Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 104 (2007) 876–887
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

Validation (in-house and collaborative) of a method based on liquid chromatography for the quantitation of 15 European-priority polycyclic aromatic hydrocarbons in smoke flavourings: HPLC-method validation for 15 EU priority PAH in smoke condensates

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Received 23 March 2006; received in revised form 2 October 2006; accepted 4 October

Abstract

A method based on high-performance liquid-chromatography with ultra-violet absorption and fluorescence detection was developed for the purpose of quantitation of 15 carcinogenic and mutagenic polycyclic aromatic hydrocarbons named by European legislation (EU priority PAHs) in the aqueous phase of primary smoke condensates (PSCs). These PSCs form the raw materials for the production of smoke flavourings to be used in or on food. The method complements a method based on gas-chromatography with mass-selective detection (GC–MS) enabling laboratories without GC–MS to perform the legally required analyses. The method was first validated in-house according to the IUPAC harmonised guideline for single-laboratory validation. Here the limit of detection values of the analytes lay between 0.1 and 1.2 μg/kg, the limit of quantification values between 0.5 and 4.0 μg/kg, and the values of the recoveries between 41% and 107%. The method thus could be used to monitor all 15 EU priority PAHs in PSC. Subsequently a collaborative trial was organised according to the IUPAC protocol for the design, conduct and interpretation of method-performance studies involving 11 laboratories of nine countries. The average values lay between 23% and 99% for the relative standard deviation of the reproducibility and between 24% and 76% for the recovery, which is showing the limits of this method in a worst case scenario.

Keywords: Polycyclic aromatic hydrocarbons (PAHs); High performance-liquid chromatography (HPLC); Smoke flavouring; Primary smoke condensate (PSC); Method validation

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) constitute a large class of organic substances containing two or more fused aromatic rings made up of carbon and hydrogen atoms. Hundreds of individual PAHs may be formed and released during the incomplete combustion or pyrolysis of wood used for smoke generation. Some decades ago, in 1970, the US-Environmental Protecting Agency identified the most frequently encountered PAHs commonly known as the 16 EPA-PAHs in environmental samples. Eight of these PAHs are known to be mutagenic and/or carcinogenic and thus give rise to serious health concern (Zedeck, 1980). Benzo[*a*]pyrene (BaP) was the first PAH to be identified as carcinogen and, as consequence, has been studied most. In spite of the fact that, according to Simko, BaP contributes only 1–20% to the total carcinogenicity found in real samples from the environment, it was often used as a marker for PAH contamination in general (Simko, 2002).

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doi:10.1016/j.foodchem.2006.10.010

The former European Commission's Scientific Committee on Food assessed 33 PAHs, confirmed the subset of eight PAHs from the EPA list mentioned above, and identified seven additional PAHs as of major concern for human health (referred to later as the EU priority PAHs). These 15 EU priority PAHs should be monitored to enable long-term exposure assessments and to verify the validity of the use of the concentrations of benzo[*a*]pyrene as a marker for a "total-PAH content" (EU, 2002).

Smoke flavourings based on the aqueous phase of condensed smoke, called primary smoke condensate (PSC), are on the market for more than hundred years. The use of smoke flavouring rather than traditional smoking was triggered by the ease of application and more efficient use of the resources (Pszczola, 1995). Also the amount of (known) harmful substances as PAHs, can be controlled easier in the flavouring than in smoke (Hattula, Elfving, Luoma, & Mroueh, 2001; Simko, Lesko, Dubravický, & Lapár, 1991). The latter is the goal of the Regulation 2065/2003 adopted by the European Council and Parliament in November 2003 laying down maximum permitted concentrations for benzo[*a*]pyrene (10 μg/kg) and benzo[*a*]anthracene (20 μg/kg) in PSC produced for human consumption (EU, 2003).

At the time being there was no validated method available for any matrix to analyse the concentration of the seven additional analytes in the group of 15 EU priority PAHs (Simon, de la Calle, Palme, Meier, & Anklam, 2005). As consequence a new multi method for the simultaneous analysis of all 15 analytes based on gas chromatography–mass spectrometry (GC–MS) was developed and validated (Simon, Palme, & Anklam, 2006a, 2006b). The draw back of this method is its incapacity of resolving the two isomeric benzo[*j*]fluoranthene and benzo[*k*]fluoranthene.

Moreover, as in case of legal conflicts, a second independent method for the confirmation of positive findings is needed, a method using high-performance liquid-chromatography with ultra-violet absorption and fluorescence detection (HPLC-UV/FLD) for the analysis of the same 15 PAHs was developed in our laboratory. The method was also validated according to the harmonised protocol for single laboratory method validation (Thompson, Ellison, & Wood, 2002). To complete the validation procedure the method was subjected to an international collaborative study designed and carried out according to the harmonised protocol for inter-laboratory method validation (Horwitz, 1995). The present work describes the method and the validation thereof. The results will be discussed with respect to the performance of the GC–MS method.

2. Materials and methods

2.1. Reagents and materials

Solvents and chemicals (potassium hydroxide, sodium sulphate, 2-propanol, methanol, cyclohexane, *n*-hexane, acetonitrile) were all of analytical grade or higher (VWR, Darmstadt, Germany). Solid phase extraction cartridges (3 ml, 0.5 g solid phase, SupelcleanTM LC-Si) were provided by Sigma–Aldrich (Bornem, Belgium).

Samples of 10 PSC were obtained from the smoke flavouring industry and used as provided or as mixture.

Attention

The following compounds are potential human carcinogens and/or mutagens. Handle these substances with utmost care and use personal protection!

