 | 0.2 | 14.9 | 0.15 | 0.50 |
| Chrysene | 0.0099 | 0.9996 | 0.0106 | 0.9990 | 112 | 1.3 | 0.1 | 1.8 | 0.2 | 13.8 | 0.13 | 0.42 |
| Benzo[*b*]fluoranthene | 0.0106 | 0.9996 | 0.0109 | 0.9959 | 92.2 | 4.1 | 0.3 | 4.8 | 0.3 | 13.7 | 0.15 | 0.50 |
| Benzo[*k*]fluoranthene | 0.0115 | 0.9994 | 0.0118 | 0.9979 | 77.8 | 4.7 | 0.3 | 5.7 | 0.4 | 14.1 | 0.19 | 0.63 |
| Benzo[*a*]pyrene | 0.0092 | 0.9986 | 0.0090 | 0.9992 | 76.8 | 2.0 | 0.2 | 3.1 | 0.2 | 15.6 | 0.15 | 0.50 |
| Indeno[1,2,3-*c,d*]pyrene | 0.0110 | 0.9995 | 0.0107 | 0.9971 | 78.4 | 2.2 | 0.2 | 3.3 | 0.2 | 16.4 | 0.19 | 0.63 |
| Dibenzo[*a,h*]anthracene | 0.0088 | 0.9984 | 0.0083 | 0.9995 | 78.8 | 1.9 | 0.2 | 2.4 | 0.2 | 15.8 | 0.19 | 0.63 |
| Benzo[*g,h,i*]perylene | 0.0102 | 0.9992 | 0.0098 | 0.9974 | 69.0 | 3.4 | 0.2 | 4.7 | 0.3 | 15.9 | 0.25 | 0.83 |

$r$ – correlation coefficient, $RSD_r$ – repeatability, $HORRAT_r$ – ratio of the $RSD_r$ to the relative standard deviation predicted from the Horwitz equation; $RSD_R$ – reproducibility, $HORRAT_R$ – ratio of the $RSD_R$ to the relative standard deviation predicted from the Horwitz equation; $rU_c$ – relative expanded uncertainty; $LOD$ – limit of detection; $LOQ$ – limit of quantification.

from 69% to 112%. At the second spiking level, 5 $\mu g \cdot kg^{-1}$, the results were also acceptable but they were slightly lower, 52.8–93.6%, with *RSD* lower than 9.3%.

Measurement uncertainty of quantification was assessed according to the Eurachem/CITAC guidelines [25] by identifying and quantifying the uncertainty components of the whole analytical process. Relative expanded uncertainty ($rU_c$) was calculated using a coverage factor of 2, which gave a confidence level of 95%. The $rU_c$ values were lower than 16.4%, thereby not exceeding the limits established by EU [21].

### Application to real samples

A number of 20 chocolate samples (6 white chocolates, 7 milk chocolates and 7 dark chocolates) were analysed for the content of polycyclic aromatic hydrocarbons. All samples were obtained from the market in 2014. Previously developed QuEChERS-DLLME procedure was used to analyse the collected samples. The levels of PAHs in investigated chocolate samples are presented in Tab. 4.

In all samples, seven PAHs were identified: acenaphthylene, fluorene, phenanthrene, anthracene, pyrene, benzo[*a*]anthracene and chrysene. Acenaphthylene was detected only in three samples of dark chocolate, with the lowest value of 1.39 $\mu g \cdot kg^{-1}$. The highest values were noticed for phenanthrene (13.5–96.6 $\mu g \cdot kg^{-1}$, with a mean of 47.5$\mu g \cdot kg^{-1}$). Within PAHs markers, only benzo[*a*]anthracene and chrysene were found in chocolates, in the ranges of 1.03–12.8 $\mu g \cdot kg^{-1}$ and 6.43–29.8 $\mu g \cdot kg^{-1}$, respectively, exceeding thereby the limit of 30 $\mu g \cdot kg^{-1}$ for the sum of 4 PAHs, established by EU [20]. This exceedance was observed in one sample of milk chocolate (36.2$\mu g \cdot kg^{-1}$) and one dark chocolate sample (38.5$\mu g \cdot kg^{-1}$). Heavy compounds, starting from benzo[*b*]fluoranthene, were not detected in any of the analysed samples.

