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Rapid determination of polycyclic aromatic hydrocarbons (PAHs) in tea using two-dimensional gas chromatography coupled with time of flight mass spectrometry

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ABSTRACT

A simple, fast, and cost effective sample preparation procedure has been developed and validated for the determination of 15+1 European Union Polycyclic Aromatic Hydrocarbons (15+1 EU PAHs) in dried tea leave samples. Based on a critical assessment of several sample extraction/clean-up approaches, the method based on the ethyl acetate extraction followed by the use of PAHs dedicated cartridges with molecularly imprinted polymers (MIPs) has been found as an optimal alternative in terms of time demands and obtained good extract purity. For the final identification/quantification of target PAHs, two dimensional gas chromatography coupled to a time-of-flight mass spectrometry (GC × GC–TOFMS) was used. The performance characteristics of the overall analytical method for individual PAHs determined at three spiking levels (0.5, 2.5 and 5 μg kg^{-1}) were in following ranges: limits of quantitation (LOQs) 0.05–0.2 μg kg^{-1}, repeatabilities 2–9%, and recoveries 73–103%. The recoveries achieved by the newly developed sample preparation procedure when employed for naturally contaminated sample ("incurred" PAHs) were comparable to those obtained by other routinely used approaches employing sonication and/or pressurised liquid extraction for sample analytes isolation. The validated method was subsequently used for the determination of selected genotoxic PAHs in 36 samples of black and green tea obtained from the Czech retail market. The levels of ΣPAH4 (sum of benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbFA) and benzo[a]pyrene (BaP)) in black and green tea leaves ranged from 7.4 to 700 μg kg^{-1} and from 4.5 to 102 μg kg^{-1}, respectively. Contamination of tested tea samples by BaP was in the range of 0.2–152 μg kg^{-1}.

1. Introduction

Tea, an aromatic infusion prepared by adding hot water to leaves of the *Camellia sinensis* plant, is one of the most widely consumed beverages in the world [1]. In addition to its specific flavour, tea is also valued for health-promoting properties associated with antioxidants and other biologically active compounds. However, under certain conditions, tea leaves may contain some organic contaminants such as pesticide residues and/or polycyclic aromatic hydrocarbons (PAHs). In the latter case, improper drying practices are typical cause of contamination by these products. Due to incomplete pyrolysis of carbonaceous materials such as wood, oil or coal, combustion gases used sometimes for direct drying may contain both gaseous and/or particles bond PAHs which are deposited on the dried tea leaves [2]. Long-distance atmospheric transportation of PAHs containing particles originated from various emission sources and, later on, their deposition on the vegetation surfaces is another conceivable source of tea leaves contamination [3].

With regard to an increasing public health concerns on the dietary exposure to genotoxic carcinogens such as PAHs, their occurrence in various food items was reviewed by the CONTAM panel of the European Food Safety Authority (EFSA) in 2008 [4]. Dried tea was identified as one of the food categories with consistently high content of PAHs. Currently, maximum levels of benzo[a]pyrene (BaP) in various foodstuffs including oils and fats, smoked meats/fish, baby food, crustaceans and mussels are specified in a Commission Regulation (EC) 1881/2006 [5]. It should be noted that according to EFSA's CONTAM panel opinion [4], BaP is not the only suitable indicator for the PAHs occurrence, since other carcinogenic/genotoxic PAHs could be detected in many samples negative for this contaminant. On this account, obtaining data on the sum of BaP, chrysene (CHR), benz[a]anthracene (BaA) and benzo[b]fluoranthene (BbFA) (PAH 4),

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alternatively, sum of BaA, BaP, BbFA, CHR, benzo[k]fluoranthene (BkFA), benzo[g,h,i]perylene (BghiP), dibenz[a,h]anthracene (DBahA), indeno[1,2,3-cd]pyrene (IP) (PAH 8), is required for the risk assessment process [4].

Although, PAHs are hydrophobic compounds, some transfer from dried tea leaves into hot water during infusion preparation cannot be avoided. Therefore, contamination extent of this commodity should be controlled by a reliable method [6]. In general terms, steps involved in the method employed for analysis of PAHs in respective food matrix fairly depend on its composition. While in most food samples, lipids are the main co-extracts when hydrophobic PAHs are isolated by low/medium polar solvents, this is not the case of tea leaves. Caffeine, various pigments and (poly)phenolics which are transferred into crude extracts are typical tea co-extracts to be removed in the following clean-up step [7]. A wide range of analytical strategies has been employed in various studies for extraction of PAHs. Pressurized liquid extraction (PLE) [2,8], supercritical fluid extraction (SFE) [9,10], microwave-assisted extraction (MAE) [1], sonication [2,3,6,9–14], saponification [2], stir bar sorptive extraction (SBSE) [15], solid phase micro extraction (SPME) [16–18] and Soxhlet extraction [9,10,19,20] are the most often applied extraction methods. In the next phase, typically two (sometimes even more) clean-up steps such as gel permeation chromatography (GPC) [2,7] combined with solid phase extraction (SPE) on silica gel [2,3,6–9,11,12,20,21], florisil [19] or C_{18} silica [8,13,14] are involved in the sample preparation process. Rather surprisingly, in only few papers concerned with a method development for analysis of PAHs in tea, full validation was documented, see overview in Table 1. For the detection and quantitation, both high performance liquid chromatography (HPLC) with ultraviolet (UV) or fluorescence detection (FLD) [6,11,12–14,17,18,21] and gas chromatography (GC) with flame ionization detection (FID) [3,10], magnetic sector mass spectrometry (MS) [2,7] or single quadrupole MS [8,9,15,16,19] have been already applied.

With respect to the lack of data on the levels of an extended range of priority PAHs in tea, the aim of the presented study was: (i) to develop and validate a simple, fast and cost-effective sample preparation procedure enabling reliable GC–MS based determination of 15+1 EU genotoxic PAHs classified by the Scientific Committee on Food (SCF) and Joint FAO/WHO Experts Committee on Food Additives (JECFA) [22] as a priority PAHs in the tea samples, and (ii) to apply this new method for the analysis of tea samples collected at the Czech market.

2. Experimental

2.1. Tea samples

The sample of black tea with low level of PAHs (BaP < 0.15 μg kg^{-1}) was used within the method optimization and validation part and in the following study on PAH levels in real tea samples 18 samples of black and 18 samples of green tea originated from the different regions of China (14), India (12), Nepal (5) and Sri Lanka (5) were tested. All of them were obtained from the Czech retail market. After the delivery into the laboratory, each sample was homogenized using a blender and stored at the room temperature until analysis.

2.2. Chemicals

Cyclohexane, *n*-hexane, (SupraSolv® quality, Merck, Germany), toluene (Merck, Germany), ethyl acetate and dichloromethane (for GC residue analysis, Scharlau, Spain) were used as supplied. Acetonitrile, magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) were delivered from Sigma Aldrich (Germany) and Lach-Ner (Czech Republic), respectively. Sea sand and aluminium oxide 90 neutral (for column chromatography) were obtained from Merck (Germany). Chloroform and acetone (analytical reagent grade, Lachema, Czech Republic) were re-distilled before use. Styrene-divinylbenzene gel (Bio-Beads® S-X3, 200–400 mesh) was purchased from Bio-Rad (USA). Silica gel (0.063–0.200 mm; Merck, Germany) was activated by heating at 180 °C for 5 h and then deactivated by addition of 2% (*w*/*w*) deionized water. SupelMIP™ SPE-PAH cartridges (50 mg/3 mL)

Table 1
An overview of analytical methods developed for determination of PAHs in dried tea leaves/tea plants.