The analytes benz[*a*]anthracene (BaA) CAS no. 56-55-3, chrysene (CHR) CAS no. 218-01-9, 5-methylchrysene (5MC) CAS no. 3697-24-3, benzo[*b*]-fluoranthene (BbF) CAS no. 205-99-2, benzo[*j*]fluoranthene (BjF) CAS no. 205-82-3, benzo[*k*]-fluoranthene (BkF) CAS no. 207-08-9, benzo[*a*]pyrene (BaP) CAS no. 50-32-8, indeno[1,2,3-*cd*]pyrene (IcP) CAS no. 193-39-5, dibenz[*a*,*h*]anthracene (DhA) CAS no. 53-70-3, benzo[*ghi*]-perylene (BgP) CAS no. 191-24-2, dibenzo[*a*,*l*]pyrene (DlP) CAS no. 191-30-0, dibenzo[*a*,*e*]-pyrene (DeP) CAS no. 192-65-4, dibenzo[*a*,*i*]-pyrene (DiP) CAS no. 189-55-9, dibenzo[*a*,*h*]-pyrene (DhP) CAS no. 189-64-0, were obtained as certified reference materials (BCR) from the Institute for Reference Materials and Measurements (IRMM) of the European Commission's Directorate General Joint Research Centre (Geel, Belgium). Cyclopenta[*cd*]pyrene (CPP) CAS no. 27208-37-3, >99.0% by GC, was manufactured on request (Biochemisches Institut für Umweltkarzinogene, Großhansdorf, Germany).

2.2. Analytical approach and range

The method described here uses the same extraction and cleanup procedure as described elsewhere (Simon et al., 2006b). However, the final solvent *n*-hexane has been replaced by acetonitrile to enable a reversed phase separation and detection on HPLC-UV/FLD (please see chapter below).

The method was tailored to control the maximum permitted limits of 10 and 20 μg/kg of BaP and BaA, respectively, in primary smoke condensate as defined by the new EU-Regulation 2065/2003 (EU, 2003). According to the IUPAC harmonised protocol (Thompson et al., 2002) the analytical ranges (target ranges) should be 50–150% of the concentration of interest. In the current approach a target range of 5–25 μg/kg was chosen for all EU priority PAHs. The sample preparation procedure resulted in a tenfold higher concentration of the analytes in the clean extract. Thus, the above mentioned concentration values of analyte in PSC would give rise to 50–250 ng/ml of analyte in the final solution to be injected into the HPLC-UV/FLD analytical system.

2.3. Spiking and sample preparation

The provided smoke condensates were tested for the presence of the 15 EU priority PAHs, but no significant

contamination could be detected (The LODs varied between 1 and 4 μg/kg PSC). Therefore spiked matrix samples were used. The spiking solutions were prepared by diluting hexane stock solutions of the individual analytes. 2-Propanol was chosen as solvent for the analytes due to its miscibility with the solvent of the stock solutions, hexane, and the matrix. Spiking was thus achieved by adding 1 ml of a solution of the analytes in 2-propanol to 10 g of PSC. The amount of analyte in the spiking formulation had been adjusted to achieve final concentrations of 0 (blank) and between 5 and 25 μg/kg PSC.

Ten grams of the liquid smoke condensate were refluxed for 30 min with alkaline methanol (3.2 g of potassium hydroxide in 32 ml of methanol) to saponify interfering compounds and ionise weak acids as for example phenol. The analytes were extracted from the methanolic solution three times with 25 ml of cyclohexane each. The organic phases were pooled and the aqueous phase was discarded. The organic phase was dried with sufficient anhydrous sodium sulphate. The organic phase was removed by rotary evaporation under reduced pressure ($T = 40$ °C, $p = 100$ mbar) to dryness. The sample was reconstituted with 500 μl of cyclohexane and transferred onto a silica cartridge activated with 2 ml of cyclohexane. The flask was rinsed with a second 500 μl of fresh cyclohexane, which were also transferred onto the cartridge. The first ml of the eluate was discarded. The analytes were eluted with 7 ml of cyclohexane. The eluate was now collected and the solvent removed under reduced pressure as above. The nearly colourless sample was re-dissolved in 1 ml acetonitrile by vortexing for 1 min and transferred to a capped amber 2 ml auto-sampler vial.

2.3.1. Instrumental analysis

A 20 μl aliquot was injected into an HPLC (1100 series, Agilent, Waldbronn, Germany) system equipped with autosampler, quaternary pump, thermostated column compartment ($T = 40$ °C), UV-diode array detector (G1315B), and FLD (G1321A). For the separation a Pinacle II reversed phase column for PAHs, 250 × 2.1 mm, 5 μm (Restek GmbH, Bad Homburg, Germany) was used. The flow of the aqueous mobile phase (acetonitrile/water) was set to 0.3 ml/min. The gradient programme for the mobile phase started with 80% acetonitrile (0 min) changing linearly to 85% (30 min), and 100% (40 min). After 60 min (still 100%) the mobile phase was changed back to the initial composition (80% ACN/20% H_2O) within 10 min and allowed to equilibrate for another 10 min. Total runtime of one analysis was thus 80 min.

The analytes were detected and quantified by monitoring the UV-absorbance at 375 nm and the fluorescence emissions simultaneously at 370 nm, 420 nm, 470 nm, and 500 nm with one common excitation wavelength of 270 nm.

2.4. Single-laboratory validation

For the (external) calibration of the system mixed standard solutions of all 15 PAHs in acetonitrile were used. Five equidistant concentrations from 50 to 250 ng/ml were measured in triplicate. The linearity of the calibration function and the homogeneity of the variances were verified and the limits of detection (LOD) and quantitation (LOQ) determined according to the German norm DIN 32645 (DIN, 1994). The protocol is implemented in software used for data processing (ValiData, Prof. Wegscheider et al., Graz, Austria).

Recovery and precision were estimated in two independent ways. Firstly, spiked blank matrix samples (c.f. collaborative trial) were analysed in duplicate at five levels spread equally over the analytical range. The recoveries were calculated from the analytical signal as the ratio between found and expected expressed in %. The mean of all values was reported. The standard deviations were calculated from the duplicate analyses and the average of all results was reported. Secondly the recoveries and their associated standard deviations were estimated from five spiked samples each at 5 and 25 μg/kg and the homogeneity of the variances were checked using the Fisher test (F-test).

