Method Development and Determination of Chlorinated Polycyclic Aromatic Hydrocarbons in Different Matrices

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Abstract

The aim of this study was to develop an analytical method, which separates selected chlorinated polycyclic aromatic hydrocarbons (Cl-PAHs) from fat, and fat-free or vegetable matrices. The method contains extraction-, cleanup-, and quantification steps. Integration of automated analysis actions, as in extraction and cleanup, should enhance the reproducibility, precision, and efficiency of the method. This was confirmed by validation of the overall analytical process. In the end, as a performance check, the developed method was applied on different matrices, e.g., tea, rice, grilled pork, and eel and predator eggs, as a non-food example. An inter-laboratory check was initiated as replacement for the lack of proficiency tests. Due to the high level of automation, both personnel and time effort are very low. In addition, the method is very robust with regard to the variability of the solvent selection and the loss of analytes by evaporation to dryness. It could be demonstrated that the developed method is applicable to different matrices with reproducible and precise results. This applies also to low-fat food and feed.

Keywords Cl-PAH · High-resolution mass spectrometry · Gas chromatography · Chlorinated polycyclic aromatic hydrocarbons

Introduction

Halogenated polycyclic aromatic hydrocarbons (X-PAHs) are classes of compounds consisting of polycyclic aromatic hydrocarbons (PAHs) with at least two aromatic rings and one halogen substituted hydrogen in the aromatic ring. Substituents could be chlorine, bromine, fluorine, and/or a mixture of these elements. A number of chlorinated organic compounds such as PCB or PCN had been produced industrially for many years. They were primarily used as non-conductive oils in capacitors or as lubricants. Residues of these substances are still detectable in the environment and food, even after a decade-long ban. In contrast, Cl-PAHs are formed during uncontrolled combustion processes in the presence of chlorine or chloride in addition to, e.g., dioxins and PAHs. According to the current state of knowledge, Cl-PAHs arise exclusively through uncontrolled anthropogenic

combustion processes or natural disasters such as volcanic eruptions and thus represent a class of contaminants that also enter the food chain through environmental inputs on various paths.

Cl-PAHs are structurally similar to other halogenated aromatic hydrocarbons such as polychlorinated dibenzodioxins, -furans (PCDD/Fs) and polychlorinated biphenyls (PCBs). These substances share a planar structure, consist of at least two annulated benzene rings, are highly soluble in fat due to the hydrophobicity, and have extremely low water solubility. However, they are less persistent in their environment compared to PCDD/Fs and PCBs because they can be easily oxidized by OH radicals in the atmosphere (Fu and Suuberg 2012). The occurrences, developments, distributions, and toxic effects of PAHs, PCDD/Fs, and PCBs have been investigated and described, extensively. Standardized analytical procedures are implemented both nationally and internationally. In addition, there are regulations and directives are setting maximum levels of these substance classes. Specifically, in the instance of X-PAHs, there are currently neither standardized methods of determination nor maximum levels. In contrast to PAHs, PCDD/Fs, and dl-PCB, health-based guidance values have not been derived.

Information on the physico-chemical properties of X-PAHs is scarcely available. Fu and Suuberg (2012) had emphasized that vapor pressure is a fundamental parameter for

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their volatility and thus also for the possibility of transporting substances across the atmospheric gas phase. It was found that doubly chlorinated PAHs had lower vapor pressure than their mono-substituted analogues. With increasing number of aromatic ring structures, steam pressure and water solubility are lowered. Fu and Suuberg (2012) demonstrated with further measurements of octanol/water coefficients that X-PAHs had significantly better fat solubility than their non-halogenated analogues. These findings were confirmed by Sun et al. (2013) who concluded that increased bioaccumulation in organisms is possible in relation to the non-halogenated PAHs, due to the superior fat solubility of the X-PAHs. Ohura et al. (2008a) established that chlorination of PAHs increased persistence in the environment. As a result, bioaccumulation in soil, animal, and vegetation is strongly in favor to non-halogenated PAHs. Appropriately, an increased cluster of the X-PAHs concentration in corresponding foodstuff is to be expected with a growing trophic level in the food chain.

In contrast to PAH, the formation mechanisms of X-PAHs in the environment and in food are still unclear. Individual experiments have indicated that formation processes of these two substance classes are fundamentally different (Ma et al. 2013). This hypothesis was again formulated by Ohura et al. (2009), after efforts to establish a correlation between PAHs and X-PAHs from surface sediments of the Shenzhen region failed.

It is unknown whether and in which concentrations halogenated polycyclic aromatic hydrocarbons found in air particles or sediments clearly affect food and feed. They are anthropogenic compounds involved in industrial processes and can be emitted into the environment. Congeners of these substances are already analytically determined to be environmental contaminants which were already isolated from various areas of the biosphere such as sediment, sludge, sea water, air, and wastewater from combustion plants (Horii et al. 2009; Sun et al. 2013). Data from the environmental sector suggests that even in intact natural areas, food contamination by X-PAHs may occur by transfer of X-PAHs loaded particles deriving from combustion plants or combustion incinerators.

In terms of human exposure, farm animals play an important role in the foodchain as they act as link between environment and humans. Through the high lipophilicity and persistence of X-PAHs, they could particularly be found in fatty foods derived from animals that were exposed to high environmental contaminants.

In addition, vegetable-based foods may also play an important role in human exposure, e.g., when they are processed from plants that are in contact to particles from the environment in form of fly ash.

In addition to environmental exposure, contamination of food may also occur by the exogenous formation of X-PAHs in industrial food processing or food preparation in the kitchen. Particularly powerful thermal processes such as

grilling or smoking may also lead to the formation of X-PAH (Viegas et al. 2012, 2014). In order to determine possible Cl-PAH exposure for consumers, sufficient validated and verified food analysis is necessary.

X-PAHs can be formed by various chemical reaction mechanisms. Formation of mono-chlorinated and maximally di-chlorinated congeners is favored (Rotard and Marinos 2009). Wang et al. (2001) showed that primarily ring systems with two to six rings were formed in experimental studies. It is important to note that the principle formation of X-PAHs during combustion is sometimes considered to take place in the presence of free chlorine or chloride (Wang et al. 2001). Combustion is the primary source of contamination in food. Depending on the conditions and the composition of the combusted materials, PAH, X-PAH, PCDD/F, and PCB are formed.