Target analytes	Sample preparation	System	Performance characteristics	Ref.
AN, BkFA, BaP	1. Microwave-assisted extraction (MAE) by DMSO 2. Liquid–liquid extraction—DMSO, *n*Hex	Spectrofluorimeter	LODs: 0.18–3.58 μg kg^{-1} Recovery: 87.1–93.1% Repeatability: 1.5–8.5% Linearity: R^2 > – Linear range: –	[1]
15+1 EU PAHs	1. ASE using nHex:Ac (1:1, *v*/*v*), 2. GPC (Bio-Beads S-X3, cHex:EtOAc (1:1, *v*/*v*), 3. SPE column (silica gel, cHex)	GC/HRMS (sector mass spectrometer)	LOQ: 0.03–0.06 μg kg^{-1} Recoveries: 75–117% Repeatability: 0.1–11% Linearity: $R^2 > 0.996$ Linear range: 1–20 μg kg^{-1}	[7]
16 EPA PAHs	1. Sonication (MeOH, 15 min) 2. in-tube solid-phase microextraction –CP-Sil 19CB, (MeCN:H_2O, 5:95, *v*/*v*)	HPLC/FLD reversed phase (MeCN:H_2O; gradient elution)	LOQ: 0.21–3.08 μg kg^{-1} Recoveries: 69.9–109.8% Repeatability: – Linearity: $R^2 > 0.997$ Linear range: 0.05–2.0 ng mL^{-1}	[17,18]
16 EPA PAHs	1. Solid-liquid extraction using cHex: DCM (1:1, *v*/*v*), 2. SPE column (silica gel, *n*Hex:DCM, 1:1, *v*/*v*)	reversed phase (MeCN:MeOH:H_2O; gradient elution) HPLC/FLD, DAD	LOQ:0.2–0.3 μg kg^{-1} Recoveries: 78–102% Repeatability: – Linearity: $R^2 > 0.97$ Linear range: –	[21]

"—" not specified in the study; LOD—limit of detection; LOQ—limit of quantitation; nHex—*n*-hexane; Ac—acetone; cHex—cyclohexane; EtOAc—ethyl acetate; MeOH—methanol; MeCN—acetonitrile; DCM—dichloromethane; DMSO—dimethylsulfoxide.
15+1 EU PAHs—15 PAHs classified as priority by the SCF and benzo[c]fluorene assessed to be relevant by the JECFA.
16 EPA PAHs—16 PAHs identified as the most frequent in environmental samples by the US Environmental Protecting Agency (EPA).

and primary secondary amine (PSA) were purchased from Supelco (USA). Water purified with a Milli-Q system (Millipore, USA) was used for the experiments.

Individual standard solutions of PAHs–benz[a]anthracene (BaA), benzo[a]pyrene (BaP), benzo[b]fluoranthene (BbFA), benzo[k]fluoranthene (BkFA), benzo[g,h,i]perylene (BghiP), dibenz[a,h]anthracene (DBahA), chrysene (CHR), indeno[1,2,3-cd]pyrene (IP), benzo[c]fluorene (BcFL), cyclopenta[c,d]pyrene (CPP), 5-methylchrysene (5-MCH), benzo[j]fluoranthene (BjFA), dibenzo[a,l]pyrene (DBalP), dibenzo[a,e]pyrene (DBaeP), dibenzo[a,i]pyrene (DBaiP), dibenzo[a,h]pyrene (DBahP) either in cyclohexane or isooctane (10 $\mu g\,mL^{-1}$) were supplied by Dr. Ehrenstorfer GmbH (Darmstadt, Germany). The purity of individual standards was not less than 98%. Eight calibration solutions (stored at $-12\,^{\circ}C$) were prepared in isooctane; they contained all PAHs mentioned above at concentration levels 0.05, 0.1, 0.5, 1, 5, 10, 50, and 100 $ng\,mL^{-1}$. Certified standard solution of labelled PAHs in nonane, US EPA 16 PAH Cocktail (acenaphthene-$^{13}C_6$ (AC-$^{13}C_6$), acenaphthylene-$^{13}C_6$ (ACL-$^{13}C_6$), anthracene-$^{13}C_6$ (AN-$^{13}C_6$), BaA-$^{13}C_6$, BaP-$^{13}C_4$, BbFA-$^{13}C_6$, BkFA-$^{13}C_6$, BghiP-$^{13}C_{12}$, DBahA-$^{13}C_6$, fluoranthene-$^{13}C_6$ (FA-$^{13}C_6$), fluorene-$^{13}C_6$ (FL-$^{13}C_6$), CHR-$^{13}C_6$, IP-$^{13}C_6$, naphthalene-$^{13}C_6$ (NA-$^{13}C_6$), phenanthrene-$^{13}C_6$ (PHE-$^{13}C_6$), pyrene-$^{13}C_3$ (PY-$^{13}C_3$)) (5 $\mu g\,mL^{-1}$), certified standard of DBaiP-$^{13}C_{12}$ (50 $\mu g\,mL^{-1}$) and DBaeP-$^{13}C_6$ (100 $\mu g\,mL^{-1}$) in nonane were supplied by Cambridge Isotope Laboratories Inc. (USA). Working standard solutions were prepared in toluene and stored at $-12\,^{\circ}C$.

2.3. Equipment

A GRINDOMIX GM 200 (Retsch, Germany) homogenizer was used for the homogenization of tea samples. An automatic shaker IKA Laboratortechnic (Germany) for the sample preparation was used. An ASE 300 system (Accelerated Solvent Extractor, Dionex, USA) was used in the first part of the study for the extraction of the samples. An automated gel permeation chromatography (GPC) system consisting of a 305 MASTER pump, fraction collector, automatic regulator of loop XL, microcomputer (software 731 PC via RS232C), dilutor 401C (Gilson, France) and a steel column 500 × 10 mm I.D. packed with Bio-Beads® S-X3, 200–400 mesh (Bio-Rad Laboratories, USA) was applied for the clean-up of crude extracts during the optimization of the extraction procedure. A centrifugal machine Rotina 35R used for centrifugation during QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) and "QuEChERS like" sample preparation procedure was supplied by Hettich Zentrifugen (Germany). Analyses of purified extracts was performed using (i) a GC 6890N gas chromatograph equipped with a programmed temperature vaporizing (PTV) injector (septumless head, CO_2 cooling) and an automatic sampler ALS 7683B Series coupled with a mass spectrometer Agilent 5975 Inert XL MS in electron ionization (EI) mode employing separation on a capillary column BPX-50 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) from SGE Analytical Science (Australia) and/or (ii) a gas chromatograph Agilent 6890N (Agilent Technologies, USA) coupled to a high-speed time-of-flight mass spectrometer Pegasus III (LECO Corp, USA) operated in EI mode. The second GC system was equipped with a split/splitless injector and a MPS 2 autosampler (Gerstel, Germany). For the separation of target analytes capillary column BPX-50 (30 m × 0.25 mm i.d. × 0.25 μm film thickness) and BPX-5 (1 m × 0.10 mm i.d. × 0.1 μm film thickness) were obtained from SGE Analytical Science (Australia). The MSD Enhanced ChemStation (Agilent Technologies, USA) and the ChromaTOF 2.32 software (LECO Corp, USA) were used for the data processing.