2.5. Collaborative trial

2.5.1. Design

The collaborative validation study was designed according to the harmonised protocol (blind duplicates, five spiked materials, and a minimum of eight participants) (Horwitz, 1995). A workshop was organised to prepare the inter-laboratory validation of the method to quantify the EU priority PAHs in PSC and held in September 2004.

Eleven laboratories from nine countries covering industrial quality control as well as private and official food control with experience in PAH analysis had agreed to participate in the collaborative trial. The participants were Chemviron Carbon GmbH, DE; General Chemical State Laboratory, GR; Laboratoire de la Repression des Fraudes de Massy, FR; Public Analyst Laboratory, IR; Scientific Institute of Public Health, BE; Österreichische Agentur für Gesundheit und Ernährungssicherheit GmbH, AT; Meat and Fat Research Institute, PL; Mastertaste, USA; Bundesforschungsanstalt für Forst und Holzwirtschaft, DE; Food Research Institute, SR; and Fraunhofer-Institut für Verfahrenstechnik und Verpackung, DE.

Each laboratory received a code for identification of the participant together with instruction guidelines, a sample receipt form to check the completeness of shipment, a method description, a results form, blank material, internal and external standard solutions, and samples (Palme, Simon, & Anklam, 2005).

In addition each laboratory received the following items:

- Ten sealed glass ampoules containing five different materials as blind duplicates (13 g each).
- Two 2 ml vials of pure standards in acetonitrile with known, but not to the participant disclosed, concentrations (UKN-samples).
- One vial with spiking solution (isopropanol, 10 ml).

- One flask (60 ml) of mixed product (blank).
- One calibration solution in hexane (20 ml).

2.5.2. Preparation of the test material

The test material used in this study was identical with the material of the collaborative validation of the GC–MS method (Simon et al., 2006a). In brief, the 15 EU priority PAHs were added to five 1 l portions of the PSC-mix to obtain five concentration levels for each analyte. Each portion will be referred to as "material" from now on. Four of the individual concentrations lay between 5 and 25 μg/kg per PAH in PSC, one was kept as blank (0 μg/kg). Special care was taken that the concentration levels for the individual PAHs were distributed randomly over the five materials to achieve individual concentration patterns in each of the five materials. For each material 11–12 ml were filled in brown glass ampoules, which were sealed by melting (referred to later as "samples"). All samples were coded individually to avoid back-tracing of numbers and comparison of results.

Additionally solutions in acetonitrile with two different concentration patterns of the 15 analytes were prepared. The respective concentrations were "unknown" for the laboratories and these solutions were also coded alike the samples and are referred to as UKN-samples (Table 1). The analysis of these solutions should allow for the separation of a calibration and instrumental bias from a bias caused by sample preparation. This set-up provided some potential for trouble-shooting in the case that conspicuous results were reported.

A solution containing 150 ng/ml of each of the 15 EU priority PAHs in 2-propanol was prepared (referred to later as spiking solution). The spiking solution in combination with blank material aimed to enable the analysts to practise the method before analysing the samples of the study. For instrument calibration a standard stock solution of the analytes (~400 ng/ml) was prepared and the participants were informed about its concentration.

2.6. Homogeneity and stability of test materials

The homogeneity and stability of the materials was assessed by analysing randomly samples using a GC–MS method (Simon et al., 2006a, 2006b) before sending the samples, during the phase of measurement, and after the results had been collected. Each sample was analysed once. The samples had been stable at room temperature within the time-frame for analysis agreed upon at the work-shop.

The homogeneity of the samples had been checked. The found relative standard deviation of the homogeneity (RSD_h) varied depending on the substance between 1.6% for BaA to 12.3% for DhP (Simon et al., 2006a). The values were close to or even lower than the estimated relative standard deviation of the in-house repeatability (RSD_i) of 1.2% for BaA to 20% for DhP for the method used (Simon et al., 2006b) and the sample materials were considered sufficiently homogeneous for the purpose of the collaborative trial.

2.7. Evaluation of the results submitted

Four laboratories resigned from the trial and one submitted the data too late. Six laboratories submitted results, which were statistically evaluated. The values reported were compared to the values expected. In case of significant deviation from the expected value(s), the respective participant was contacted to check the results and to find out about potential sources of the error.

The results were statistically evaluated according to the harmonised protocol on collaborative method validation (Horwitz, 1995). As according to the harmonised protocol the minimum required number of valid results is eight and only six results were available, no outlier test was performed.

Table 1
Concentrations of the EU priority PAHs in the respective materials (Mat no.)

Analyte	Mat 1 (μg/kg)	Mat 2 (μg/kg)	Mat 3 (μg/kg)	Mat 4 (μg/kg)	Mat 5 (μg/kg)	UKN 1[a] (ng/L)	UKN 2[a] (ng/L)
BaA	24.3	17.4	11.7	Blank	19.8	190	199
CPP	22.2	10.5	15.2	18.3	Blank	110	97
CHR	9.7	Blank	5.7	15.8	23.5	78	165
5MC	14.4	24.6	Blank	11.0	6.2	64	150
BbF	Blank	23.9	16.3	10.7	19.2	164	65
BjF	9.7	Blank	6.0	13.8	22.0	144	57
BkF	5.1	12.1	22.0	9.4	Blank	111	219
BaP	5.3	20.2	Blank	8.8	11.4	156	67
IcP	5.4	Blank	23.9	10.9	13.3	156	105
DhA	24.7	Blank	19.8	11.1	15.2	116	207
BgP	22.0	10.5	15.4	18.5	Blank	185	97
DlP	Blank	17.2	19.7	24.4	11.5	155	73
DeP	10.8	Blank	23.4	16.4	13.3	137	245
DiP	10.4	15.4	21.2	11.8	Blank	118	239
DhP	11.4	12.9	22.2	Blank	17.5	128	228

[a] UKN X = solutions with (unknown) (=known, but not disclosed to the participants) concentrations of the 15 EU priority PAHs in acetonitrile.

