Application Note

Food Testing and Agriculture

Determination of 19 Polycyclic Aromatic Hydrocarbon Compounds in Salmon and Beef

Using Captiva EMR—Lipid Cleanup by GC/MS/MS

Authors

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Abstract

This Application Note presents the development and validation of a multiresidue method for the analysis of polycyclic aromatic hydrocarbon (PAH) residues in salmon and beef. The method uses liquid extraction, followed by Agilent Captiva EMR—Lipid cleanup, and analysis by GC/MS/MS. Salmon or beef samples were extracted using solid/liquid extraction (SoLE) followed by Captiva EMR—Lipid cleanup. The cleaned sample eluent was then back-extracted using isooctane to remove water prior to GC/MS/MS analysis. The extraction efficiency of PAHs from the fatty food matrices was improved using a two-step SoLE with a mixture of ethyl acetate and acetonitrile. Agilent Captiva EMR—Lipid cartridges provided efficient and selective cleanup of the sample matrix, and the developed method was verified in both salmon and beef. The results showed that all the tested PAH compounds achieved acceptable recovery results based on European Commission regulation (recoveries of 50 to 120 %), RSD <20 %, and calibration curves from 1 to 500 ng/g in salmon and beef with R^2 >0.99. The matrix co-extractive residue removal efficiency measured by gravity was 60 % in salmon and 92 % for beef.

Introduction

PAHs are a large class of ubiquitous and toxic compounds characterized by a thermodynamically stable fused aromatic ring structure. These compounds are naturally found in crude oils and coal, but can also be formed through food processing. PAH compounds can be classified according to the number of condensed aromatic rings, as light (2–3 rings) or heavy (4–6 rings) PAHs. The heavy PAHs are more stable and toxic than the lighter ones. The U.S. Food and Drug Administration (FDA) requires PAH analysis at low-ppb levels in seafood[1]. The European Commission (EC) specified the criteria for the methods of analysis of four heavy PAH compounds: benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, and chrysene down to a limit of quantitation (LOQ) of 0.9 µg/kg and limit of detection (LOD) of 0.3 µg/kg for each PAH[2].

PAHs are highly lipophilic compounds, and tend to bio-accumulate in fatty foods such as fish, meat, oil, and milk. The main challenge for the analysis of PAHs in fatty food matrices is to isolate the analytes of interest from the bulk presence of lipid compounds in the food matrix. This challenge includes the efficient extraction of PAHs from fatty matrix, then the selective removal of the unwanted fatty matrix co-extractives. Common sample preparation techniques include Soxhlet extraction[3], solid/liquid extraction with ultrasound-assisted extraction[4], pressurized solvent extraction[5], and QuEChERS extraction[6]. These techniques can be coupled with cleanup procedures such as solid phase extraction[7] (SPE) or gel permeation chromatography[8].

Agilent Enhanced Matrix Removal—Lipid (EMR—Lipid) dSPE cleanup has gained considerable attention since its introduction in 2015. The EMR—Lipid dSPE sorbent selectively interacts with the unbranched hydrocarbon chains of lipids, leaving bulky target analytes in solution for subsequent analysis. This selective interaction makes it ideal for multiclass, multiresidue analysis in fatty food matrices. Captiva EMR—Lipid cartridges require less water for sorbent activation (20 %) compared to the traditional Bond Elut EMR—Lipid (50 %). This change simplifies the workflow, and improves the recoveries of hydrophobic compounds during cleanup[9].

This study investigates sample preparation using Captiva EMR—Lipid cartridge pass-through cleanup for the analysis of 19 PAH compounds in salmon and beef by GC/MS/MS. This method was developed to improve the limitations of a previous method using Bond Elut EMR—Lipid dSPE cleanup in PAH determination in food[10]. Table 1 shows the classification, Log P value, retention time, and MS/MS transitions for the tested pesticides.

Table 1. List of PAHs for analysis, Log P value, retention time (RT), and MS/MS conditions.