2.4. Analytical methods

2.4.1. Isolation

Four sample preparation procedures characterized below were tested within this study:

(i) Sonication
An amount of 2 g of homogenized tea was placed into an Erlenmeyer flask and sonicated three times using 100, 50 and 30 mL of extraction solvent mixture, each for 20 min, with two different solvent mixtures: *n*-hexane:dichloromethane (1:1, *v*/*v*) and *n*-hexane:acetone (3:2, *v*/*v*). The crude extract was then filtered through the anhydrous sodium sulphate and evaporated using a rotary vacuum evaporator (RVO) at 35 °C and the remaining few drops of solvent were carefully removed by a gentle stream of nitrogen. The residues were re-dissolved in 5 mL of chloroform for the subsequent GPC clean-up step.

(ii) Pressurized liquid extraction (PLE)
An amount of 2 g of homogenized tea was mixed with 10 g of magnesium sulphate and transferred into the 33 mL extraction cell. The remaining volume was completely filled up with sea sand and the cell was then placed into the ASE 300 system. The same extraction mixtures as those tested for sonication-assisted extraction were used. PLE conditions were earlier optimised for this type of matrix and the sample preparation was in house validated. The experimental set-up of PLE was as follows: 2 static extraction cycles (5 min), the pressure 1500 psi (10.34 MPa), extraction temperature 100 °C, flush volume 60% of the extraction cell volume and purge of cell by nitrogen 60 s. The extracts were evaporated as in the previous procedure. The residue was dissolved in 5 mL of chloroform.

(iii) Extraction by shaking
For a solvent extraction employing shaking the method published earlier by Danyi et al. [21] was used. An amount of 1 g of tea was extracted three times for 15 min with 15 mL of mixture of cyclohexane:dichloromethane (1:1, *v*/*v*) using an automatic shaker. The extraction step was performed in the presence of 0.5 g primary–secondary amine (PSA) and 1 g deactivated aluminium oxide (deactivation by addition of 5% of distilled water). The crude extract was centrifuged (11,000 rpm, 5 min), the organic phase was evaporated using a rotary vacuum evaporator and the remaining few drops of solvent were carefully removed by a gentle stream of nitrogen. The dry residue was reconstituted with 5 mL of chloroform.

(iv) QuEChERS
Extraction method QuEChERS, developed originally for pesticide residues analysis [23], was used for analytes isolation. An amount of 2 g of tea was mixed with 10 mL of distilled water and 10 mL of acetonitrile or ethyl acetate (the latter solvent was found as optimal) in a polypropylene tube and then, the suspension was shaken vigorously for 1 min. Subsequently, inorganic salts, 4 g of magnesium sulphate plus sodium chloride (either 1 or 2 g) were added. The tube was shaken once again for 1 min, then centrifuged for 5 min and, finally, an aliquot of 5 mL from the upper organic layer was taken and evaporated by RVO, remaining few drops of solvent were carefully eliminated under a gentle stream of nitrogen. The dry residue was reconstituted either with 5 mL of chloroform for GPC or in 2.5 mL of cyclohexane for SPE clean-up on MIPs cartridges.

2.4.2. Clean-up

To assess the efficiency of an extraction step, conventional GPC followed by silica SPE was firstly employed as a 'reference' clean-up procedure. Afterwards, when isolation of target analytes was

optimized, a new SPE clean-up approach was tested and time and labour demanding GPC followed by SPE was replaced by a single step SupelMIP™ SPE-PAH column purification.

(i) *Clean-up using GPC followed by SPE on silica columns.*
For the GPC employing Bio-Beads S-X3 gel and chloroform as a mobile phase with a flow rate 0.6 mL min^{-1}, was used. 2 mL of the crude extract were injected. The fraction corresponding to the elution volume of 16–32 mL was collected. The eluate was then evaporated using a RVO followed by a gentle stream of nitrogen. The residue obtained in the first clean-up step (GPC) was dissolved in 1 mL of *n*-hexane and transferred onto 1 g in-house prepared silica gel mini-column pre-conditioned with 6 mL of *n*-hexane:dichloromethane (3:1, *v*/*v*) and 3 mL of *n*-hexane. For elution of target analytes, 10 mL of *n*-hexane:dichloromethane (3:1, *v*/*v*) were used. The eluate was evaporated, re-dissolved in 0.25 mL of toluene containing ^{13}C-labelled PAHs (2 ng mL^{-1}) used as syringe standards, prior to GC–MS analysis, the sample was transferred into a glass vial for a subsequent GC–MS analysis.

(ii) *Clean-up using SupelMIP™ SPE-PAH column*
The commercial SPE cartridge (50 mg/3 mL) was conditioned with 1 mL of cyclohexane, then, the residue left after ethyl acetate evaporation (optimized ethylacetate method) and re-dissolved in 2.5 mL cyclohexane was loaded on the top of cartridge. Following washing with 3 mL of cyclohexane, PAHs were eluted with 6 mL of the elution solvent were used. Two different solvents ethyl acetate and dichloromethane were tested for elution within the method development experiment. For the final approach the dichloromethane was selected as the best choice. The eluate was evaporated, re-dissolved in 0.25 mL of toluene with ^{13}C-labelled PAHs (used as syringe standards) and transferred into a glass vial for a subsequent GC–MS analysis.

2.5. GC–MS analysis

Optimization experiments were performed using an Agilent 6890 GC system coupled to a quadrupole mass spectrometer Agilent 5975 Inert XL and target analytes were separated using BPX-50 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness). The GC conditions were as follows: oven temperature program: 110 °C (5.4 min), @ 50 °C min^{-1} to 350 °C (5.8 min). Carrier gas was helium with initial flow 2 mL min^{-1} in constant pressure mode. Programmed-temperature vaporiser (PTV) injection: solvent vent mode; injection volume 4 × 4 μL; vent time: 3.4 min; vent flow: 50 mL min^{-1}; vent pressure 50 psi (345 kPa); initial temperature: 50 °C (3.4 min); inlet rating velocity: 400 °C; final inlet temperature: 350 °C.

The MS detector was operated under the following conditions: quadrupole temperature: 150 °C; ion source temperature: 230 °C; transfer line temperature: 320 °C. The ions (*m*/*z*) selected for the monitoring of target PAHs are shown in Table 2.

2.6. GC × GC–TOFMS analysis

A method validation and subsequent analysis of real tea samples was performed using an Agilent 6890 GC system coupled to the high speed time-of-flight mass spectrometer Pegasus III. Target analytes were separated on BPX-50 capillary column (30 m × 0.25 mm i.d. × 0.25 μm film thickness) and BPX-5 (1 m × 0.1 mm i.d. × 0.1 μm film thickness) in the 1st and 2nd dimension, respectively. The GC conditions were as follows: oven temperature program: 1st dimension: 80 °C (4.3 min), @ 30 °C min^{-1} to 220 °C, @ 2 °C min^{-1} to 240 °C (0 min) and @ 10 °C min^{-1} to 360 °C (15 min); 2nd dimension: temperature offset 10 °C; carrier gas helium with a ramped flow 1.3 mL min^{-1} (19 min) @ 50 mL min^{-1} to 2 mL min^{-1} (16 min); PTV injection: solvent vent; injection volume 1 × 8 μL; vent time: 2.3 min; vent flow: 50 mL min^{-1}; vent pressure 50 psi; initial temperature: 50 °C (2.3 min); inlet rating velocity: 400 °C; final inlet temperature: 300 °C.