3. Results and discussion

3.1. Results of development and single-laboratory validation of the method

The initial HPLC parameters were taken from a method published (DIN, 2002) and adapted to separate and detect the 15 EU priority PAHs on the analytical column in use in a minimum of run time. The use of internal standard compounds was considered (e.g., benzo[*b*]chrysene), but, due to the residual probability of distortion of results by natural contamination with the compound, the approach was discarded.

The wavelengths of the maximum fluorescence quantum yields were determined for each analyte for excitation and emission in the respective ranges of 200–300 nm and 300–550 nm (Table 2). The quantum yield varied moderately with the change of the excitation wavelength and the maxima were not very pronounced. Additionally, the excitation maxima of the different compounds lay relatively close to each other and for all analytes one single excitation wavelength (270 nm) was chosen. The exception was CPP (abbreviations of the analytes are given above), for which virtually no fluorescence was detectable within that range. The maxima of the emission spectra showed more diversity for the various analytes and therefore four wavelengths were chosen for the detection. The choice described above was dictated by the limitations of the detector, which allows for a simultaneous monitoring of maximum four emissions and one excitation wavelengths (or vice versa).

The PAH CPP did not show any fluorescence (data not shown). It was therefore detected by UV-absorption spectroscopy. Additionally to the detection problem a partial overlap of the retention times of BaA and CPP was observed (Fig. 1(a)). The analysis of the UV-absorbance spectra of both analytes showed that at 375 nm the absorbance of BaA was already negligible while still a signal could be observed for CPP (Fig. 1(b)).

Table 2
Fluorescence maxima for absorption (between 200 and 300 nm) and emission (300–550 nm) and wavelengths used for detection in order of elution

Analyte	Maximum absorption wavelength (nm)	Maximum emission wavelength (nm)	Absorption[a] or emission wavelength used for detection (nm)
CPP	405 (weak)	–	375[a]
BaA	280	396	420
CHR	268	376	370
5MC	268	384	370
BjF	240 (weak)	505	500
BbF	256	440	420
BkF	244	424	420
BaP	264	412	420
DhA	292	404	420
DlP	272	428	420
BgP	296	420	420
IcP	248	492	500
DeP	280	404	420
DiP	292	440	470
DhP	260 (300)	456	470

[a] For CPP UV-absorption was used for detection.

The used respective UV-absorption and emission wavelengths for detection of the respective analytes are also given in Table 2.

When comparing the method performance characteristics, it was important to keep in mind the effect of the sam-

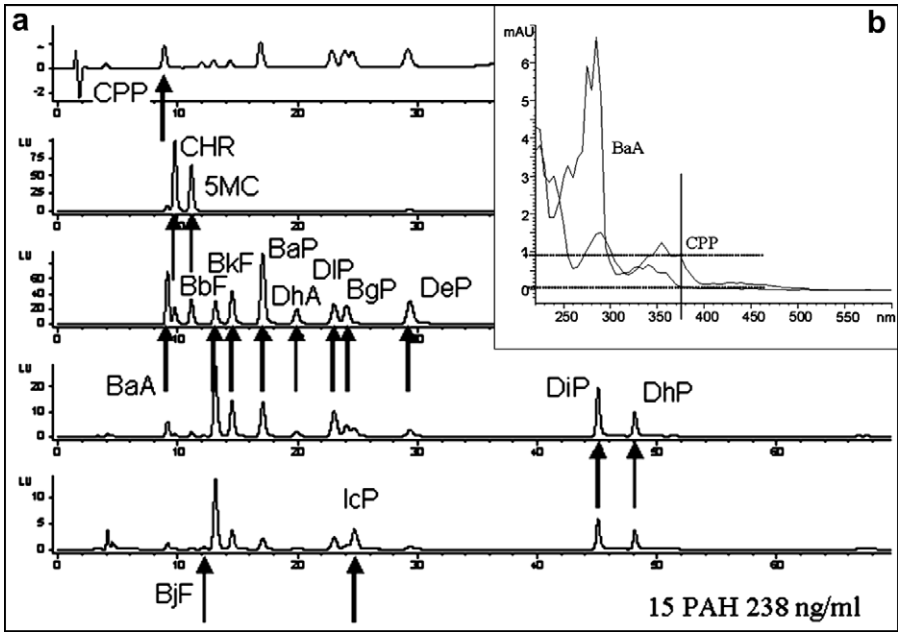

Fig. 1. (a) Chromatogram of an analysis with the 15 relevant PAHs. Red arrows indicate the peaks used for quantitation and detection. (b) UV-absorbance spectra of CPP and BaA (overlaid). (For interpretation of this figure in colour, the reader is referred to the web version of this article.)

ple preparation leading to a tenfold higher concentration of the analytes in the final extract. This was the case, because 10 g of sample were extracted and the content was dissolved in 1 ml of solvent. To distinguish between the respective phases in the text the concentrations are expressed in μg/kg (original sample) and ng/ml (final extract).

The linearity of the calibration was confirmed for all analytes in pure standard solution and in spiked matrix samples over the range of 50–250 ng/ml and 5–25 μg/kg, respectively. The data agreed well with a linear regression and the plots of residuals showed no obvious patterns. The homogeneities of the variances were found using the same set of data. LODs and LOQs for pure analyte solutions lay between 1.4 and 12 and 5–40 ng/ml depending on the analyte. Taking into account the concentration effect of the sample preparation the LODs and LOQs would be around or below 1 μg/kg and 4 μg/kg PAH in PSC, respectively, for all analytes. The blank matrix was tested for potential interferences and only traces below the respective LODs were found for some of the analytes (data not shown). The values for the LODs and LOQs were confirmed by the analysis of spiked matrix samples (see above). To confirm the LODs six samples were spiked with 1.5 μg/kg PAH in PSC. The relative standard deviations of the detector responses (RSD_ds) were mostly at or below 6.5%, except for CPP (17%) and BjF (19%). The higher RSD_d values for the latter two analytes could be explained by the overall weaker detector response for these compounds resulting in a low signal-to-noise ratio at this concentration.

The values of the first experiment assessing the recovery lay between 70% and 110% and showed no trends for all analytes except for DiP and DhP, which showed both increasing values for recovery with higher levels of spiking (data not shown). The effect was stronger for DhP. In both cases the trend was statistically not significant, however, to verify the observation a second experiment was conducted (see below).