The total contents of PAHs ranged from 42.6 $\mu g \cdot kg^{-1}$ to 150 $\mu g \cdot kg^{-1}$. White chocolates were characterized by the lowest total sum of PAHs (42.6–122 $\mu g \cdot kg^{-1}$, mean 68.8 $\mu g \cdot kg^{-1}$) followed by milk chocolates (48.7–147 $\mu g \cdot kg^{-1}$, mean 95.8 $\mu g \cdot kg^{-1}$) and dark chocolates (87.8–150 $\mu g \cdot kg^{-1}$, mean 113 $\mu g \cdot kg^{-1}$). However, statistically signifi-

**Tab. 4.** Content of polycyclic aromatic hydrocarbons in samples of chocolates.

| Samples | Content [$\mu g \cdot kg^{-1}$] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acp | Flu | Ant | Phen | Pyr | B[*a*]a | Chr | B[*b*]f | B[*k*]f | B[*a*]p | I[*cd*]p | D[*ah*]a | B[*ghi*]P | ΣPAHs | Σ(4 PAHs) |
| White chocolate | | | | | | | | | | | | | | | |
| 1 | nd | 24.8 ± 1.2 | 56.1 ± 1.4 | nd | 41.6 ± 2.0 | nd | nd | nd | nd | nd | nd | nd | nd | 122 | – |
| 2 | nd | 19.6 ± 1.3 | 50.7 ± 1.7 | nd | 11.8 ± 1.1 | nd | nd | nd | nd | nd | nd | nd | nd | 82.1 | – |
| 3 | nd | nd | 57.3 ± 3.7 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd | 57.3 | – |
| 4 | nd | 7.17 ± 0.47 | 16.8 ± 0.8 | 6.54 ± 0.35 | 16.4 ± 0.6 | nd | 17.1 ± 0.3 | nd | nd | nd | nd | nd | nd | 64.0 | 17.1 |
| 5 | nd | 12.9 ± 0.9 | 13.5 ± 0.4 | 2.89 ± 0.11 | 5.88 ± 0.45 | nd | 7.47 ± 0.11 | nd | nd | nd | nd | nd | nd | 42.6 | 7.47 |
| 6 | nd | 5.47 ± 0.69 | 27.2 ± 0.3 | 8.45 ± 0.71 | 3.00 ± 0.25 | nd | nd | nd | nd | nd | nd | nd | nd | 44.2 | – |
| Milk chocolate | | | | | | | | | | | | | | | |
| 7 | nd | 27.3 ± 1.9 | 61.2 ± 0.5 | 5.84 ± 0.24 | 44.2 ± 0.4 | nd | 8.71 ± 0.30 | nd | nd | nd | nd | nd | nd | 147 | 8.71 |
| 8 | nd | 21.3 ± 1.6 | 56.3 ± 0.6 | nd | 36.4 ± 1.0 | nd | nd | nd | nd | nd | nd | nd | nd | 114 | – |
| 9 | nd | 6.60 ± 0.56 | 20.3 ± 1.9 | 6.10 ± 0.34 | 6.80 ± 0.54 | 1.03 ± 0.1 | 7.85 ± 0.45 | nd | nd | nd | nd | nd | nd | 48.7 | 8.88 |
| 10 | nd | 21.0 ± 0.7 | 51.3 ± 2.0 | nd | 12.8 ± 0.6 | nd | nd | nd | nd | nd | nd | nd | nd | 85.1 | – |
| 11 | nd | 21.2 ± 0.9 | 50.6 ± 1.6 | nd | 12.2 ± 0.9 | nd | nd | nd | nd | nd | nd | nd | nd | 83.9 | – |
| 12 | nd | 13.1 ± 0.8 | 31.7 ± 0.7 | 5.12 ± 0.20 | 17.5 ± 1.1 | nd | 28.4 ± 1.1 | nd | nd | nd | nd | nd | nd | 95.8 | 28.4 |
| 13 | nd | 19.1 ± 1.3 | 24.0 ± 1.5 | 20.8 ± 0.7 | 17.7 ± 1.1 | 6.45 ± 0.4 | 29.8 ± 1.0 | nd | nd | nd | nd | nd | nd | 117 | 36.2* |
| Dark chocolate | | | | | | | | | | | | | | | |
| 14 | 6.61 ± 0.51 | 17.0 ± 1.0 | 41.8 ± 0.8 | 15.4 ± 0.7 | 20.8 ± 0.7 | 12.8 ± 1.1 | 25.7 ± 1.2 | nd | nd | nd | nd | nd | nd | 140 | 38.5* |
| 15 | nd | 17.7 ± 1.1 | 45.9 ± 2.7 | 10.6 ± 1.0 | 13.6 ± 0.5 | nd | nd | nd | nd | nd | nd | nd | nd | 87.8 | – |
| 16 | nd | 23.3 ± 1.6 | 54.7 ± 0.6 | nd | 13.6 ± 0.9 | nd | 6.50 ± 0.25 | nd | nd | nd | nd | nd | nd | 98.1 | 6.50 |
| 17 | nd | 20.8 ± 1.9 | 58.3 ± 4.7 | 6.65 ± 0.51 | 12.6 ± 0.7 | nd | nd | nd | nd | nd | nd | nd | nd | 98.4 | – |
| 18 | 6.62 ± 0.45 | 13.5 ± 1.1 | 96.6 ± 2.6 | 21.0 ± 1.5 | 12.2 ± 1.0 | nd | nd | nd | nd | nd | nd | nd | nd | 149 | – |
| 19 | 1.39 ± 0.11 | 16.2 ± 0.9 | 68.5 ± 3.1 | 6.84 ± 0.50 | 7.23 ± 0.52 | nd | 6.43 ± 0.50 | nd | nd | nd | nd | nd | nd | 106 | 6.43 |
| 20 | nd | 28.7 ± 0.8 | 67.1 ± 1.6 | nd | 43.1 ± 1.4 | nd | nd | nd | nd | nd | nd | nd | nd | 138 | – |