Table 2
Ions selected for GC–MS analysis of target PAHs.

Group	Analytes	Quantitation ion (*m*/*z*)	Confirmation ion (*m*/*z*)
PAHs	**BcFL**	216	215
	BaA, CHR	228	226
	CPP	226	228
	5-MCH	242	241
	BbFA, BkFA, BjFA, BaP	252	253
	IP, BghiP	276	277
	DBahA	278	276
	DBalP, DBaeP, DBaiP, DBahP	302	303
^{13}C-PAHs	**BaA-$^{13}C_6$, CHR-$^{13}C_6$**	234	232
	BbFA-$^{13}C_6$, BkFA-$^{13}C_6$	258	259
	BaP-$^{13}C_4$	256	257
	IP-$^{13}C_6$	282	283
	DBahA-$^{13}C_6$	284	282
	BghiP-$^{13}C_{12}$	288	289
	DBaeP-$^{13}C_6$	308	307
	DBaiP-$^{13}C_{12}$	314	313

The MS detector was operated under the following conditions: mass range: *m*/*z* 45–750 u; ion source temperature: 250 °C; transfer line temperature: 280 °C; detector voltage: 1950 V; acquisition rate: 100 spectra/s. The ions (*m*/*z*) selected for the quantitation are shown in Table 2.

The quantification of target analytes according to their height was performed using an eight points calibration curve. For elimination of potential injection inaccuracies, syringe standards were used as follows: corresponding ^{13}C-labelled analogues for PAHs and for those PAHs for which ^{13}C-labelled standards were not available, following ^{13}C-PAHs were used for the quantification: $^{13}C_4$-BaP for BjFA, $^{13}C_{12}$-DBaiP for DBahP and DBalP, $^{13}C_6$-CHR for CPP and 5MC and $^{13}C_6$-FA for BcFL.

3. Results and discussion

The aim of this study was to find and optimize a fast and reliable analytical procedure for determination of 15+1 EU PAHs in tea (dry tea leaves). As a 'reference', our routine, in-house validated method employing extraction by *n*-hexane:acetone mixture (3:2, *v*/*v*) supported by sonication for obtaining crude extract and followed by two clean-up steps was used. Firstly, part of co-extracts (mainly pigments) by GPC on BioBeads S-X3 was removed and subsequently, SPE on silica gel mini column provides final purification. However, such sample preparation procedure needs about 2.5 h per one sample, thus one-day sample throughput was 6 samples only. With regards to the growing demands for number of samples to be analyzed within various studies/surveillance programmes concerned with tea quality, a rapid and simple extraction approach for isolation of 15+1 EU PAHs from this matrix had to be searched. The experimental design of the 'new' method development, including parameters that were tested/optimized, is shown in Fig. 1. In the paragraphs below, individual phases of our study together with facts taken into account are described in a detail.

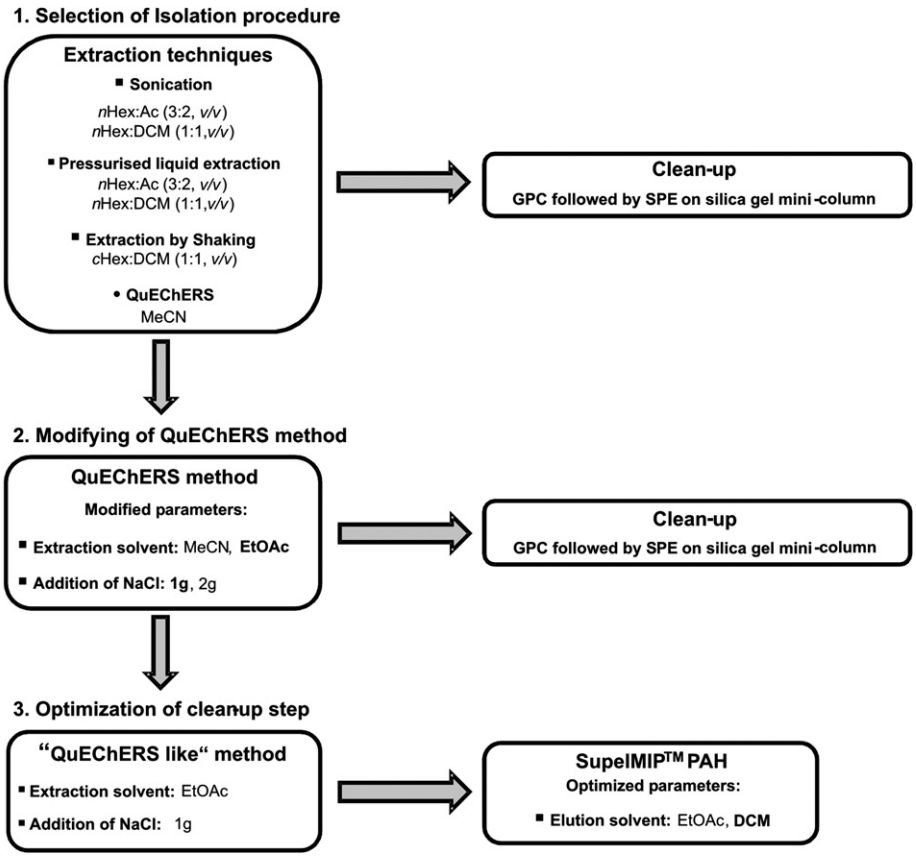

Fig. 1. Simplified overview of the tested extraction procedures together with the parameters that were subject of optimization. nHex—*n*-hexane; Ac—acetone; EtOAc—ethyl acetate; MeCN—acetonitrile, cHex—cyclohexane.

3.1. Selection of a suitable extraction approach

The efficiency of 15+1 EU PAHs of four conceivable extraction procedures was critically assessed:

(i) Extraction supported by sonication, either with *n*-hexane-dichloromethane (1:1, *v*/*v*) or *n*-hexane-acetone (3:2, *v*/*v*) mixtures—an approach routinely used in our laboratory for isolation of PAHs from tea,
(ii) PLE employing either *n*-hexane-dichloromethane (1:1, *v*/*v*) or *n*-hexane-acetone (3:2, *v*/*v*) mixtures as an efficient and a solvent saving automated extraction procedure often used for isolation of PAHs from dry matrices including tea [2,8];
(iii) Shaking with cyclohexane:dichloromethane (1:1, *v*/*v*) mixture—extraction technique often used in studies concerned with analysis of PAHs in dried plant matrices [21];
(iv) QuEChERS—an easy and efficient partition-based method that was primarily introduced in pesticide residue analysis in fruit/vegetable [23].

The extraction efficiencies achieved by these tested approaches when employed for analysis of tea samples spiked at level 2.5 μg kg^{-1} are summarized in Table 3. Slightly lower recoveries (55–81%) were obtained for the sonication using *n*-hexane: dichloromethane (1:1, *v*/*v*), shaking in cyclohexane: dichloromethane and PLE employing both of the tested solvent mixtures. On the other hand, sufficient recoveries 70–120%, were achieved for majority of the target PAHs by sonication with *n*-hexane: acetone (3:2, *v*/*v*), and QuEChERS, without any significant differences among these two sample preparation approaches. For further experiments, the QuEChERS procedure was selected as a fast, simple and high throughput approach which is becoming, in addition to analysis of pesticide residues, a widely used 'gold standard' for many contaminant groups [24–27]. Worth to notice, that adding water together with organic solvent, obviously represents an important way to modify dried tea leaves texture (some swelling occurs as the result of wetting) thus enabling accessibility of hydrophobic analytes for extraction.