The by the second experiment estimated values for recovery were between 50% and 120% for all analytes, except for DiP at 25 μg/kg PSC (41%). The recoveries for 5 μg/kg and 25 μg/kg varied within the 95% interval (±2 SD) of normal distributions and the statistically insignificant trends observed in the first experiment were not confirmed. Both estimates fulfilled the minimum requirements given for BaP by Commission Directive 2005/10/EC (EU, 2005). The homogeneity of the variances for the recovery could also be confirmed for most of the analytes with the exceptions of DeP and DhP. Reasons for this could be a poor re-dissolution of the analytes when reconstituting the evaporated extract with acetonitrile after the SPE cleanup. This explanation is corroborated by the fact that when using hexane the variances of the recoveries were homogenous (Simon et al., 2006b).

Summarising the results of both experiments it can be said that measured values for the dibenzopyrenes DeP, DiP, and DhP must be interpreted with especially taking into account the limits given by the poor repeatability. The single-laboratory performance characteristics (LOD, LOQ, analytical range, recovery, and precision) of the method are summarised in Table 3.

In general the single-laboratory performance characteristics of the HPLC-UV/FLD method resemble the respective values of the GC–MS method closely. The LOD values of the analytes lay between 0.1 and 1.2 μg/kg, the LOQ values between 0.5 and 4.0 μg/kg, and the values of the recoveries between 41% and 107%. The average values of the RSD of the recoveries were for the HPLC-UV/FLD method between 3% and 21%, which did also agree with the respective results of the GC–MS method.

Table 3
Single-laboratory method performance characteristics for pure standard solutions and the recovery from matrix (*Note:* 10 ng/ml ≡ 1 μg/kg PSC!)

Analyte	Retention time (min)	LOD (ng/ml)	LOQ (ng/ml)	Range (ng/ml)	SD (ng/ml)	RSD (%)	Recovery at 5 μg/kg		Recovery at 25 μg/kg	
							(%)	SD[a]	(%)	SD[a]
CPP	9.0	2.8	10	50–250	1.9	1.3	84	1.6	75	12
BaA	9.1	1.5	5.5	50–200	1.0	0.7	94	2.4	89	13
CHR	9.7	1.4	5.2	50–250	1.0	0.6	96	4.5	93	14
5MC	11.1	1.4	5.0	50–250	0.9	0.6	92	2.0	94	18
BjF	12.2	5.0	18	50–250	2.9	1.9	94	4.6	85	14
BbF	13.1	5.2	18	50–250	1.3	0.9	92	1.6	85	14
BkF	14.5	4.4	15	50–250	1.2	0.8	92	1.3	82	12
BaP	17.1	2.6	9.3	50–250	1.8	1.2	96	1.9	79	14
DhA	19.9	3.0	11	50–200	2.1	1.4	91	1.9	80	17
DlP	23.0	2.1	7.7	50–250	1.8	1.2	91	1.4	77	20
BgP	24.1	2.9	10	50–250	1.9	1.3	91	1.5	83	5
IcP	24.7	4.0	14	50–250	1.5	1.0	92	6.7	71	15
DeP	29.4	4.1	14	50–250	4.3	2.9	86	1.0	66	13[b]
DiP	45.1	4.2	15	50–250	1.8	1.2	79	3.3	41	22
DhP	48.1	12	40	50–250	8.1	5.4	107	14	53	28[b]

[a] SD = RSD, because it is already expressed in % recovery.
[b] The variances were not homogeneous at 5 and 25 μg/kg PSC (50 and 250 ng/ml extract).

3.2. Results of the collaborative validation

3.2.1. Homogeneity and stability of the test material

The concentrations of the analytes in the samples were shown to be stable within the agreed timeframe of 30 days for analysis with the exception of CPP. The concentrations of this analyte decreased in all materials (data not shown). The observation of instability of CPP in solution is in agreement with previous findings in another laboratory (Seidel, 2004).

Because the participating laboratories were requested to store the samples at 4 °C while the data mentioned above relate to the storage at room temperature (~22 °C) and the laboratories were required to finalise their measurements after 30 days of receipt of the samples, the material could be considered to have maintained its homogeneity during the course of the study.

3.2.2. Results submitted

Six laboratories submitted results within in a period of 38 days, which were evaluated. The valid results (900 data points in total) were tested for statistical outliers using Cochran and Grubbs tests. To evaluate the results visually and to double check the numerical removal of outliers, the data were also displayed graphically ordered by material and analyte in two different views: first as an analytical result versus laboratory plot with indication of the statistical mean (mean and range) and second as first result versus second result plot of blind duplicates (Youden-plot). The mean-and-range graphics helped to find outliers with respect to the between laboratory reproducibility. This is shown for BaA as example in Fig. 2(a). The Youden-plots were used to find outliers in the dataset of the within-laboratories repeatability values (Fig. 2(b)). Both ways of presentation were thus complementary and confirmed finally the numerical identification of statistical outliers.

The relative repeatability standard deviation (RSD_r) varied – substance and concentration dependent – between 9.3% for DeP at 16.4 μg/kg in PSC and 83% for CPP at 18.3 μg/kg in PSC. The RSD_rs average values lay for the individual analytes between 14% and 54%. The relative reproducibility standard deviation (RSD_R) was between 13% for BaA at 24.3 μg/kg in PSC and 111% for DhP at 12.9 μg/kg in PSC. The averages of the RSD_R values were between 23% and 99% (Tables 4 and 5).