In our current study, an analytical method was developed which allows reliable detection of selected Cl-PAHs in fatty, fat-free, and plant matrices. The validation of the method was used to assess the quality and reliability of the analytical results.

Material and Methods

Chemicals

On the basis of expected exposure by formation of X-PAHs as mentioned above and with reference to the availability of standard substances, congeners were selected for method development as shown in Fig. 1. Picograde *n*-hexane, acetone, toluene, dichloromethane, and ethyl acetate were purchased from Promochem LGC Standards GmbH, Wesel Germany. Ph Eur absolute ethanol was purchased from Th. Geyer, Berlin GmbH, Berlin, Germany. Assorted chlorinated polycyclic aromatic hydrocarbons have been chosen for this project on the basis that the formation in environmental and food samples is already demonstrated. ^{13}C labeled standards were used as internal standards for quantification. $^{13}\text{C}_6$ -1-Chloropyrene (99%; 50 µg/mL in toluene), $^{13}\text{C}_6$ -7-Chlorobenz[a]anthracene (99%; 50 µg/mL in toluene), $^{13}\text{C}_6$ -7,12-Dichlorobenz[a]anthracene (99%; 50 µg/mL in toluene) was supplied from Promochem LGC Standards GmbH Wesel, Germany. $^{13}\text{C}_6$ -9-Chlorophenanthrene (10 mg) was synthesized and supplied from Campro Scientific GmbH, Berlin, Germany. For samples, an internal standard spiking mixture was used (100 ng/mL each analyte), and calibration was performed with individual stock solutions, by dilution with toluene. The spiking mixture was stored at 8 °C. Native standards 1-chloropyrene (50 µg/mL in toluene), 7-chlorobenz[a]anthracene (50 µg/mL in toluene), 7,12-dichlorobenz[a]anthracene (50 µg/mL in toluene), 9-chlorofluorene (50 µg/mL in toluene), 9-chlorophenanthrene

Fig. 1 Overview of used/analyzed substances

(50 µg/mL in toluene), 2-chloroanthracene (50 µg/mL in toluene), 3-chlorofluoranthene (50 µg/mL in toluene), and 6-chlorobenz[a]pyrene (50 µg/mL in toluene) were supplied from LGC Standards GmbH, Wesel, Germany. Individual stock solutions (5 µg/mL) were prepared in toluene and stored at 8 °C. Standard mixtures with various concentrations were prepared from these individual stock solutions and were used for calibration and validation.

As drying agent for the extraction, performed by using ASE 350, Isolute HM-N was used and purchased from Separtis GmbH, Grenzach-Wyhlen, Germany. Silica 60 (0.063–0.2 mm) and alumina 90 (neutral activity level 1) were purchased from Merck GmbH, Darmstadt, Germany. For column cleanup, the silica and alumina were 3% deactivated with ultrapure water. Ultrapure water with 18.2 mΩ/cm resistivity was obtained from a Milli-Q-System from Merck, Darmstadt, Germany. The carbon column for Cl-PAH fractionation was purchased from Campro Scientific GmbH, Berlin, Germany. For GPC column, Bio-Beads S-X3 (40–80 µm bead) were purchased from Bio-Rad Laboratories, Düsseldorf, Germany.

As reference-gas for the HRMS perfluorotributylamine (FC43 or PFTBA) was purchased from MasCom Technologies GmbH, Bremen, Germany.

Instrumentation

HRGC-HRMS (DFS)

High-resolution mass spectrometer (HRMS) determinations were carried out using a double-focusing mass spectrometer DFS (Thermo Scientific; Bremen, Germany) in connection with a gas-chromatograph 6890N (Agilent, Santa Clara, USA) and a TriPlus sampler (Thermo Scientific, Bremen, Germany). For instrument control and data processing, the Excalibur Software (Thermo Scientific, Bremen, Germany) was used. The TargetQuan Software (Thermo Scientific, Bremen, Germany) was implemented for quantification purpose. The chromatographic separation was performed with an Agilent DB-5MS column (60 m length, 0.25 mm i.d., and 0.25-µm film; Agilent Technologies; Santa Clara, USA) and completed by use of Helium 5.0 (Linde GmbH, Berlin, Germany) as a carrier gas with a 1.2-mL per minute flow rate and a gradient oven program in constant flow mode (Table 1). Mass spectrometer was operated using an EI source, performing with ionization energy of 35 eV at a temperature of 280 °C. The mass spectrometric resolution set was $R > 10,000$. FC 43 (MasCom, Bremen, Germany) was used as reference for continual mass calibration. The instrument ran on multiple-ion detection mode (MID) using assorted mass fragments for quantification and qualification (Table 2).

Cleanup

Cleanup Was Divided into Two Steps

Step one: the analytes from fat separation, therefore, were analyzed using the PrepLink gel permeation chromatography (GPC) system (J2 Scientific, Columbia, MO, USA). This separation was achieved using two inline low pressure columns (76 cm length and 3 cm i.d.) filled with Bio-Beads S-X3 material (Bio-Rad, Berlin,

Table 1 Gradient GC oven program

Temperature program	Rate (°C/min)	Temperature (°C)	Step time (min)
Start		120	2
Ramp 1	50	180	0
Ramp 2	5	250	0
Ramp 3	20	350	15

Table 2 Selected mass fragments for ms detection

Analyte	Quan-trace (m/z)	Qual-trace (m/z)
¹³ C ₆ -9-Cl-Fluorene	171.0390	206.0589
9-Cl-Fluorene	165.0699	200.0387
9-Cl-Phenanthrene	212.0387	214.0358
2-Cl-Anthracene	212.0387	214.0358
3-Cl-Fluoranthene	236.0387	238.0358
1-Cl-Pyrene	236.0387	238.0358
¹³ C ₆ -1-Cl-Pyrene	242.0589	244.0559
7-Cl-Benz[a]anthracene	262.0544	264.0514
¹³ C ₆ -7-Cl-Benz[a]anthracene	268.0745	270.0716
7,12-Cl ₂ -Benz[a]anthracene	296.0154	298.0125
¹³ C ₆ -7,12-Cl ₂ -Benz[a]anthracene	302.0355	304.0326
6-Cl-Benz[a]pyrene	286.0544	288.0514

Germany). The chromatographic separation was carried out with isocratic elution at a flow rate of 5 mL per minute and a dichloromethane/*n*-hexane (50/50; v/v) solvent mix. A special two-dimensional (2D) column switch program was developed (Table 3) for separating fat amounts up to 4 g.