3.2. Optimization of the QuEChERS extraction followed by SPE MIPs column

As soon as the QuEChERS extraction was identified as a promising option, more detailed search for its optimal setting was initiated. In line with the original QuEChERS approach, acetonitrile was the first tested extraction solvent. The practical problem, we experienced, was a relatively long time needed for a careful evaporation of partitioned acetonitrile phase that has to be carried out to enable solvent exchange prior to a subsequent clean-up step. At this stage, it was decided to replace acetonitrile by ethyl acetate possessing not only polarity more relevant to hydrophobic PAHs (while polarity index of acetonitrile is 5.8, ethyl acetate is rather less polar, their polarity index value is 4.4), but also representing a cheap solvent, easy to evaporate and posing a minimal workplace hazard.

Also, the effect of the amount of NaCl added to induce the transfer of analytes into organic phase during a partition step was studied. The results of this part of experiments are shown in Fig. 2. To illustrate the effect of tested parameters, seven representative analytes were selected: (i) BaA, CHR, BbFA and BaP that represent PAH4 identified by EFSA as the most suitable indicators of food contamination by carcinogenic/genotoxic PAHs; (ii) BcFL, the last added analyte to 15+1 EU PAHs, and (iii) BghiP and DBaeP representing most heavy 6-ring PAHs. The obtained results

Table 3
The efficiency of the tested extraction techniques employed for the isolation of PAHs from black tea sample, PAHs spike 2.5 μg kg^{-1}, each (n=6).

	Sonication				**PLE**				**Extraction by shaking**		**QuEChERS**	
	*n*Hex:Ac (3:2, *v*/*v*)		*n*Hex:DCM (1:1, *v*/*v*)		*n*Hex:Ac (3:2, *v*/*v*)		*n*Hex:DCM (1:1, *v*/*v*)		cHex:DCM (1:1, *v*/*v*)		MeCN 1 g NaCl	
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
5-MCH	85	8	68	5	73	2	78	5	70	5	94	4
BaA	77	2	55	6	62	2	72	6	64	7	81	4
BaP	94	8	72	8	72	4	79	6	74	4	87	4
BbFA	84	6	67	5	79	3	80	6	68	5	98	6
BcFL	63	4	55	4	70	6	74	2	61	4	74	9
BghiP	91	5	74	9	73	4	73	7	87	5	95	6
BjFA	89	5	72	7	81	3	76	4	79	8	95	3
BkFA	86	7	71	6	71	5	76	3	78	5	96	2
CHR	72	3	58	4	65	4	75	9	77	11	80	5
CPP	76	2	61	5	65	8	75	4	70	4	86	5
DBaeP	79	4	79	7	76	7	81	3	87	13	93	3
DBahA	87	2	78	9	74	5	73	6	80	6	98	2
DBahP	83	6	74	5	79	3	71	9	86	5	86	4
DBaiP	84	2	72	6	78	6	73	6	83	9	86	8
DBalP	85	7	75	5	79	5	76	6	83	9	94	4
IP	90	2	77	11	75	3	74	6	90	5	93	5

RSD—relative standard deviation; PLE—pressurized liquid extraction; nHex—*n*-hexane; cHex—cyclohexane, Ac—acetone; DCM—dichloromethane; MeCN—acetonitrile.

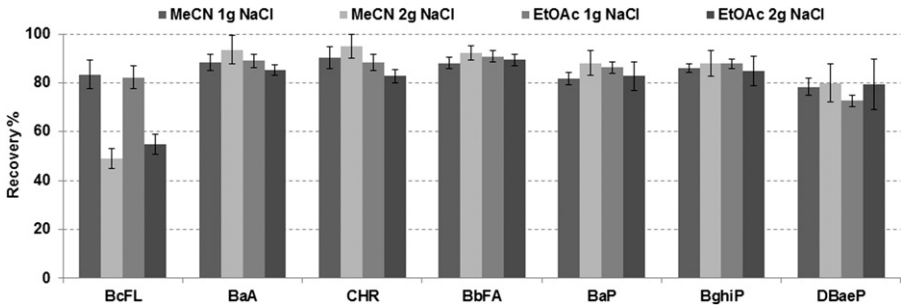

Fig. 2. The effect of the extraction solvent and amount of added NaCl on the extraction efficiency of selected representatives of PAHs expressed as mean recovery, tea spiked at level 2.5 μg kg^{-1}(each analyte) 'error bars' represent repeatability of the procedure expressed in percentages (n=6); MeCN—acetonitrile; EtOAc—ethyl acetate.

document the possibility to substitute acetonitrile by ethyl acetate. As regards NaCl addition, no significant differences in recoveries for most of the target PAHs were obtained for salt addition of 1 and 2 g. The only exception was BcFln recovery of which was slightly lower when higher amount of NaCl was added. For the follow-up experiments, addition of 1 g of NaCl was preferred.

3.3. Selection of elution solvent for MIPs cartridge

In the next phase, the choice of an optimal purification step which would enable removing most of co-extracts was investigated. As mentioned earlier, tea is a very complex matrix containing high amounts of extractable pigments, polyphenols, methyl xanthines, such as caffeine, purines and various phenolic acids [7]. Although a part of these co-extracts was left in an aqueous phase during QuEChERS partition, some less polar matrix components were transferred into ethyl acetate layer, thus contributing to a high chemical noise when introduced into the GC–MS system. To achieve sufficiently low detection limits, additional purification of a crude ethyl acetate extract was unavoidable. As mentioned in introduction, GPC followed by SPE is the most common clean-up set-up. However, this two-steps approach is rather time and labour demanding, therefore a suitable alternative enabling higher sample throughput was searched.

The use of commercial SupelMIP™ SPE cartridges employing molecularly imprinted polymer (MIP) with molecular recognition elements engineered to bind structurally related PAHs with a high selectivity was identified as a challenging option for removing residues of tea matrix. It is assumed that multiple interactions (such as hydrogen bonding, ionic, van der Waals or hydrophobic forces) taking place between the target analytes and MIP cavities are responsible for their binding [28]. Operatively, the MIP SPE technique is very similar to the traditional SPE performed on non-specific supports; usual steps of column conditioning, sample loading, column washing and analyte elution are carried out [29].