The Horwitz Ratio (HORRAT) is a measure for the quality of the results for the reproducibility and should be smaller than two (Horwitz, Britton, & Chirtel, 1998). It is calculated as the ratio between found and an empirically derived RSD_R. In general it can be stated that a HORRAT larger than two indicates problems of method with the respective analyte. In the present study it was found to be higher than two for more than one concentration for CCP, BjF, BgP, DeP, DiP, and DhP. For CHR, BkF, IcP, and DhA the value of two was superseded for only one concentration. As the GC–MS method employs

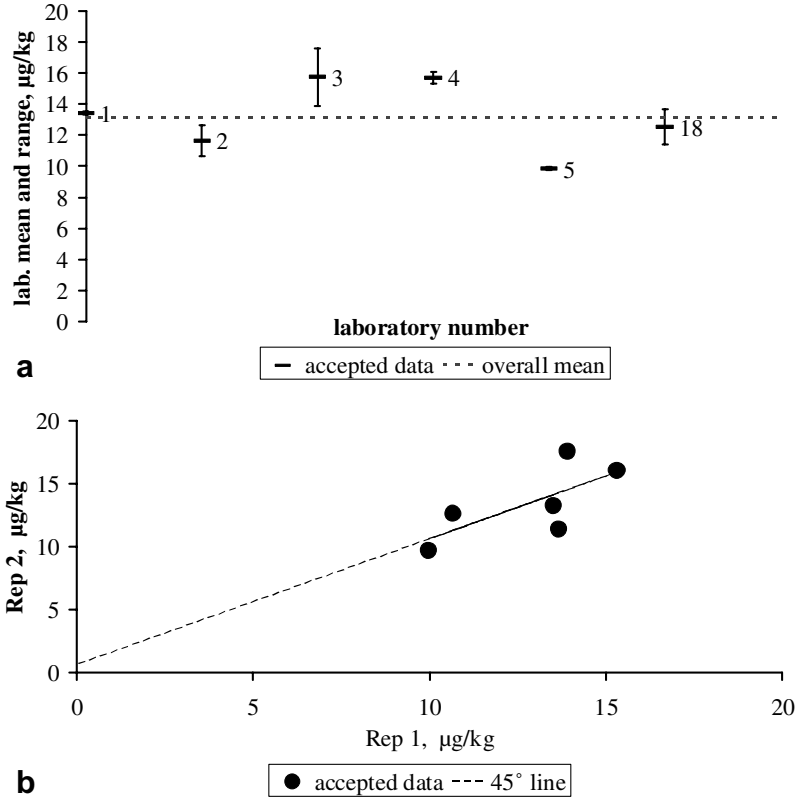

Fig. 2. (a) Mean-and-range presentation of the data for BAA in PSC, material 2 (b) Youden-plot of the data received for BAA in PSC, material 2.

Table 4
Detailed results of the collaborative trial (6 laboratories)

Analyte	Spiked value (μg/kg)	Mean value (μg/kg)	s_r[a] (μg/kg)	RSD_r (%)[a]	s_R[a] (μg/kg)	RSD_R (%)[a]	HORRAT	Recovery (%)
BaA	0.0	3.6	–	–	–	–	–	–
	11.7	8.1	2.0	25	3.0	37	1.6	69
	17.4	12.6	4.2	33	4.8	38	1.7	73
	19.8	13.1	1.4	11	2.5	19	0.8	67
	24.3	18.0	2.4	13	2.4	13	0.6	74
CCP	0.0	0.8	–	–	–	–	–	–
	10.5	2.4	1.1	46	2.0	85	3.7	22
	15.2	4.5	2.3	51	3.7	82	3.6	30
	18.3	4.9	4.1	83	4.1	83	3.6	27
	22.2	6.1	2.2	36	3.3	55	2.4	27
CHR	0.0	0.6	–	–	–	–	–	–
	5.7	5.0	2.0	40	2.0	40	1.7	88
	9.7	7.6	1.1	14	1.3	17	0.7	78
	15.8	11.5	3.0	26	3.0	26	1.1	72
	23.5	15.8	9.1	58	9.1	58	2.5	67
5MC	0.0	1.1	–	–	–	–	–	–
	6.2	4.3	1.7	40	1.7	40	1.7	68
	11.0	6.7	0.9	14	1.3	20	0.9	61
	14.4	10.8	1.4	13	1.4	13	0.6	75
	24.6	17.8	1.8	10	3.0	17	0.7	72
BbF	0.0	1.8	–	–	–	–	–	–
	10.7	7.8	2.5	33	2.5	33	1.0	73
	16.3	10.4	1.9	18	2.1	20	0.6	64
	19.2	10.5	3.7	35	5.1	49	1.5	55
	23.9	15.2	2.6	17	3.6	24	0.8	64
BjF	0.0	1.0	–	–	–	–	–	–
	6.0	4.8	2.1	43	2.6	53	2.3	80
	9.7	7.8	1.3	16	1.3	16	0.7	80
	13.8	8.5	7.1	83	7.1	83	3.6	62
	22.0	14.6	2.4	16	3.9	27	1.2	66
BkF	0.0	0.9	–	–	–	–	–	–
	5.1	3.7	0.6	17	0.6	17	0.7	73
	9.4	5.0	2.3	46	2.5	50	2.2	53
	12.1	8.5	1.3	15	2.2	26	1.1	70
	22.0	15.1	2.9	19	3.7	24	1.1	69
BaP	0.0	0.9	–	–	–	–	–	–
	5.3	3.5	0.4	13	0.8	22	0.9	66
	8.8	5.5	1.0	19	1.3	24	1.0	63
	11.4	7.5	1.0	14	1.5	20	0.9	66
	20.2	13.5	1.7	13	3.3	25	1.1	67
IcP	0.0	0.5	–	–	–	–	–	–
	5.4	2.8	1.2	42	1.4	51	2.2	51
	10.9	5.8	0.7	12	1.3	22	1.0	53
	13.3	7.3	1.4	18	3.1	43	1.9	55
	23.9	14.9	3.4	23	3.4	23	1.0	62
DhA	0.0	1.4	–	–	–	–	–	–
	11.1	5.7	1.1	19	1.6	19	0.8	51
	15.2	8.2	1.8	22	3.7	45	2.0	54
	19.8	11.0	2.6	24	3.7	34	1.5	56
	24.7	13.3	3.8	28	7.1	53	2.3	54
BgP	0.0	2.3	–	–	–	–	–	–
	10.5	8.6	6.3	73	7.0	82	3.6	82
	15.4	10.9	1.7	16	17.0	16	0.7	71
	18.5	10.6	6.5	60	6.9	65	2.8	57
	22.0	14.5	2.5	17	2.7	19	0.8	66
DlP	0.0	0.8	–	–	–	–	–	–
	11.5	6.7	2.8	41	2.8	41	1.8	58