Step two: an automatic solid-phase column method was developed by using the PowerPrep System (FMS, Watertown, MA, USA) as cleanup for analyte fractionation. A specified developed alumina-silica-column (Fig. 2), in combination with a carbon column (Campro Scientific, Berlin, Germany) was implemented in conjunction with various solvents for analyte fractionation in multiple steps. An adapted instrument method was used (Table 4) to perform these fractionation steps.

Extraction

The automated accelerated solvent extraction system ASE 350 (Thermo Scientific, Bremen, Germany) was used for the extraction of all homogenized and dried samples. A total of 100-mL stainless steel cells and 250-mL sample bottles were

Fig. 2 Combined alumina-silica-column for PowerPrep system

used to extract higher sample weights. Optimal extraction results (with the highest fat and analyte yields) were obtained by using dichloromethane/*n*-hexane (50/50; v/v) solvent mixture. The instrument parameters for ASE extraction are shown in Table 5.

Sample Preparation

Grindomix 200 (Retsch, Haan, Germany) with stainless steel container and gravity top was used to grind and homogenize each sample. A freeze dryer Delta 2-24 LCS (Martin Christ, Osterode am Harz, Germany) was used for drying every examined sample matrix. The instrument parameters for lyophilisation are shown in Table 6.

Results

Optimization of the HRGC-HRMS Analysis

The selectivity and specificity of low-resolution mass spectrometry (LRMS) should increase when using high-resolution mass spectrometry (HRMS). Limit of detection should remarkably decrease when applying HRMS. For quantification of analytes in the ultra-trace range, the scanning operation of the mass spectrometer was not sensitive enough to enter the lower pg/μL range. For this reason, multiple-ion detection

Table 3 2D - GPC program

Time window	Phase	System configuration
0–25 min	Dump phase 1	Only column 1
25–50 min	Dump phase 2	Column 1 and 2 in a row
50–110 min	Collect phase	Column 1 and 2 in a row
110–111 min	Rinse phase	Column 1 and 2 in a row

Table 4 PowerPrep program

Step	Flow (mL/min)	Volume (mL)	Description
1	10	20	Flush bypass with <i>n</i> -hexane
2	10	50	Flush multicolumn with <i>n</i> -hexane
3	10	20	Flush carbon column with <i>n</i> -hexane
4	5	50	Sample injection
5	10	50	Elute from multicolumn with <i>n</i> -hexane
6	10	12	Change to <i>dc</i> m/ <i>n</i> -hexane 3:7
7	10	90	Elute both columns with <i>dc</i> m/ <i>n</i> -hexane 3:7
8	10	12	Change to toluene
9	10	90	Elute carbon column via backflush with toluene
10	10	20	End (flush bypass with <i>n</i> -hexane)

mode (MID) was used. This resulted in loss of the option for structural analysis. However, the detection strength increased by a factor of up to 1000 which is achieved by a better signal-to-noise ratio (S/N).

The larger the masses of the selected fragments were, the more specific was the assignment to the analyte. In case of halogenated compounds, it is advantageous to produce molecular ions. In order to determine the optimum ionization energy for obtaining predominantly molecular ions, mass spectra of the analytes used were recorded at different ionization energies. A maximum yield of molecular ions for all analytes was achieved at an ionization energy of 35 eV (Fig. 3). Regulation (EU) 2017/644 stipulates that the deviation of the determined ionic ratio from the theoretical/calculated ionic ratio for an analyte must not be higher than 15%. From the analytical point of view, it is important to consider whether a deviation of 15% for concentrations in the lower pg/ μ L range is too narrow. Through the decreasing ion concentrations, ionic ratios can be generated, which are outside the required 15% and are still not affected analytically. Considering this analytical point, it was decided to increase the limit for the deviation of the calculated value ion ratio from 15 to 20%. Mass spectra of the

individual analytes and the calculated theoretical masses led to the selection of mass traces for the MID method in high-resolution mode already shown in Table 2. The selection of a correspondingly high mass spectrometric resolution can lead to significant increases in sensitivity. In the field of low-resolution mass spectrometry, as it is used in quadrupole devices, it can be due to the partly insufficient resolution, to signal superimposition and increased ground noise. Especially in the case of analytes with negative mass defect, the use of high resolution can lead to a clearly reduced ground noise and an improved signal resolution of two chromatographically superimposed peaks. The loss of transmission when using high-resolution in the case of double-focusing sectoral devices increased the signal-to-noise ratio and led to lower detection limits. The application of a mass spectrometric resolution of $R = 10,000$ as required for dioxin analysis by Regulation (EU) 2017/644 was adopted for the use of Cl-PAH analysis. Following the MS parameter optimization, the chromatographic parameters had to be improved. Tests with two different GC columns (DB-5MS and HT-8 PCB) showed equal results. The DB-5MS was chosen for further analysis.

After optimization of the temperature program, an elution time of the first substance 9-Cl-fluorene at 11 min was achieved. 6-Cl-Benz[a]pyrene eluted after 27.7 min as the latest substance. Without matrix influence, all peaks were separated from each other chromatographically and the runtime

Table 5 Accelerated solvent extraction parameters

Parameter	Setting
ASE cell	100-mL stainless steel
Collection bottle	250-mL bottle with septa
Solvent	Dichloromethane/ <i>n</i> -hexane 1:1
Static flush volume	40%
Static cycles	3
Duration of static cycle	15 min each
Oven temperature	100 °C
Nitrogen flush time	900 s
System pre-flush volume	5 mL
Amount of system flush cycles	1
Oven preheat time	0 min.

Table 6 Instrument method for lyophilisation

Drying step	Phase	Time	Temperature of bowl	Vacuum
1	Load		−25 °C	0.370 mbar
2	Main drying	00:15 h	−25 °C	0.370 mbar
3	Main drying	00:20 h	−23 °C	0.370 mbar
4	Main drying	00:30 h	−20 °C	0.370 mbar
5	Main drying	01:30 h	+20 °C	0.370 mbar
6	Main drying	12:00 h	+20 °C	0.370 mbar
7	Post drying	12:00 h	+25 °C	0.370 mbar

Fig. 3 Optimized ionization energy

of 35 min was very short for the complete analysis on a 60-m column. The baking time at the end of the program could be extended if necessary. Due to the short overall chromatography time, a peak broadening for 6-Cl-benz[a]pyrene could have been counteracted (Fig. 4).