For the optimisation of a SupelMIP™ SPE clean-up, ethyl acetate (recommended in the application note) and dichloromethane were tested as elution solvents. First, the clean-up procedure described in the application note was tested [28]. In order to obtain an efficient clean-up effect, a washing step using 3 mL of cyclohexane instead of 1 mL recommended in the original procedure was included in the SPE procedure to remove more interfering compounds from the sorbent (no breakthrough of heavy PAHs (5–6 rings) occurred, slightly lower recoveries of 4-rings PAHs (BaA and CHR) may occur. The purification effect achieved by SupelMIP™ columns is illustrated in Fig. 3. The examples chromatograms of spiked black tea sample (spiking level 2.5 μg kg^{-1}) extracted by the optimized QuEChERs method followed by cleaned-up procedures using SupelMIP™ SPE and 2 different elution solvents, (A) ethyl acetate and (B) dichloromethane, are documented here. Finally,

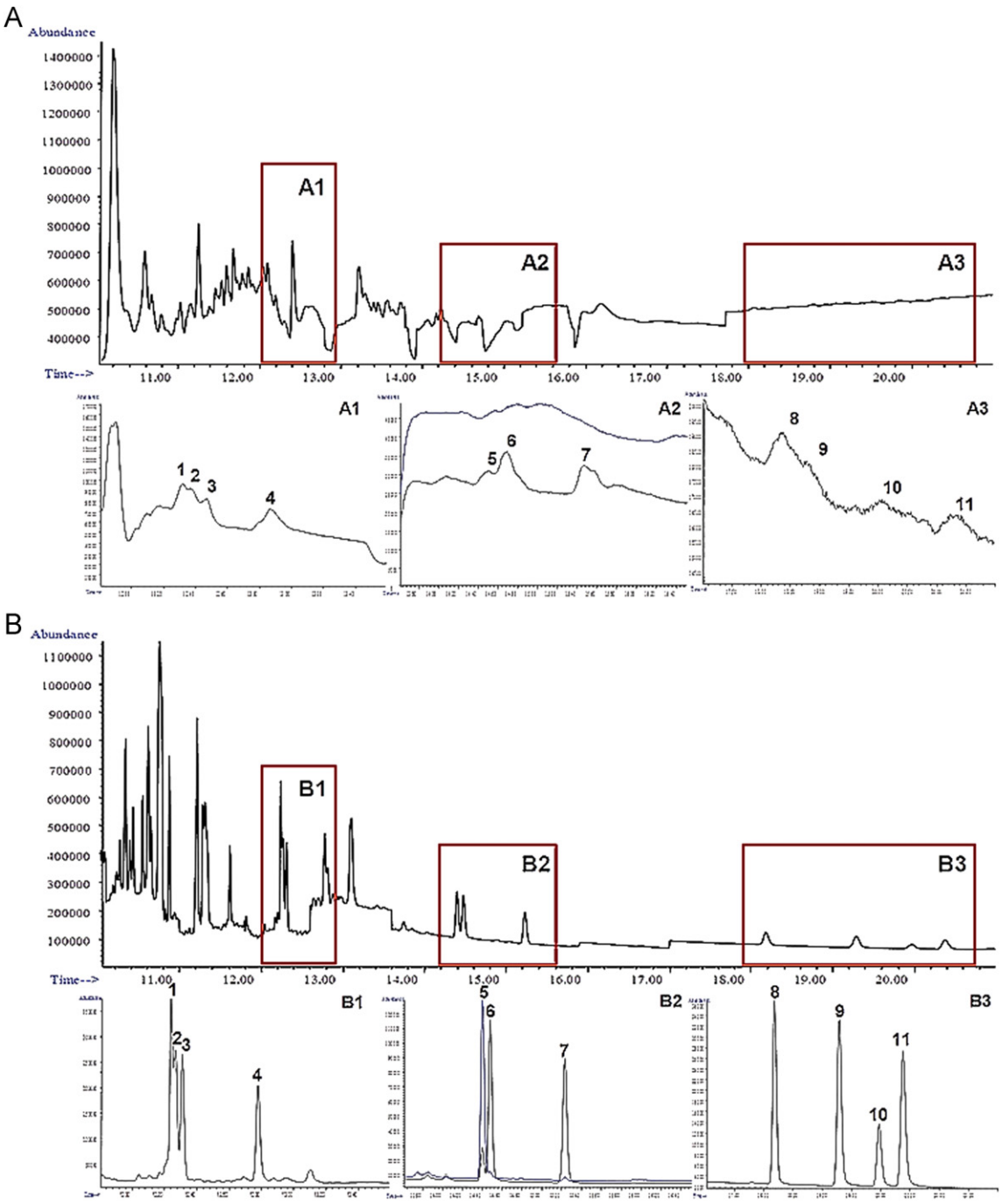

Fig. 3. Comparison of a GC–MS chromatogram of extract purified on MIPs SPE column using (A) ethyl acetate and (B) dichloromethane as an elution solvent in TIC and in SIM (A1,B1) *m*/*z* 252; (A2,B2) *m*/*z* 276, 278 and (A3,B3) *m*/*z* 302. 1-BbFA; 2-BkFA; 3-BjFA; 4-BaP; 5-DBahA; 6-IP; 7-BghiP; 8-DBalP; 9-DBaeP; 10-DBaiP; 11-DBahP

dichloromethane was chosen as an alternative to ethyl acetate since it is less polar and often used as an elution solvent suitable for crude extract purification. For the purpose of identification/quantification of target analytes during the optimization experiments a single quadrupole GC–MS was used because of the data handling is simpler compared to those from GC × GC–TOFMS. Fig. 3A illustrates the presence of a huge amount of interfering compounds that disturb the identification of all target analytes when ethyl acetate was used as elution solvent. Significantly higher purification effect was achieved when dichloromethane was used. In this case only a few co-extracts were eluted at the beginning of the chromatogram where may complicate identification of only BcFL.

3.4. Comparison of different clean-up procedures

As already mentioned, two different clean-up techniques were compared within our study (i) a routinely used GPC on BioBeads S-X3 using chloroform as a mobile phase followed by SPE on in-house prepared 1 g silica cartridges and (ii) SupelMIP™–PAH SPE. As expected, more co-extracts were removed from the crude extract using GPC followed by silica gel SPE (see Fig. 4A and B). This approach is an excellent clean-up procedure for the removal of a variety of co-extracts. However, as also documented in Fig. 4A and B, comparing clean-up by both techniques, both are sufficient enough to enable the analysis of PAHs in tea extracts. In attempt to minimize the time needed for the crude extract clean-up,

achieving at the same time satisfactory recoveries, SPE on SupelMIP™ was evaluated as the best choice for the purification of tea samples. Compared to the conventional approach, up to 10 samples can be processed within one hour. Moreover, the volume of the chlorinated solvents and consumption of other chemicals was significantly reduced.