Values are expressed as mean ± standard deviation, $n = 3$.
Acp – acenaphthylene; Flu – fluorene; Phen – phenanthrene; Ant – anthracene; Pyr – pyrene; B[*a*]a – benzo[*a*]anthracene; Chr – chrysene; B[*b*]f – benzo[*b*]fluoranthene; B[*k*]f – benzo[*k*]fluoranthene; B[*a*]p – benzo[*a*]pyrene; I[*cd*]p – indeno[1,2,3-*c,d*]pyrene; D[*ah*]a – dibenzo[*a,h*]anthracene; B[*ghi*]P – benzo[*g,h,i*]perylene, PAHs – polycyclic aromatic hydrocarbons. nd – not detected, **(*)** – values exceeding established limits.

cant differences (calculated by t-test) were found only between the means for white and dark chocolates.

In general, the results presented in this study were higher but in the same order of magnitude as the results reported by other authors. In the study conducted in Germany [1], it was observed that PAHs in none of the analysed samples exceeded the maximum levels set in European Union for the sum of 4 PAHs in cocoa beans and its products (35 $\mu g \cdot kg^{-1}$) [20]). Benzo[*a*]pyrene means in the samples of milk chocolate and dark chocolate were 0.70 $\mu g \cdot kg^{-1}$ (fat basis) and 0.57 $\mu g \cdot kg^{-1}$ (fat basis), respectively, with the means of 4 PAHs 10.11 $\mu g \cdot kg^{-1}$ (fat basis) and 5.88 $\mu g \cdot kg^{-1}$ (fat basis). In a study on the level of PAHs in chocolate candies [9], total PAHs contents were found to range from 2.70 $\mu g \cdot kg^{-1}$ to 235.91 $\mu g \cdot kg^{-1}$ with a mean content of 67.62 $\mu g \cdot kg^{-1}$. In all samples, light PAHs were mostly detected, being present at relatively high contents. Benzo[*a*]pyrene content was found to range from 0.35 $\mu g \cdot kg^{-1}$ to 12.76 $\mu g \cdot kg^{-1}$ with mean content at 1.62 $\mu g \cdot kg^{-1}$. In another study on PAHs levels in chocolate samples carried out in Germany [6], the benzo[*a*]pyrene contents in chocolate ranged between 0.07 $\mu g \cdot kg^{-1}$ and 0.63 $\mu g \cdot kg^{-1}$. The highest PAHs contents were found for chrysene (0.83–2.09 $\mu g \cdot kg^{-1}$). The sum contents of the analysed compounds were between 1.3 $\mu g \cdot kg^{-1}$ and 6.9 $\mu g \cdot kg^{-1}$. Finally, in an Irish survey [26] 18 samples, involving milk chocolate, dark chocolate and chocolate-coated biscuits were examined for the levels of PAHs. The levels of PAHs ranged from 1.13 $\mu g \cdot kg^{-1}$ to 3.87 $\mu g \cdot kg^{-1}$ (fresh weight basis), with chrysene being the dominant PAH in the pattern.

## CONCLUSIONS

The findings of this study indicate that the combination of QuEChERS with DLLME is a rapid and effective sample treatment that provides a sufficiently clean extracts with acceptable compound recoveries and good validation parameters. Thus, our approach appears to be a novel and attractive procedure for analysis of these organic contaminants. Analysis of real samples demonstrated that chocolate samples were contaminated mostly with light PAHs. However, in 2 samples of chocolate, the sum of 4 PAHs markers slightly exceeded the limits established by European Union. The total sum of PAHs was significantly higher in dark chocolates in comparison to samples of white chocolate. The content of PAHs in milk chocolate was between these two values, however, any significant differences were not found. The three heaviest PAHs were not identified in any of the analysed samples.