Optimization of the Cleanup

The integration of automated analysis steps in the method, such as extraction and cleanup, should optimize reproducibility, precision, and efficiency of the analytical method.

Fig. 4 Optimized mass fragmentogram

GPC

The purification of the sample extract was necessary in order to remove mainly fat and other accompanying substances, which were extracted from the examination matrix. A fat disruption within the scope of the cleanup by sulfuric acid led to considerable loss of analytes. Accordingly, another cleanup method had to be used. Gel permeation chromatography (GPC) is among others a common method for separating lipophilic sample components, e.g., DIN EN 16215:2012 for dioxins and PCBs. The advantage of GPC lies in its high degree of automation options. Based on massive problems with the use of ethyl acetate/cyclohexane (50/50; v/v) as GPC eluent and the present good extraction results with dichloromethane/*n*-hexane (50/50; v/v), this eluent mixture was used for GPC cleanup. After initial calculations for the detection sensitivity of Cl-PAHs over the whole procedure, it was shown that a fat quantity of 1 g would not be sufficient to achieve the desired detection strength. For this reason, the system of two-dimensional GPC (2D-GPC) has been applied. The 2D-GPC allowed a significantly higher amount of fat to be applied to the separation columns (up to 4 g of fat at once). From the technical point of view, in 2D-GPC, two standard separation columns are switched in series and connected to one another by a valve (partial separating bed doubling). An elution profile of fat was prepared with an injection of 4 g of fat onto the first separation column. For this purpose, 4 g of fish oil was dissolved in 10 mL of dichloromethane/*n*-hexane (50/50; v/v) and injected onto the GPC column 1. At 5-min intervals, the fractions were collected after the separation column, reduced in volume to dryness and balanced. This resulted in the fat elution profile, shown in Fig. 5, and demonstrated that fat was completely eluted off the column after 32 min.

An analyte elution profile was prepared afterwards. For this purpose, 10 mL of GPC eluent was spiked with 100 µL of Cl-PAH standard. This standard contained eight native Cl-PAHs with a concentration of 100 pg/µL each. Subsequently, this spiked solution was applied to the GPC system. After a first dump phase of 25 min on column 1, column 2 was connected in series. Before switching to column 2, already 95% of the fat was eluted. Dump phase 2 followed for 20 min before

Fig. 5 GPC fat elution profile

fractions of 25 mL each was collected in 5-min intervals. The mass spectrometric evaluation revealed the elution profiles shown in Fig. 6.

Finally, after a total runtime of 120 min, an X-PAH fraction was obtained, completely separated from fat. The final volume was 330 mL.

Additional Fractionation Using Silica-Alumina-Carbon Columns

The separation of large-molecular-weight matrix components could be achieved easily by using 2D-GPC. This did not apply to low molecular weight interfering substances. Predetermined and established in the analysis of organic substances is the separation of low molecular weight compounds by using column chromatographic separation processes with aluminum oxide (alumina), silica gel (silica), and activated carbon [DIN EN 16215:2012, LFGB L13.00.33, and LFGB L13.00.34]. To optimize this cleanup step and for further implementation in automated routine analysis, a combination of a silica and alumina column (combi column), shown in Fig. 2, with a subsequently switched active carbon column was used. This included the automation of the retention and elution processes by a flow injection system (PowerPrep). PowerPrep is a computer-aided system, originally developed for routine analysis of dioxins. Performing cleanup using this system can be divided into sections as follows: sample application, retention of analytes on the combi column, removal of interfering substances, elution of analytes from the combi column, retention of analytes at activated carbon column, removing further interfering substances, and elution of analytes from activated carbon column. For the optimized method, the program was used as shown in Table 4. Finally, a purified 90 mL X-PAH fraction was obtained.

Optimized Overall Method

To check the overall method, recovery attempts without matrix were carried out. For this purpose, five standard solutions were used over the complete analysis procedures. By the use of standard solutions, no drying or homogenization was necessary. Therefore, it was decided to abandon these steps and to start directly with the extraction. Five 100 mL ASE cells were filled with purified diatomaceous earth, each spiked with 100 µL of a standard solution which contains 100 pg/µL per analyte. Finally, 100 µL of the internal standard solution (IS) was added per cell. This IS consisted of three ¹³C₆-labeled analytes with levels of 100 pg/µL each. Extraction was fulfilled by the use of ASE with a mixture of dichloromethane/*n*-hexane (50/50; v/v) as extracting agent. The extracts were collected in 250-mL glass bottles and then transferred quantitatively into 250-mL round flasks. Using the rotary evaporator, the extracts were reduced in volume to approximately

Fig. 6 GPC analyte elution profile

1 mL at 32 °C water bath temperature and an applied vacuum from 200 mbar. Subsequently, the scorched extracts were quantitatively converted into GPC vials and diluted (50/50; v/v) with a mixture of dichloromethane/*n*-hexane to a final volume of 10 mL. This step was followed by the 2D-GPC, in which the Cl-PAH fraction was collected in a 500-mL round bottom flask per fraction. The volume of the X-PAH fraction was again reduced on a rotary evaporator at 32 °C to approximately 5 mL. This extract was quantitatively converted with *n*-hexane in a 25-mL pointed flask and filled up to a final volume of about 20 mL. The following step was the automated combi column cleanup by applying PowerPrep. The obtained Cl-PAH fraction was collected in a 100-mL Kuderna Danish flask and concentrated to approximately 0.5 mL by using the rotary evaporator. This fraction was solved in toluene. The concentrated extract was quantitatively transferred to a GC vial and reduced to a final volume of 100 µL by using a nitrogen stream. This extract was measured at the HRGC-HRMS. As reference solution for determination of the recovery, respectively 100 µL of the abovementioned standard solutions (native and labeled) was transferred into a GC vial and applied to a final volume of 100 µL. This solution was set to 100%

reference value. The result of the recoveries of the overall procedure is presented in Table 7.