(*continued on next page*)

Table 4 (*continued*)

Analyte	Spiked value (μg/kg)	Mean value (μg/kg)	s_r[a] (μg/kg)	RSD_r (%)[a]	s_R[a] (μg/kg)	RSD_R (%)[a]	HORRAT	Recovery (%)
	17.2	10.5	2.7	26	3.7	35	1.5	61
	19.7	12.3	2.5	20	2.7	22	1.0	63
	24.4	11.6	2.8	24	3.7	32	1.4	47
DeP	0.0	0.5	–	–	–	–	–	–
	10.8	4.8	1.1	24	2.6	55	2.4	44
	13.3	5.9	1.4	23	3.6	61	2.6	44
	16.4	5.9	0.5	9.3	3.8	64	2.8	36
	23.4	8.9	4.1	46	6.4	71	3.1	38
DiP	0.0	0.7	–	–	–	–	–	–
	10.4	3.0	0.8	25	2.2	73	3.2	29
	11.8	2.7	0.3	11	2.4	88	3.8	23
	15.4	5.2	1.8	34	5.0	95	4.1	34
	21.2	6.3	1.4	22	5.0	79	3.4	30
DhP	0.0	0.6	–	–	–	–	–	–
	11.4	2.7	0.7	26	2.2	81	3.5	24
	12.9	3.5	1.4	40	3.9	111	4.8	27
	17.5	4.2	1.5	35	4.0	96	4.2	24
	22.2	4.3	3.0	69	4.6	107	4.7	19

[a] s_r – repeatability standard deviation, RSD_r – relative repeatability standard deviation, s_R – reproducibility standard deviation, RSD_R – relative reproducibility standard deviation.

Table 5
Mean method precision data from collaborative trial

Analyte	Spiked value (μg/kg)	Mean value (μg/kg)	SD_r[a] (μg/kg)	RSD_r[a] (%)	SD_R[a] (μg/kg)	RSD_R[a]	HORRAT	Rec (%)
BaA	18.3	13.0	2.5	20	3.2	27	1.2	71
CCP	16.5	4.5	2.4	54	3.3	76	3.3	27
CHR	13.7	10.0	3.8	34	3.9	35	1.5	76
5MC	14.1	9.9	1.5	19	1.9	23	1.0	69
BbF	17.5	11.0	2.7	26	3.3	31	1.0	64
BjF	12.9	8.9	3.2	40	3.7	45	1.9	72
BkF	12.2	8.0	1.8	24	2.2	29	1.3	66
BaP	11.4	7.5	1.0	14	1.7	23	1.0	65
IcP	13.4	7.7	1.7	24	2.3	35	1.5	55
DhA	17.7	9.6	2.3	23	4.0	38	1.6	54
BgP	16.6	11.2	4.3	42	8.4	45	2.0	69
DlP	18.2	10.3	2.7	28	3.2	33	1.4	57
DeP	16.0	6.4	1.8	25	4.1	63	2.7	41
DiP	14.7	4.3	1.1	23	3.7	84	3.6	29
DhP	16.0	3.7	1.7	42	4	99	4.3	24

[a] s_r – repeatability standard deviation, RSD_r – relative repeatability standard deviation, s_R – reproducibility standard deviation, RSD_R – relative reproducibility standard deviation.

the same steps for sample preparation and the instrument repeatabilities are comparable, the authors suspect that the reconstitution with acetonitrile rather than with hexane after the solid phase clean-up is the most vulnerable point of the here presented method. A small variation at this step could lead to a large difference in the result.

The mean values of the recoveries found in each laboratory were calculated for each material. Depending on the analyte the values lay between 19% (DhP) and 88% (CHR). The average values for recovery of the five materials were between 24% (DhP) and 76% (CHR) with DhP, DiP, and CPP being the exceptions. For DhP and DiP the low recovery was probably due to adsorption of the analyte to the walls of the sample containers and/or the glassware used during the reconstitution of the extract with acetonitrile after sample clean-up.

For CPP the low average recovery had clearly to be attributed to the loss of analyte during the time passed between the date of preparation and analysis of the respective sample. The mean laboratory recovery for CPP of 27% fit roughly with the residual concentration of about 30% of the initial value found in the materials at our laboratory around 40 days after production (data not shown). This finding indicates that the real recovery found by the laboratories was close to 90% for this analyte. The results are summarised in Tables 4 and 5.

On the other hand the results from the UKN-samples (clean solvent solutions of the analytes) showed that parts

of the variability were not related to matrix effects, but rather intrinsically to the analyte and/or the instrumental part of the analysis. For example, RSD_R values for CPP were as high as 40% for both samples, which might be explained by the low stability of the analyte in solution (Seidel, 2004). A similar high RSD_R value of 45% was found for DhP. As this compound was already hardly soluble in hexane (<8 mg/l at 20 °C, data not shown) and with acetonitrile being a weaker solvent for hydrophobic compounds in general, we concluded that a loss of analyte by adsorption to the walls of the container could not be excluded as the underlying cause of the effect. The same might be true to a lower extent for DiP, which showed solubility in hexane between an estimated 8 and 20 mg/l at 20 °C (data not shown). The higher variation for IcP was believed to indicate interferences from the weak signal originated from BgP, which was not eliminated completely by chromatographic separation. During the in-house validation this did not appear to be a problem, but a future development of this method shall account for this effect. For DhA the reason for the high variance remained unclear (Table 6).