PAH Compound (Abbreviation)	Log P	RT (min)	First MS/MS (*m/z*)	CE (V)	Second MS/MS (*m/z*)	CE (V)
Naphthalene (Na)	3.3	6.12	128.1 → 102.1	20	128.1 → 78	20
Acenaphthylene (Ac)	3.9	8.28	152.1 → 126	30	152.1 → 150.1	50
Fluorene (F)	4.2	9.21	166.1 → 165	50	165.1 → 164.1	20
Phenanthrene (Pa)	4.5	11.50	178.1 → 152.1	25	178.1 → 176.1	50
Anthracene (A)	4.5	11.65	178.1 → 176.1	50	178.1 → 152.1	25
Pyrene (P)	4.9	15.61	202.0 → 202.0	50	202.0 → 200.0	50
Benzo[c]fluorine (BcF)	5.4	16.62	215.8 → 214.8	50	215.8 → 212.8	50
Benz[a]anthracene (BaA)	5.9	19.29	228.1 → 226.1	30	226.1 → 224.1	35
Chrysene (Ch)	5.9	19.45	228.1 → 226.1	30	226.1 → 224.1	40
5-Methylchrysene (5MeCh)	6.4	20.73	241.8 → 240.8	50	241.8 → 238.8	50
Benzo[b]fluoranthene (BbF)	6.4	22.52	252.1 → 226.1	30	252.1 → 252.1	50
Benzo[k]fluoranthene (BkF)	6.4	22.59	252.1 → 252.1	50	252.1 → 250.1	50
Benzo[j]fluoranthene (BjF)	5.7	22.69	251.7 → 251.7	50	251.7 → 249.7	50
Benzo[e]pyrene (BeP)	6.4	23.66	251.8 → 251.8	50	251.8 → 249.8	50
Benzo[a]pyrene (BaP)	6.4	23.81	252.1 → 250.1	50	125.1 → 124.1	10
Perylene	6.4	24.21	252.1 → 252.1	50	252.1 → 250.1	50
Dibenzo[a,h]anthracene (DBahA)	7.1	27.68	277.8 → 277.8	50	277.8 → 275.8	50
Indo[1,2,3-cd]pyrene (IP)	7.0	27.78	277.0 → 277.0	50	276.0 → 274.0	50
Benzo[g,h,l]perylene (BghIP)	6.6	29.39	275.8 → 275.8	50	275.8 → 273.8	50

Experimental

Chemicals and reagents

PAH and IS standards were from Ultra-Scientific (North Kingstown, RI, USA) or Agilent. HPLC-grade acetonitrile (ACN), acetone, and ethyl acetate (EtOAc) were from Honeywell (Muskegon, MI, USA). Reagent-grade isooctane was from Sigma-Aldrich (St. Louis, MO, USA).

Solutions and standards

The two PAH stock solutions were in acetone at 2,000 µg/mL or 500 µg/mL. The working solution was prepared from the stock solutions at 4 µg/mL in acetone. A spiking solution was then freshly prepared at 1 µg/mL in acetone for daily sample spiking. An IS working solution containing five IS compounds was prepared in acetone at 20 µg/mL. Both working solutions were stored in amber glass vials in a refrigerator at 4 °C for one month.

The 20:80 EtOAc/ACN extraction solvent and 16:64:20 ACN/EtOAc/water elution solution were prepared and stored at room temperature.

Equipment and material

The study was performed using an Agilent 7890B GC coupled with an Agilent 7000D triple quadrupole GC/MS. The GC system was equipped with an electronic pneumatic control (EPC), a multimode inlet (MMI) with air cooling, an Agilent 7693A automatic liquid sampler (ALS), and a backflush system based on a purged Ultimate union controlled by an AUX EPC module. Agilent MassHunter workstation software was used for data acquisition and analysis.

Sample preparation equipment included:

- Centra CL3R centrifuge (Thermo IEC, MA, USA)
- Multi Reax Test Tube Shaker (Heidolph, Schwabach, Germany)
- 2010 Geno/Grinder (Metuchen, NJ, USA)
- Pipettes and repeater (Eppendorf, NY, USA)
- Agilent positive pressure manifold 48 processor (PPM-48) (p/n 5191-4101)
- Captiva EMR—Lipid cartridge, 3 mL, 300 mg (p/n 5190-1003)
- Ceramic homogenizers (p/n 5992-9312)

Instrument conditions

The GC/MS/MS instrument conditions were established based on a previously published method[11]. Table 2 lists the conditions of GC/MS/MS operation.

Table 2. 7890B and 7000D GC/MS/MS conditions.

Parameter	Value
Column 1	J&W DB-EUPAH, 30 m × 0.25 mm, 0.25 µm (p/n 122-9632), Front MM inlet to Aux EPC 4
Column 2	J&W Silcotek deactivated tubing, 1.36 m × 0.15 mm, 0 µm (p/n 160-7625-5), Aux EPC 4 to MSD
Carrier gas	Helium
Mode	Constant flow
Column 1 flow	1.1063 mL/min
Column 2 flow	1.942 mL/min
Injection volume	2 µL pulsed splitless
Inlet liner	4 mm id Ultra Inert liner single taper w wool (p/n 5190-2293)
Oven temperature program	80 °C hold for one minute, Ramp to 200 °C by 25 °C/min, Then to 335 °C by 8 °C/min, Hold for 9.325 minutes
Max oven temperature	340 °C
Run time	32 minutes
Backflush conditions	Two minutes post run 335 °C oven temperature 50 psi aux EPC pressure, and 2 psi inlet pressure
Transfer line temperature	320 °C
Source temperature	EI source, 320 °C
Quadrupole temperature	150 °C
Data monitoring	Dynamic MRM mode
Solvent delay	Three minutes

Figure 1 shows typical MRM chromatograms for each PAH compound in the fortified salmon samples at the level of 1 ng/g using the established GC/MS/MS conditions.