Validation of the Method

In the first part of validation, an “analytical-related system design test” was carried out, exclusively from the measurement of standard solutions. These solutions could be used for the basic calibration and the determination of the measurement precision. The measurement precision was calculated from the relative standard deviation obtained by multiple measurements of an analytical standard solution and demonstrated the precision of the measuring instrument. It shows the correlation between an analyte concentration and a corresponding measured signal. For this purpose, a standard solution with a concentration of 100 pg/µL per analyte was measured ten times. The generated signals were integrated for each analyte, and the signal area values were calculated. By determining the standard deviation and the mean value, the coefficient of variation could be determined from the data pool of each individual analyte from the multiple measurements. Prior to the statistical evaluation, however, it was to be ensured that the data used was normally distributed and free from outliers. For

Table 7 Recovery of analytes

Analyte	Sample 1 (%)	Sample 2 (%)	Sample 3 (%)	Sample 4 (%)	Sample 5 (%)	Mean (%)	RSD (%)
9-Cl-Phenanthrene	76	72	63	75	75	72	6
2-Cl-Anthracene	76	74	64	74	73	72	6
3-Cl-Fluoranthene	93	97	88	92	96	93	4
1-Cl-Pyrene	98	101	95	96	98	98	2
7-Cl-Benz[a]anthracene	97	101	93	95	98	97	3
7,12-Cl ₂ -Benz[a]anthracene	100	100	99	100	100	100	1
6-Cl-Benz[a]pyrene	74	77	78	87	81	79	5

normal distribution testing, the Anderson-Darling test was used. The test according to Grubbs was used to test for outliers. The data for measurement precision (RSD) for the individual analytes is shown in Table 8. Except for the 9-Cl-fluorene, all RSD values were below 10%, which corresponded to the device specifications of the equipment manufacturer as well as for the typical concentration range of 100 pg. The distribution of the RSDs over the individual analytes turned out very uniform, from which it was concluded that the injections of the automatic sampler were constant and the individual mass traces were separated very precisely from the mass filter. For the basic calibration, 10 calibration support points were used at equidistant intervals.

Linear Range

In order to determine the linear range, standard solutions were prepared of stock and calibration solutions and were measured by using a mass spectrometer. The obtained Cl-PAH solutions had concentrations of 0.1; 1.0; 10; and 100 pg/ μ L. On the basis of the adjustment test according to Mandel, it was possible to determine whether in the selected concentration the calibration function was linear or rather a function of second degree. The statistical tests showed for all analytes the best fit for linear calibration within the selected range.

Working Range

The working range determines the range of concentration to be routinely worked with. This had to be within the linear range and should be selected as far as possible to cover a maximum concentration range. The aim was to achieve the desired concentration range with the minimum number of standards. The number of calibration solutions should not be less than three in order to make a minimal statement about the linearity. For the determination, the Cl-PAHs showed an

optimal working range between 100 fg/ μ L and 100 pg/ μ L. This range did not apply to all analytes, but accounts for all analytes of the linear and the working range.

Detection Limit According to DIN 32 645

Determination of the detection limit according to DIN 32 645:2008 results in additional relevant data for assessing the quality of the calibration function because the detection limit was determined from data of the calibration function. For this purpose, 10 calibration standard solutions in equidistant concentration intervals had been manufactured and measured. The calibration range should be within the range of a decade and the smallest calibration standard should be close to the detection limit. On the basis of this method, the relative process standard deviation was obtained. This was the basis for calculating the detection limit and a necessary quantity in the assessment of the performance of the calibration function. It combines information on precision and sensitivity (DIN 32645: 2008). To determine these data, ten standard solutions with concentrations from 1 to 10 pg/ μ L in 10 equidistant concentration intervals were prepared and measured (Table 8).

Detection Limit from the Smallest Calibration Standard

Due to the low experience with the method, the few collected data and the elaborately determined detection limit from the signal-to-noise ratio, the lowest calibration standard of the current used standard row was set as the detection limit. By incorporating sample processing steps in the calculation of the results, a detection limit in pg/g fat could be achieved. The calculated fat-based detection limits were calculated from a theoretical fat weight of 3 g and a fixed final sample extract volume of 100 μ L.

Table 8 Statistical data

Analyte	Instrument precision	Recovery whole method		Calibration		Limit of determination	Recovery lab-to-lab check	Expanded measurement uncertainty
		Mean	RDS	Lowest std	Highest std			
				(pg/ μ L)	(pg/ μ L)	DIN 32 645	(%)	(%)
9-Cl-Phenanthrene	8	72	6	0,1	100	1.21	76	18
2-Cl-Anthracene	8	72	6	0,1	100	0.77	93	18
3-Cl-Fluoranthene	7	93	4	0,1	100	1	97	12
1-Cl-Pyrene	5	98	2	0,1	100	0.64	99	6
7-Cl-Benz[a]anthracene	6	97	3	0,1	100	0.71	97	9
7,12-Cl ₂ -Benz[a]anthracene	5	100	1	1	100	0.66	95	3
6-Cl-Benz[a]pyrene	5	79	5	1	100	0.64	70	15

Accuracy

To determine the accuracy of a method, certified reference materials (CRM) are predestined. For lack of CRM, a separate reference material had to be produced from spiked matrix samples. In parallel, the repeatability was determined from these samples. The recovery should be based on the requirements of the dioxin and residue analysis in the ppb range. Recoveries between 60 and 120% are accepted in the ppt level (Commission: commission decision 2002/657/EC 2002). In the end, six fish oil samples were spiked with 100 µL of the native Cl-PAH standard, each at a concentration of 100 pg/µL per analyte. The spiked samples were subjected to the complete analytical procedure and measured at the HRGC-HRMS.

Repeatability

To obtain reliable repeatability statistical data, multiple determinations were required under conditions as follows: one sample, same inspector, one device, identical reagents, and 1 day (Kromidas 2011). The corresponding statistical data was determined in parallel from the samples for the determination of the accuracy. The recoveries of all analytes ranged from 72 to 100% (Table 8) and met the requirements of Commission: commission decision 2002 /657/EC 2002 and those for dioxin analysis limits (60–120%). Experience has shown that the standard deviation in such a complex process after such a short development period is about twice as high. On the basis of these data, it was shown that the developed method based on spiked matrix samples provided accurate and correct results.