Compared to the values of the method performance parameters of the GC–MS method (given in parenthesis), the HPLC-UV/FLD showed less favourable trends. The RSD_r values were between 11% and 83% (5–20%), the RSD_R between 16% and 85% (10–52%), the HORRAT ratios between 0.7 and 3.7 (0.4–2.3), and the recoveries between 22% and 81% (41–89%). These data insinuated a better performance of the GC–MS than the HPLC-UV/FLD method, whereat the better reproducibility of the GC–MS method originated in the use of internal standards. Nevertheless, this picture had to be seen as provisional, because the number of six results evaluated in this study was not sufficient to match the criteria of the minimum eight valid results given by the harmonised protocol (Horwitz, 1995).

Table 6
Results from the analysis of the UKN samples

Analyte	Spiked value (μg/kg)	Mean value (μg/kg)	s_r[a] (μg/kg)	RSD_r[a] (%)	s_R[a] (μg/kg)	RSD_R[a] (%)	HORRAT (RSD_R/23)	Recovery (%)
BaA	189	204	2	1	10	5	0.2	108
	200	210	4	2	11	5	0.2	105
CPP	111	103	2	2	44	42	1.8	93
	97	91	6	7	37	40	1.8	94
CHR	79.0	81	3	4	5	6	0.3	103
	165	168	3	2	7	4	0.2	102
5MC	64	66	2	4	4	6	0.3	103
	150	153	3	2	6	4	0.2	102
BbF	164	167	4	2	6	4	0.2	102
	65	67	2	3	2	4	0.2	103
BjF	143	156	13	8	13	8	0.4	109
	56	60	2	4	6	9	0.4	105
BkF	111	122	5	4	8	6	0.3	109
	219	234	6	2	8	4	0.2	107
BaP	155	156	4	3	8	5	0.2	101
	66	67	5	7	6	9	0.4	100
IcP	155	151	35	23	35	23	1.0	97
	105	109	10	10	10	10	0.4	104
DhA	116	119	1	1	18	15	0.7	103
	207	198	3	2	51	26	1.1	96
BgP	186	192	7	4	10	5	0.2	103
	97	102	4	4	6	6	0.3	105
DlP	155	170	5	3	16	9	0.4	110
	73	83	5	6	10	12	0.5	113
DeP	138	141	3	2	4	3	0.1	103
	246	254	7	3	11	4	0.2	103
DiP	118	115	5	5	23	20	0.9	98
	240	238	10	4	45	19	0.8	99
DhP	129	118	7	6	53	45	2.0	91
	229	210	23	11	97	46	2.0	92

[a] s_r – repeatability standard deviation, RSD_r – relative repeatability standard deviation, s_R – reproducibility standard deviation, RSD_R – relative reproducibility standard deviation.

4. Conclusion

Commission Regulation (EC) No. 627/2006 lays down the minimum performance criteria for the analysis of PAHs in smoke condensate. The maximum values for benzo[*a*]-pyrene and benzo[*a*]anthracene of the relative repeatability standard deviations are 20% and of the relative reproducibility standard deviations 40%. The respective values of the method described are all below these maxima. For the other analytes, no legal conflict could be expected at the time being as only for benzo[*a*]pyrene and benzo[*a*]anthracene maximum permitted contents are given by legislation.

The single-laboratory validation of the here described method has shown that the method is suitable for quantitation of BaP and BaA in PSC between 50% and 150% of the maximum permitted concentration levels of 10 and 20 μg/kg, respectively. The method can also be used to monitor all 15 EU priority PAHs in PSC above their LOD of 0.14–1.2 μg/kg in PSC, respectively. However, the recoveries for DiP and DhP are rather low while the variance of the repeatability is high at the 25 μg/kg level for all compounds and the method should be scrutinised for improvement on this part.

The results of the collaborative trial showed quite poor recoveries for the analytes CPP, DeP, DiP, and DhP and more work is needed to improve the method in this aspect. Additionally, for CPP, DeP, DiP, DhP, and DeP, the HORRATs were above 2 indicating that the values for RSD_R were too high for these analytes. One major cause for these problems seemed to be the relative unstable matrix. It can thus be assumed that the outcome of the collaborative trial reflected a worst case scenario for the method performance. Nevertheless, there is room for improvement. In a further development the use of internal standards, e.g., of fluorinated compounds, should be considered.

The described method is complementary to the GC–MS method and the second validated method for the analysis of the recently identified 15 EU priority PAH. The method enables manufacturers of smoke flavourings and control bodies to analyse PSC for these PAHs to ensure that these and derived products are safe for the use in food and thus for human consumption.

Acknowledgement

We thank the following collaborators for participation in this study:

Kirsten Brune (Chemviron Carbon GmbH, DE), Dimitrios Chrysafidis (General Chemical State Laboratory, GR), Laurence Delaire (Laboratoire de la Repression des Fraudes de Massy, FR), Chris Griffin (Public Analyst Laboratory, IR), Vincent Hanot (Scientific Institute of Public Health, BE), Heribert Holzer (Östereichische Agentur für Gesundheit und Ernährungssicherheit GmbH, AT), Mieczyslaw. W. Obiedzinski (Meat and Fat Research Institute, PL), Sreekumar Ramakrishnan (Mastertaste, USA), Martina Schirmacher (Bundesforschungsanstalt für Forst und Holzwirtschaft, DE), Božena Sklaršová (Food Research Institute), and Gerd Wolz (Fraunhofer-Institut für Verfahrenstechnik und Verpackung, DE).

We also thank Antoine M. Bouttereux, Forest Smoke Europe, Le Chesnay, France; Barry Welch, Mastertaste, Gloucestershire, UK; Bernward Wosnitza, Chemviron Carbon GmbH, Bodenfelde, Germany; Gary Underwood, Red Arrow Products Co. LLC, Manitowoc, USA; Jean-Jacques Weiland, Sofral, Strasbourg, France; Gwendolina Vercoutere, Flandor Flavors International, Zulte, Belgium; Ian Gatfield, Symrise, Holzminden, Germany; Peter Henriksen, Brøste, Lyngby, Denmark; Sam Crace, Forest Flavors International, Glasgow, USA for supply of the PSC materials.

Last, but not least, we want to thank Thomas Wenzl for critical reading of the manuscript.

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