## REFERENCES

1. Raters, M. – Matissek, R.: Quantitation of polycyclic aromatic hydrocarbons (PAH4) in cocoa and chocolate samples by an HPLC-FD method. Journal of Agricultural and Food Chemistry, *62,* 2014, pp. 10666–10671. DOI: 10.1021/jf5028729.
2. Ciecierska, M. – Obiedziński, M. W.: Polycyclic aromatic hydrocarbons in vegetable oils from unconventional sources. Food Control, *30*, 2013, pp. 556–562. DOI: 10.1016/j.foodcont.2012.07.046.
3. Farhadian, A. – Jinap, S. – Abas, F. – Sakar, Z. I.: Determination of polycyclic aromatic hydrocarbons in grilled meat. Food Control, *21*, 2010, pp. 606–610. DOI: 10.1016/j.foodcont.2009.09.002.
4. Wenzl, T. – Simon, R. – Kleiner, J. – Anklam, E.: Analytical methods for polycyclic aromatic hydrocarbons (PAHs) in food and the environment needed for new food legislation in the European Union. Trends in Analytical Chemistry, *25*, 2006, pp. 716–725. DOI: 10.1016/j.trac.2006.05.010.
5. Polycyclic aromatic hydrocarbons in food. Scientific opinion of the panel on contaminants in the food chain (Question N° EFSA-Q-2007-136) Adopted on 9 June 2008. EFSA Journal, *724*, 2008, pp. 1–114. DOI: 10.2903/j.efsa.2008.724.
6. Ziegenhals, K. – Speer, K. – Jira, W.: Polycyclic aromatic hydrocarbons (PAH) in chocolate on the German market. Journal für Verbraucherschutz und Lebensmittelsicherheit, *4*, 2009, pp. 128–135. DOI: 10.1007/s00003-009-0478-1.
7. Misnawi: Effect of cocoa bean drying methods on polycyclic aromatic hydrocarbons contamination in cocoa butter. International Food Research Journal, *19(4)*, 2012, pp. 1589–1594. <http://worldcocoafoundation.org/wp-content/files_mf/misnawi2012postharvestdrying632kb.pdf>
8. Kumari, R. – Chaturvedi, P. – Ansari, N. G. – Murthy, R. C. – Patel, D. K.: Optimization and validation of an extraction method for the analysis of polycyclic aromatic hydrocarbons in chocolate candies. Journal of Food Sciences, *71*, 2012, pp. 34–40. DOI: 10.1111/j.1750-3841.2011.02488.x.
9. Kumari, R. – Patel, D. K. – Chaturvedi, P. – Ansari, N. G. – Chandra Murthy, R.: Solid phase micro extraction combined with gas chromatography-mass spectrometry for the trace analysis of polycyclic aromatic hydrocarbons in chocolate. Analytical Methods, *5*, 2013, pp. 1946–1954. DOI: 10.1039/C3AY26296K.
10. QuEChERS. A mini-multiresidue method for the analysis of pesticide residues in low-fat products. Stuttgart: Chemisches und Veterinäruntersuchungsamt, 2015. <http://quechers.cvua-stuttgart.de/pdf/reality.pdf>