Robustness

Typical disturbances of analytical methods are deviating volumes within the sample processing steps, incorrect solvents, excessively high temperatures, or evaporation of the analyte solution to dryness. Within the framework of method development, it was already possible to establish the selection of solvent in the evaporation process. Massive analyte losses up to the complete loss of individual substances were observed, when the solvent mixture ethyl acetate/*n*-hexane (50/50; v/v) was used. The same was observed when sulfuric acid was used for fat disruption as a following cleanup step. No significant differences were observed during the extraction step by using different solvents for extraction (except ethyl acetate/cyclohexane). The reduction in volume of sample extracts up to dryness using the rotary evaporator showed no effects with respect to analyte recoveries. The obtained recoveries for the different analytes were in the range of 69% up to 117% applied on variable solvents (Table 8).

Lab-to-Lab Check

In order to check the accuracy of the overall method and due to the lack of proficiency tests, external spiked matrix samples with unknown analyte contents were measured. Therefore, spiked fish oil samples were used. All determined values were in a recovery range between 70 and 99% and thus within the required 60 to 120% in ultra-trace analysis (Commission Regulation (EU) 2017/644, 2017). With the exception of the 9-Cl-phenanthrene and the 6-Cl-benz[a]pyrene, all results yield in recoveries above 95% (Table 8).

Measurement Uncertainty

Due to the small number of data, it was not possible to calculate the measurement uncertainty using the application of the “ISO/IEC Guide 98-3:2008” (2008). In many routine laboratories, the required precision and accuracy data for the calculation of the measurement uncertainty was obtained by means of control cards and proficiency tests. In view of the small number of data and the still limited experience with the application of the analytical method itself, measurement uncertainty was calculated from the 6-fold repeatability experiment measurement data in our study. The extended measurement uncertainty was calculated using the coverage factor of 3 to provide a wider coverage of 99% (Eppe et al. 2015) and is presented in Table 8. The measurement uncertainties were small despite the high coverage factor in comparison to values from the dioxin analysis, but the data was comparable in magnitude (mean value control card for PCDD/F at the National Reference Laboratory (NRL) for Dioxins and PCB in food and feed at the BfR). After statistical verification of individual steps and the overall procedure, the method was then applied to the analyses of various foods and feeds. It was investigated whether the method was robust enough to examine different matrices without major changes in the individual method steps.

Analysis of Selected Matrices

For the application of the developed method applied to common food and feed samples, various matrices were examined. Test samples were selected if occurrence of Cl-PAHs was expected. It is known that there are various foods which contain higher concentrations of dioxins, PCBs, and PAHs which are formed during processing. This is due either to the higher fat content or to the surface properties, which could adsorb contaminated particles. Depending on the position in the food chain, bioaccumulation also plays an increased role.

Similar mechanisms of formation are suggested to be responsible for the generation of X-PAHs; the same food and feed matrices were selected. In addition, food was also selected if consumed in major quantities. It is likely that either high

concentrations of organic contaminants like X-PAHs in food matrices or high consumption of food or both is of special interest regarding consumer's health protection. Analysis of the chosen food matrices will be described below.

Analysis of Mussels

Mussels from Great Britain were examined. The samples were specifically drawn from the Irish Sea and a lake near Perth where increased levels of PAHs and PCBs were already known. Comparable to cod liver, mussels are not generally consumed by a majority of consumers. Concerning the Cl-PAH concentrations, 1-Cl-pyrene and 9-Cl-phenanthrene dominated in a comparable ratio of approximately 1.5:1 in both samples. In the Irish Sea sample, 2-Cl-anthracene, 3-Cl-fluoranthene, and 7,12-Cl₂-benz[a]anthracene were additionally detectable, whereas only 3-Cl-fluoranthene and 7-Cl-Benz[a]anthracene could be detected at the sample derived from the lake near Perth. The distribution of the individual Cl-PAHs in the mussel samples is shown in Fig. 7.

Analysis of Green Tea

In recent years, consumption of green tea has increased throughout Europe, presumably due to suggested beneficial health effects of green tea ingredient's properties. On the basis of various publications on increased Cl-PAH content in environmental samples such as fly ash and other particulate matter in air (Nilsson and Ostman 1993; Ohura et al. 2004, 2005, 2008b, 2009;

Ohura 2007; Ma et al. 2013) and due to its high consumption, green tea was chosen as a matrix for analysis of X-PAH. A green tea from China as well as a jasmine tea from Thailand were examined. The green tea was a gun powder tea from a local Asian market in Berlin, Germany. The jasmine tea was purchased directly in Thailand. In both samples, the most dominant congener was 9-Cl-phenanthrene, which was about 300 pg/g per sample and constituted about half of the total concentration of the few congeners measured. In contrast to the other measured matrices, 6-Cl-benz[a]pyrene was detectable in both tea samples. The distribution of the individual Cl-PAH in the tea samples is shown in Fig. 8.

Analysis of Rice

In Asia, Germany, and throughout Europe, rice is consumed and implemented as a major component of daily nutrition. In 2012, detection of Cl-PAHs in rice from China was published for the first time (Ding et al. 2012). In their study, rice samples from various regions of China were examined. Mean concentrations of 0.5 ng/g wet weight (ww) of 9-Cl-phenanthrene and 0.4 ng/g ww of 2-Cl-anthracene were determined (Ding et al. 2012). In our study, varieties of rice were analyzed as follows: Thai jasmine premium long-grain rice, Thai jasmine rice, parboiled long-grain rice, traditional basmati rice, and long-grain rice. Concentrations of 6-Cl-benz[a]pyrene were determined in Thai jasmine premium long-grain rice (1.8 pg/g) and in Thai jasmine rice (0.9 pg/g); 9-Cl-phenanthrene and 2-Cl-anthracene were not detected in any of the samples in

Fig. 7 Cl-PAH concentrations in pg/g fat in mussels

Fig. 8 Cl-PAH concentrations in pg/g in green teas

contrast to the findings by Ding et al. (2012). Detected concentrations of X-PAHs in our samples were much lower (250–500-fold) as described by Ding et al. (2012) and were all close to the limits of detection.