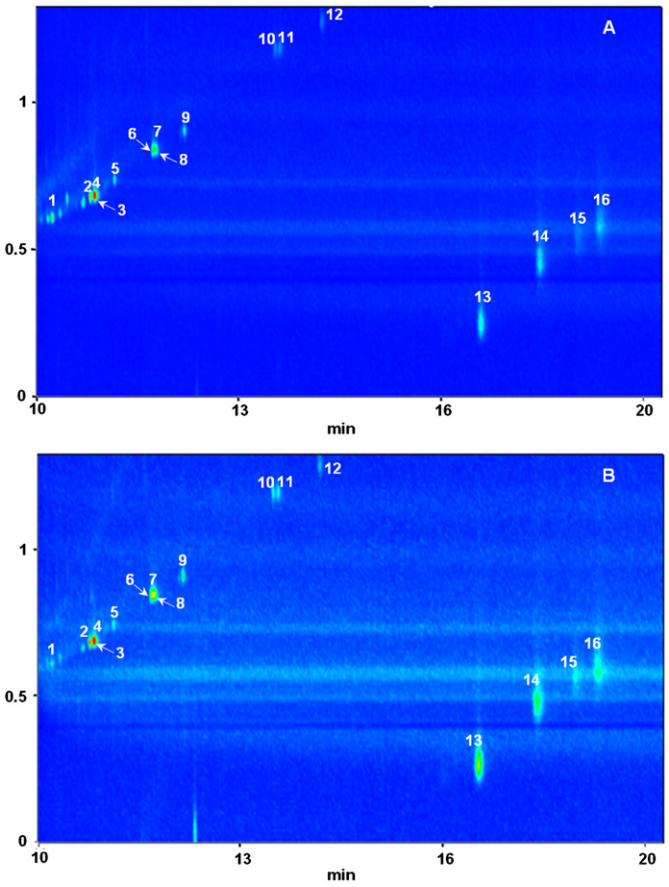

Fig. 4. An example of a GC × GC–TOFMS chromatogram of PAHs in the tea sample extracts purified (A) using GPC followed by SPE, (B) on SupelMIP™ PAHs. 1-BcFL; 2-BaA; 3-CHR; 4-CPP; 5-5-MCH; 6-BbFA; 7-BkFA; 8-BjFA; 9-BaP; 10-DBahA; 11-IP; 12-BghiP; 13-DBalP; 14-DBaeP; 15-DBaiP; 16-DBahP.

3.5. Validation of the developed method

Validation experiments were carried out in order to assess the accuracy of the optimized sample handling procedure (i.e., 2 g tea sample shaken with 10 mL water for 1 min, addition of 10 mL ethyl acetate and 1 g NaCl and shaking of 1 min, after evaporation, clean-up of crude extract using SPE on SupelMIP™). For the final identification/quantification of the target analytes, GC × GC–TOFMS was used. This technique was selected since it enabled obtaining lower LOQs and better separation of critical pairs of isomeric PAHs compared to one dimensional GC with quadrupole analyser. Since tea or similar herbal matrices with certified concentrations of PAHs (certified reference material—CRM) are not commercially available, the newly developed method was validated by the analysis of spiked black tea sample. To cover influence of the concentration, approach covering spiking at three different levels (0.5, 2.5 and 5 μg kg^{-1}) was applied with six replicates for each of the spiking levels. In the batch of every 6 samples the procedural blank was included. The performance characteristics obtained within the validation are summarized in Table 4. Recovery of all the target analytes were in the range of 73–103%. The repeatability of the procedure, expressed as the relative standard deviation (RSD, %) was satisfactory for all target analytes. In general, RSDs for most of the analytes did not exceed 10% and ranged from 2 to 12% for all PAHs. Using GC × GC–TOFMS the LOQs of the optimized sample preparation method were between 0.05 and 0.2 μg kg^{-1}. As shown in Table 4 the linearity of the calibration curves were in the range 0.05–100 ng mL^{-1} with correlation coefficients (R^2) ≥ 0.998 for most of the target PAHs.

High peak capacity provided by GC × GC, enabled separation of otherwise well-known critical groups of PAHs represented by: (i) BaA, CPP, and CHR; (ii) BbFA, BjFA, and BkFA; and (iii) DBahA, IP, and BghiP. The only remaining problematic pair was CHR/Tri, which have the same quantification mass (m/z). When injecting equal concentrations of these analytes, apexes of their peaks were under employed GC × GC conditions partly resolved (20%). However, in case of significantly lower concentration of one of them, the detection of separated peak apexes was not possible anymore. Nevertheless, some broadening of co-elution band can be observed. Fortunately, in real life samples the concentrations of CHR/Tri do not differ significantly. To avoid unacceptable overestimation of CHR concentration, measurement of peak height instead of peak area was used for quantification.

Table 4
Performance characteristics of the optimal method proposed for the preparation of tea samples, validation at three spiking levels (n=6 each).

	Level I (0.5 μg kg^{-1})		Level II (2.5 μg kg^{-1})		Level III (5 μg kg^{-1})		LOQ (μg kg^{-1})	Linear range (ng mL^{-1})	Correlation coefficient (R^2)
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)			
5-MCH	85	2	84	4	90	3	0.2	0.1–100	0.9995
BaA	86	4	80	2	84	5	0.05	0.05–100	0.9981
BaP	88	3	93	10	92	5	0.05	0.05–100	0.9994
BbFA	98	2	93	5	103	2	0.05	0.05–100	0.9988
BcFL	73	4	82	8	82	4	0.2	0.1–100	0.9997
BghiP	76	2	83	6	81	3	0.05	0.05–100	0.9992
BjFA	87	5	88	4	95	6	0.05	0.05–100	0.9986
BkFA	93	3	94	5	88	2	0.05	0.05–100	0.9963
CHR	88	3	84	9	89	3	0.05	0.05–100	0.9985
CPP	84	6	88	12	84	5	0.05	0.05–100	0.9993
DBaeP	75	3	84	6	82	4	0.2	0.1–100	0.9969
DBahA	88	4	83	2	84	7	0.05	0.05–100	0.9978
DBahP	79	5	79	7	84	6	0.2	0.1–100	0.9993
DBaiP	77	6	80	7	82	8	0.2	0.1–100	0.9978
DBalP	85	5	91	8	84	5	0.2	0.1–100	0.9980
IP	86	4	92	8	92	4	0.05	0.05–100	0.9989

Repeatability was calculated as a relative standard deviation (RSD, %), n=6.

Table 5
Levels of 15+1 PAHs (μg kg^{-1}) and repeatabilities of measurements (n=3) obtained by procedures involving (A) sonication with clean-up using GPC followed by silica SPE, (B) pressurized liquid extraction with clean-up using GPC followed by silica SPE and (C) new method followed by SupelMIP™ SPE clean-up.

Analyte	Sonication, *n*Hex:Ac (3:2, *v*/*v*)		PLE, *n*Hex:Ac (3:2, *v*/*v*)		New method	
	(μg kg^{-1})	RSD %	(μg kg^{-1})	RSD %	(μg kg^{-1})	RSD %
5-MCH	0.49	6	0.38	8	0.52	5
BaA	4.88	4	3.98	5	4.64	12
BaP	1.69	4	1.23	6	1.73	6
BbFA	3.49	6	2.76	3	3.49	7
BcFL	1.22	8	0.92	7	1.03	9
BghiP	1.35	5	1.08	5	1.39	4
BjFA	3.09	7	2.39	6	3.00	6
BkFA	3.69	5	2.88	4	3.57	8
CHR	11.5	8	9.76	6	10.2	10
CPP	1.25	4	1.04	10	1.32	5
DBaeP	0.43	5	0.31	7	0.50	4
DBahA	0.40	5	0.32	6	0.48	6
DBahP	n.d.	–	n.d.	–	n.d.	–
DBaiP	n.d.	–	n.d.	–	n.d.	–
DBalP	n.d.	–	n.d.	–	n.d.	–
IP	1.22	4	0.95	8	1.33	6

n.d.—not detected; relative standard deviation (RSD, %), n=3.

Table 6
Levels of 15+1 EU PAHs (μg kg^{-1}) determined in the set of black and green tea samples obtained at the Czech retail market.