11. Mohammadi, A. – Ghasemzadeh-Mohammadi, V. – Haratian, P. – Khaksar, R. – Chaichi, M.: Determination of polycyclic aromatic hydrocarbons in smoked fish samples by a new microextraction technique and method optimisation using response surface methodology. Food Chemistry, *141*, 2013, pp. 2459–2465. DOI: 10.1016/j.foodchem.2013.05.065.
12. Melo, A. – Mansilha, C. – Pinho, O. – Ferreira, I. M. P. L. V. O.: Analysis of pesticides in tomato combining QuEChERS and dispersive liquid–liquid microextraction followed by high-performance liquid chromatography. Food Analytical Methods, *6*, 2013, pp. 559–568. DOI: 10.1007/s12161-012-9469-4.
13. Bresin, B. – Piol, M. – Fabbro, D. – Mancini, M. A. – Casetta, B. – Del Bianco, C.: Analysis of organochlorine pesticides residue in raw coffee with a modified "quick easy cheap effective rugged and safe" extraction/clean up procedure for reducing the impact of caffeine on the gas chromatography-mass spectrometry measurement. Journal of Chromatography A, *1376*, 2015, pp. 167–171. DOI: 10.1016/j.chroma.2014.12.016.
14. Karami-Osboo, R. – Shojaee, M. H. – Miri, R. – Kobarfard, F. – Javidnia, K.: Simultaneous determination of six fluoroquinolones in milk by validated QuEChERS-DLLME HPLC-FLD. Analytical Methods, *6*, 2014, pp. 5632–5638. DOI: 10.1039/C4AY00848K.
15. Chen, L. – Yin, L. – Song, F. – Liu, Z. – Zheng, Z. – Xing, J. – Liu, S.: Determination of pesticide residues in ginseng by dispersive liquid–liquid microextraction and ultra high performance liquid chromatography–tandem mass spectrometry. Journal of Chromatography B, *917–918*, 2013, pp. 71–77. DOI: 10.1016/j.jchromb.2012.12.034.
16. Cunha, S. C. – Cunha, C. – Ferreira, A. R. – Fernandes, J. O.: Determination of bisphenol A and bisphenol B in canned seafood combining QuEChERS extraction with dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry. Analytical and Bioanalytical Chemistry, *404*, 2012, pp. 2453–2463. DOI: 10.1007/s00216-012-6389-5.
17. Andraščíková, M. – Hrouzková, S. – Cunha, S. C.: Combination of QuEChERS and DLLME for GC-MS determination of pesticide residues in orange samples. Food Additives and Contaminants A, *30*, 2013, pp. 286–297. DOI: 10.1080/19440049.2012.736029.
18. Cunha, S. C. – Fernandes, J. O.: Assessment of bisphenol A and bisphenol B in canned vegetables and fruits by gas chromatography-mass spectrometry after QuEChERS and dispersive liquid-liquid microextraction. Food Control, *33*, 2013, pp. 549–555. DOI: 10.1016/j.foodcont.2013.03.028.
19. Arroyo-Manzanares, N. – Huertas-Pérez, J. – Gámiz-Gracia, L. – García-Campañan, A. M.: A new approach in sample treatment combined with UHPLC-MS/MS for the determination of multiclass mycotoxins in edible nuts and seeds. Talanta, *115*, 2013, pp. 61–67. DOI: 10.1016/j.talanta.2013.04.024.
20. Commission Regulation (EU) No 835/2011 of 19 August 2011 amending Regulation (EC) No 1881/2006 as regards maximum levels for polycyclic aromatic hydrocarbons in foodstuffs. Official Journal of the European Union, *L 215/4*, 2011, pp. 4–8. DOI: 10.3000/17252555.L_2011.215.eng.
21. Commission Regulation (EU) No 836/2011 of 19 August 2011 amending Regulation (EC) No 333/2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs. Official Journal of the European Union, *L 215/4*, 2011, pp. 9–16. DOI: 10.3000/17252555.L_2011.215.eng.
22. Sadowska-Rociek, A. – Surma, M. – Cieślik, E.: Determination of PAHs in cocoa samples using d-SPE with different sorbents. Journal of Microbiology, Biotechnology and Food Sciences, *4*, 2015, pp. 135–137. DOI: 10.15414/jmbfs.2015.4.special3.135-137.
23. Farajzadeh, M. A. – Khoshmaram, L. – Nabil, A. A. A.: Determination of pyrethroid pesticides residues in vegetable oils using liquid–liquid extraction and dispersive liquid–liquid microextraction followed by gas chromatography–flame ionization detection. Journal of Food Composition and Analysis, *34*, 2014, pp. 128–135. DOI: /10.1016/j.jfca.2014.03.004.
24. Liu, S. – Xie, Q. – Chen, J. – Sun, J. – He, H. – Zhang, X.: Development and comparison of two dispersive liquid–liquid microextraction techniques coupled to high performance liquid chromatography for the rapid analysis of bisphenol A in edible oils. Journal of Chromatography A, 1295, 2013, pp. 16–23. DOI: 10.1016/j.chroma.2013.04.054.
25. Ellison, S. L. R. – Williams, E. (Ed): Eurachem/CITAC guide: Quantifying uncertainty in analytical measurement. 3rd edition. Leoben : Eurachem, 2012. ISBN 978-0-948926-30-3. <https://www.eurachem.org/images/stories/Guides/pdf/QUAM2012_P1.pdf>
26. Investigation into levels of polycyclic aromatic hydrocarbons (PAHs) in food on the Irish market. Dublin : Food Safety Authority of Ireland, 2006. <https://www.fsai.ie/uploadedFiles/PAH_levels.pdf>

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