Analysis of Fat Grilled Pork Belly or Comb

Barbeque and grilling meat, poultry, and vegetables are common forms of food preparation, especially during the summer season. It is well-known that home cooking, such as grilling, roasting, and smoking, particularly charcoal grilled or barbecued foods can lead to high concentrations of PAHs (EFSA 2008). The fat content of the food, as well as the use of marinated food, can influence the formation of processing contaminants. Taking into account the environmental parameters of the charcoal grill, such as high temperatures, fat, coal, and salt, the formation of X-PAHs would be possible analog to the “de novo synthesis” of PCDD/F, PCB, and PCN in thermal processes (Maetzing 2001; Weber et al. 2001; Takasuga et al. 2004). The calculated sums of the Cl-PAH concentrations of the individual marinated samples (pork belly–marinated: 84.8 pg/g fat and marinated pork comb: 67.7 pg/g fat) were above the sum of the non-marinated sample (53.8 pg/g fat). The congener pattern of the grilled meat was dominated by 9-Cl-phenanthrene and 3-Cl-fluoranthene. These two congeners contributed most to the total concentration. The concentration of the 9-Cl-phenanthrene was approximately five times higher than that of the 3-Cl-fluoranthene in the marinated samples. Concentrations of 9-Cl-phenanthrene (27 pg/g fat) and 3-Cl-fluoranthene (26 pg/g fat) were similar in the non-marinated samples. 1-

Cl-pyrene (11.4 pg/g fat) and 3-Cl-fluoranthene (13.9 pg/g fat) were also detectable in the marinated sample. In none of the samples, 6-Cl-benz[a]pyrene was detectable. The distribution of the individual Cl-PAH in the grilled pork samples is shown in Fig. 9.

Analysis of Fresh Eel

Eels (*Anguilla anguilla*), are rich in fat. Depending on the freshwater region, these benthophagous fishes can absorb and accumulate contaminants from sediment (PAHs, PCBs, and dioxins) if contaminated. Due to the abovementioned characteristics of eels, they are suitable as bioindicators for environmental contamination with lipophilic substances. From some freshwater regions in Germany for example increased sediment contamination is known (BfR Opinion No. 027/2010 2010).

Four samples from the “Niederhavel” area in Berlin were taken for this purpose. All samples were aggregate samples, each consisting of three fishes. Predominantly, three congeners were detected as main components. In all samples, 9-Cl-phenanthrene dominated and was the only analyte found in all samples. Furthermore, 6-Cl-benz[a]pyrene was detectable in all samples, except in sample 4. Only low concentrations of 3-Cl-fluoranthene were also detected. Except 3-Cl-fluoranthene and 7-Cl-benz[a]anthracene, all other analytes were detected in sample 1. The respective sums of the contents of these two most heavily loaded samples were approximately the same order of magnitude but were differently composed. Only the samples 2 and 3 had the same congener composition

Fig. 9 Cl-PAH concentrations in pg/g in grilled pork

but different concentrations. The overall sum of Cl-PAHs in the eel samples was between 16 and 26 pg/g fat.

Analysis of Raptor Eggs

In addition to food matrices, raptor eggs were included in the analysis, as eggs from predator birds can be a good indicator for environmental fat-soluble pollutants (von der Trenck 2012). Hence, measureable levels of Cl-PAHs will be expected, and the validity of the analysis can be proofed in another matrix than food. This could extend the application of the method for other questions than food safety, e.g., applicability for environmental samples. Cl-PAHs in egg fat were determined from various raptors (eagle owl, osprey, and peregrine falcon) derived from southern Germany.

In addition, as the tested eggs are similar in composition to chicken eggs, the method for analysis of raptor eggs could be also directly applied for chicken eggs. In addition, due to their environmental dependent contamination, they do not have to be additionally spiked as would be the case for chicken eggs. A robust determination method of Cl-PAHs in eggs in general would be desirable due to the relevance for consumer protection regarding repeated exceeding of maximal levels of organic contaminants in chicken eggs.

Besides 6-Cl-benz[a]pyrene as main component, 9-Cl-phenanthrene was present in all samples. Small amounts of 1-Cl-pyrene could be detected in peregrine falcon as well as in eagle owl samples. Exclusively, in an eagle owl sample, 3-Cl-fluoranthene was determined. Comparing Cl-PAH contents of eggs of the three included bird species, peregrine falcon eggs were the most heavily contaminated samples with concentrations almost twice as high as those in eagle owl and osprey

eggs with regard to all congeners. Again, 6-Cl-benz[a]pyrene was the most abundant congener. Its contribution to the determined concentration of 36.8 pg/g fat for the sum of both congeners was more than two-thirds. The highest value for the sum of the Cl-PAH was found in the peregrine falcon egg with an amount of 56.5 pg/g fat, followed by the eagle owl egg with 36.8 pg/g of fat and the osprey egg with 30.5 pg/g fat.

Discussion

While there are comprehensive methods for the determination of organic contaminants and residues such as PAHs or chlorinated substances such as PCBs and dioxins in environmental and food matrices, the field for the determination of halogenated PAHs is hardly illuminated at all. The first attempts to develop determination methods for the detection of Cl-PAHs have already been made in the environmental sector. Due to the low concentrations, only isolated and mainly mono-chlorinated substances could be detected in these environmental studies. These compounds served as indicator substances for the present work in order to enable a first foray towards the detection of Cl-PAHs in food. The small number of Cl-PAHs examined in this work represents the starting point for the development of new methods for determining halogenated PAHs in foods. Compared to the hundreds of possible congeners of chlorinated or halogenated PAHs, the set of eight compounds examined in the present study is only a spearhead for the opening of a new field of analysis to be considered in future studies. The expansion of the determination method to include other chlorinated or brominated PAHs can be based on the results of the present study.

The method described here is suitable to expand the spectrum of organic contaminant analysis by Cl-PAHs. Compared to Ni and Guo (2013), who used soxhlet extraction for 48 h per food sample and a solvent volume of at least 200 mL per sample, with the automated accelerated solvent extraction system (ASE 350), we were able to reduce the extraction time per sample to 1.5 h. Similarly, solvent consumption could be lowered down to 150 mL per sample and unattended extraction was possible overnight. The method used by Ding et al. (2013), who used several liquid-liquid extraction steps, also had a considerable higher consumption of solvents. Timesaving is an additional advantage of our method. Using the ASE resulted in shorter evaporation- and solvent exchange times due to the lower volumes of solvents.