Analyte	BLACK TEA (n=18)				GREEN TEA (n=18)			
	Mean	Median	90% Percentile	(min–max)	Mean	Median	90% Percentile	(min–max)
5-MCH	1.3	0.4	2.9	(0.2–5.3)	1.0	0.9	1.7	(0.2–2.6)
BaA	31.4	3.5	55.5	(1.4–196.1)	11.2	4.7	26.8	(0.7–28.3)
BaP	21.8	1.4	33.4	(0.2–151.7)	6.7	2.9	16.8	(0.2–17.9)
BbFA	20.7	4.1	35.5	(0.9–123.2)	10.8	11.8	18.3	(0.7–23.9)
BcFL	5.2	1.7	9.8	(0.7–20.3)	1.9	1.6	2.7	(<0.3–5.5)
BghiP	9.8	1.3	20.6	(0.4–54.4)	5.5	2.0	13.6	(0.6–15.7)
BjFA	10.5	2.9	15.9	(0.7–59.2)	5.2	3.2	8.8	(0.8–17.5)
BkFA	13.1	4.3	29.2	(1.0–63.2)	8.5	5.9	16.3	(0.9–24.0)
CHR	41.9	10.4	69.0	(3.9–229.0)	21.2	19.9	37.2	(2.9–42.4)
CPP	7.5	3.4	17.3	(<0.3–32.3)	6.0	4.7	10.5	(<0.3–17.1)
DBaeP	0.9	0.4	1.5	(n.d.–5.3)	1.0	0.5	2.2	(n.d.–2.9)
DBahA	4.5	0.5	10.4	(0.2–29.7)	0.9	0.7	2.0	(0.2–2.3)
DBahP	0.2	–	–	(n.d.–1.6)	–	–	–	n.d.
DBaiP	0.3	–	0.0	(n.d.–3.1)	0.1	–	0.4	(n.d.–0.5)
DBalP	0.6	–	0.8	(n.d.–4.3)	1.0	0.1	2.2	(n.d.–5.6)
IP	7.5	1.2	16.8	(0.2–39.4)	5.5	2.3	12.6	(0.4–12.9)
∑PAH4	115.9	17.1	167.7	(7.4–699.9)	49.4	47.3	98.3	(4.5–102.3)
∑15+1PAH	175.9	33.7	268.2	(12.7–988.3)	83.7	82.4	154.4	(10.3–159.7)

n.d.—not detected; than 50% values < LOD, median not calculated.

Table 7
The comparison of results generated within presented study with similar studies conducted worldwide in the recent 15 study.

Tea type	Sampling market	Number of samples	ΣPAH4 (min-max, μg.kg^{-1})	BaP (min-max, μg.kg^{-1})	Ref.
Black tea	**Czech Republic**	**18**	**7.4–699.9**	**0.2–151.7**	**This study**
	China	4	–	5.6–22.2	[1]
	Japan	4	21.9–205.1	5.3–73.2	[6]
	Germany	11	9.0–44.6	0.8–14.1	[7]
	China	2	–	20.1, 246.0	[9]
	China	1	493.3	39.7	[12]
	Austria	4	5.0–103.7	0.4–5.9	[17]
Green tea	**Czech Republic**	**18**	**4.5–102.3**	**0.2–17.9**	**This study**
	China	4	–	n.d. –23.0	[1]
	Japan	2	3.0, 3.8	15.5, 23.4	[6]
	Germany	11	12.3–167.9	1.6–32.6	[7]
	China	1	–	6.8	[9]
	China	2	25.2, 31.3	n.d.	[12]
	Germany	2	59.4, 101.2	7.4, 9.7	[17]
	Austria	1	73.7	3.1	[19]

"–" not analyzed, ΣPAH4-sum of BbFA, BaP, BaA, CHR, n.d.—not detected.

Due to the lack of the tea certified reference materials, the efficiency of the new validated method was assessed by its comparison with the routinely used sonication method and PLE extraction, both followed by GPC clean-up on the naturally contaminated black tea sample. As shown in Table 5, lower PAH levels compared to sonication were obtained when PLE for the extraction was used. Repeatabilities and findings achieved by sonication and the "QuEChERS like" method almost identical, but slightly lower recoveries for BcFL, BaA and CHR using the new extraction procedure were obtained. This may be caused by the use of a larger amount of solvent (3 mL) in washing step in sample clean-up to remove more interfering compounds from the sorbent.

3.6. Levels of PAHs in tea samples

Following successful method validation, the survey focused on the assessment of PAH levels both in black and green tea leaves was performed on altogether 36 tea samples (18 green and 18 black) of a different origin represented by China, India, Nepal, Sri Lanka (all samples were collected at the Czech market in 2011); the aggregated data are shown in Table 6. In all analysed samples at least 10 of 16 target PAHs exceeded limits of quantitation (LOQs). Comparing average PAHs levels in green and black tea samples, the latter ones were clearly more contaminated, probably due to drying processes employed during production. The highest BaP content was found in black Assam tea (152 $\mu g\,kg^{-1}$), while the maximum level determined in green tea Pu-Erh was lower by one order of magnitude (17.9 $\mu g\,kg^{-1}$). As regards the presence of "heavy" dibenzopyrenes, DBaiP and DBahP in only 1 sample of green and 2 samples of black tea were detected. Regarding contamination patterns, these largely varied among the samples, nevertheless, no distinct trend characterizing country/area of origin was identified.

The results of this study were subsequently compared with the PAHs findings reported for black/green tea in similar studies conducted worldwide, see Table 7 (the data on contamination of other types of tea such as herbal tea, Mate, etc. were not included). As shown in Table 7, the mean PAHs content in black tea collected at the German market was rather lower compared to that found in Czech samples. The highest contamination of this commodity was reported by Lin et al. [12] in China tea, where the content of BaP was 246 $\mu g\,kg^{-1}$.

4. Conclusions

A new simple sample preparation 'QuEChERS-like' procedure followed by SPE on molecularly imprinted polymers (MIPs) clean-up, that has been developed and extensively validated in this study, represents a challenging alternative for control of 15+1 EU PAHs content in dried tea leaves. Target analytes are extracted from wetted sample by ethyl acetate, their quantitative transfer into organic layer is supported by added inorganic salts. Tailor-made SupelMIP™ PAH cartridges enable an effective isolation of PAHs from most of matrix components contained in a complex crude extract. To achieve a maximum resolving power for critical PAH groups and minimize interfering chemical noise, originated by residual matrix, comprehensive GC × GC–TOFMS represents the best option. Low LOQs in the range 0.05–0.20 $\mu g\,kg^{-1}$ can be achieved not only thanks to an introduction of a relatively large sample equivalent (32 mg tea) onto the GC column by means of LV–PTV, but also due to peaks compression during the analyte transfer onto the second dimension column. The performance characteristics achievable by this method (i.e., the recoveries in the range 73–100% and repeatabilities 2–12%) comply with criteria required for the official control of BaP in the Regulation 333/2007/EC which might be generically applied also for other PAHs.

Compared to the 'classic', until now the most widely used approaches consisting typically from time-consuming extraction (such as Soxhlet extraction), followed by two steps clean-up, (typically GPC followed by SPE), the new procedure enables significant labour reduction and substantially increased sample throughput: one analyst can prepare 10 samples for the final instrumental determinative analysis in less than 1 h.

The small survey that was conducted in the final phase of our study (18 black and 18 of green tea samples) showed detectable PAHs in all analyzed samples. The BaP content ranged from 0.20 up to 152 $\mu g\,kg^{-1}$, the sum of 15+1 EU PAHs was in the range 10.3–988 $\mu g\,kg^{-1}$.

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