Subedi and Usenko (2012) described an integrated cleanup within the extraction step. This is not practical for Cl-PAHs for reasons as follows: When using alumina without activated carbon in the ASE cell, the Cl-PAHs already eluted with *n*-hexane from alumina, which resulted in no improvement of the cleanup. Experiments with alumina and activated carbon in the ASE cell showed that while rinsing the activated carbon with toluene in the direction of flow, the Cl-PAHs did not completely elute. Flushing with toluene against flow direction was necessary to elute the Cl-PAHs from activated carbon, completely. However, a flow reversal was not possible using the ASE. For this reason, extraction and activated carbon cleanup had to be performed in two different steps. The development of a 2D-GPC method for fat separation from the sample extract allowed reduced workload and lead to reproducible results. In relation to the standard GPC used for fat separation, as described, e.g., by Kannan and colleagues (Kannan et al. 2008) or by Jira et al. (2008), the 2D-GPC was able to separate 4 g of fat compared to only 1 g of fat per analysis. This resulted in a time gain of about 2 h and a solvent saving of 50% per sample. The use of ethyl acetate/cyclohexane (50/50; v/v) as eluent was not possible at the GPC, since it leads to a massive loss of analyte when the solvent was removed by vacuum in the rotary evaporator. It appears to be possible that acetic acid residues from ethyl acetate can lead to a degradation of some Cl-PAH congeners. In order to avoid this, the GPC method was developed with dichloromethane/*n*-hexane (50/50; v/v) as eluent. The use of high-resolution mass spectrometer resulted in a considerable increase of the detection strength compared to low-resolution mass spectrometry. This could be proved in a direct comparison by using a mussel sample with a resolution of $R = 1000$ (representing the low-resolution quadrupole MS) and $R = 10,000$ (the high-resolution sectoral MS). Figure 10 shows retention periods of analytes at high- and low-resolution, respectively. The baseline ground noise by a set resolution of $R = 1000$ was much higher than at a resolution of $R = 10,000$. This was confirmed by the corresponding signal-to-noise ratios (S/N). At a resolution of $R = 1000$, an S/N of 3/1 was

determined, whereas at a resolution of $R = 10,000$, an S/N of 229/1 was calculated. By using the high-resolution mass spectrometry, a significantly higher S/N ratio was obtained. This resulted in low detection limits and could be confirmed by means of the basic calibration and the calculated detection limit dealing with German DIN 32 645 standard (2008). In matrix-free standard solutions, determination limits were found to be in the range of 1 pg/ μ L based on German DIN 32645 standard and of 0.1 pg/ μ L with respect to the selection of the smallest calibration standard. Sun et al. (2011) were able to establish a limit of quantification, which was based on selection of the smallest calibration standard of 5 pg/ μ L. In comparison, the detection limit achieved in this work in relation to the German DIN 32 645 (2008) standard was increased by a factor of five. If the smallest calibration standard is compared to the detection limit, it is even lower by a factor of 50 than obtained by Sun and colleagues. Despite the double column length (60 m) of the developed and optimized temperature program, a significantly shorter chromatography time was achieved compared to that described by Ding et al. (2012) for the determination of Cl-PAHs in rice. The analysis time and the optimized method developed in this study have been reduced from 51 min for the completely resolved analyte signals as described by Ding et al. to 36 min in our method. The precision of the results of Cl-PAHs in rice measured by Ding et al. (2012) led to relative standard deviations of approximately 70%. According to the method developed in this study, average relative standard deviations of 10% were determined.

In our experiments, we used a coverage factor of 3 to calculate extended measurement uncertainty and to provide a wider coverage of 99% (Eppe et al. 2015, see Table 8). Even using the coverage factor of three to obtain the approximated extended measurement uncertainty, a dimension was reached, which is still significantly below that of the method described by Ding et al. (2013). The developed method is applicable to different matrices and results in reproducible and precise results. This even applies to low-fat food and feed. It can be used for the analysis of food and environmental samples of scientific and for regulatory interest. Due to the high level of automation, both personnel and time effort are very low. In addition, the method is very robust with regard to the variability of the solvent selection and the loss of analytes by evaporation to dryness.

Currently, there are no maximum levels or health-based guidance values on Cl-PAHs. Chlorinated congeners have a higher toxicological potential relative to their non-chlorinated counterparts (Sun et al. 2013). The maximum levels for the sum of dioxins and dl-PCBs are in the pg (WHO₂₀₀₅-TEQ)/g range, while those for PAHs are in the ng/g range. The determined contents for the sum of Cl-PAHs from the examined

Fig. 10 Reduced ground noise depending on ms resolution

samples were in the lower to middle pg/g range and were not calculated on the basis of toxicity equivalent factors (TEQ factors). A correlation between PCB and Cl-PAH or between dioxin and Cl-PAH concentrations within the samples was not apparent. To sum up, we established a new analytical method for determination of chlorinated PAHs in food and environmental samples. The method proved to be time-, material-, and personal-saving. The method should be validated in a broad-scale sent as laboratory comparison.

Conclusion

The results obtained in this work show that selected chlorinated polycyclic aromatic hydrocarbons (Cl-PAH) can be reliably detected from fat and fat-free or vegetable matrices in the lower picogram range. An automated 2D-GPC method

has been developed, which allows to apply a significantly higher amount of fat to the separation columns (up to 4 g of fat at once) to achieve lower detection limits. The effective parameters on efficiency of the developed automated multi-layer solid-phase extraction and isotope dilution high-resolution mass spectrometry (ID-HRMS) were investigated and optimized. Further advantages of the proposed method were significant solvent- and time savings, caused by an unattended automated overnight cleanup procedure. The predominant combined use of automated analysis instruments like accelerated solvent extraction (ASE), 2D-GPC, multilayer solid-phase extraction, and ID-HRMS leads to a robust determination method with reproducible results and good accuracy. Ultimately it was shown that this method, which describes the determination of analytes about which little is known so far and focuses on food matrices, is applicable to be used for routine operations.

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Compliance with Ethical Standards

Conflict of Interest Carsten Schoernick declares that he has no conflict of interest.

Anja Lueth declares that she has no conflict of interest.

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