Sample preparation

The deep sea caught salmon and organic beef were purchased from a local grocery store, chopped into small pieces, and stored at −20 °C. The frozen samples were homogenized with dry ice using a mechanical grinder.

The homogenized sample was then weighed (2.5 g) into 50-mL centrifuge tubes, and spiked as necessary with standard and IS solutions. Salmon and beef samples were then prepared using the procedure shown in Figure 2, featuring three major parts:

1. Sample extraction by a two-step solid-liquid extraction(SoLE), in light blue box
2. Sample extract cleanup using Captiva EMR—Lipid cartridges, in light purple box
3. Post treatment for water removal using isooctane back-extraction (BE), in light green box

The entire workflow introduced a four-fold dilution of the original sample concentration.

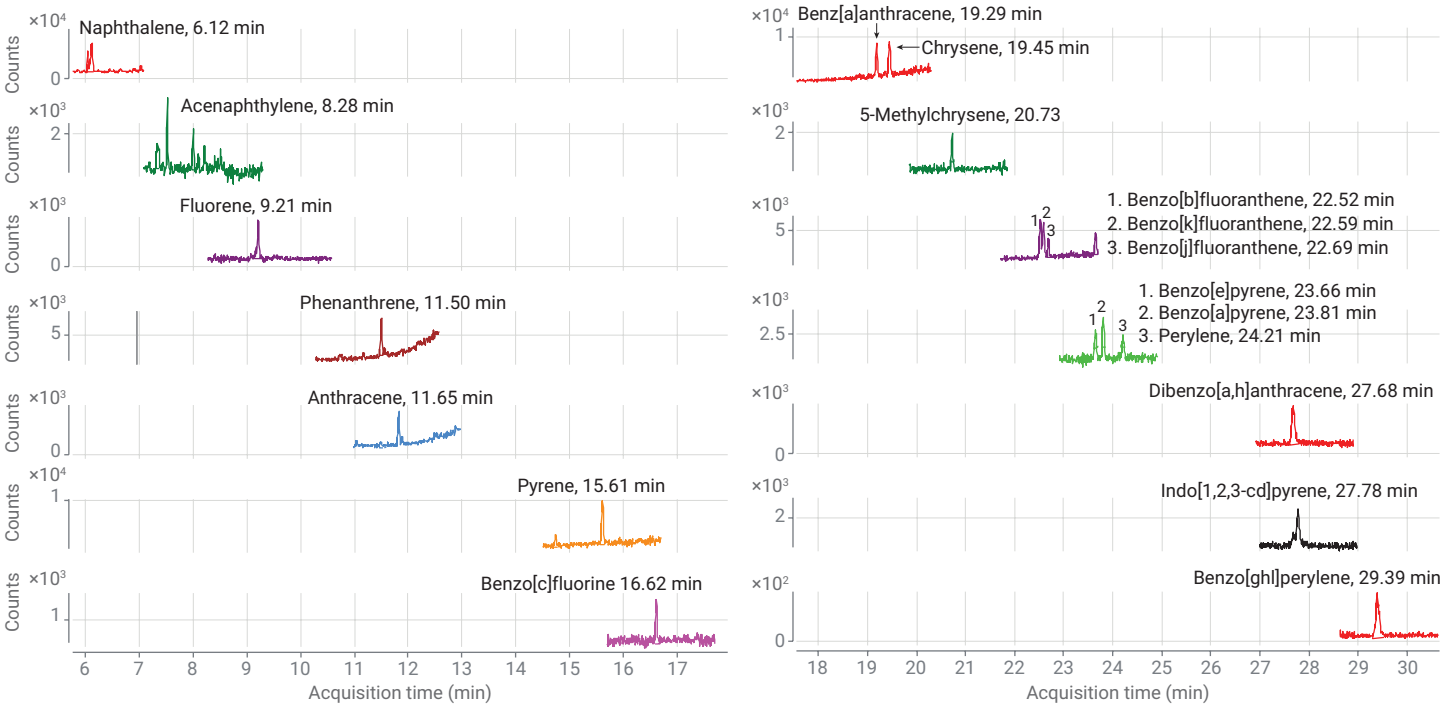

Figure 1. GC/MS/MS MRM chromatogram of PAHs in the fortified salmon sample at the level of 1 ng/g.

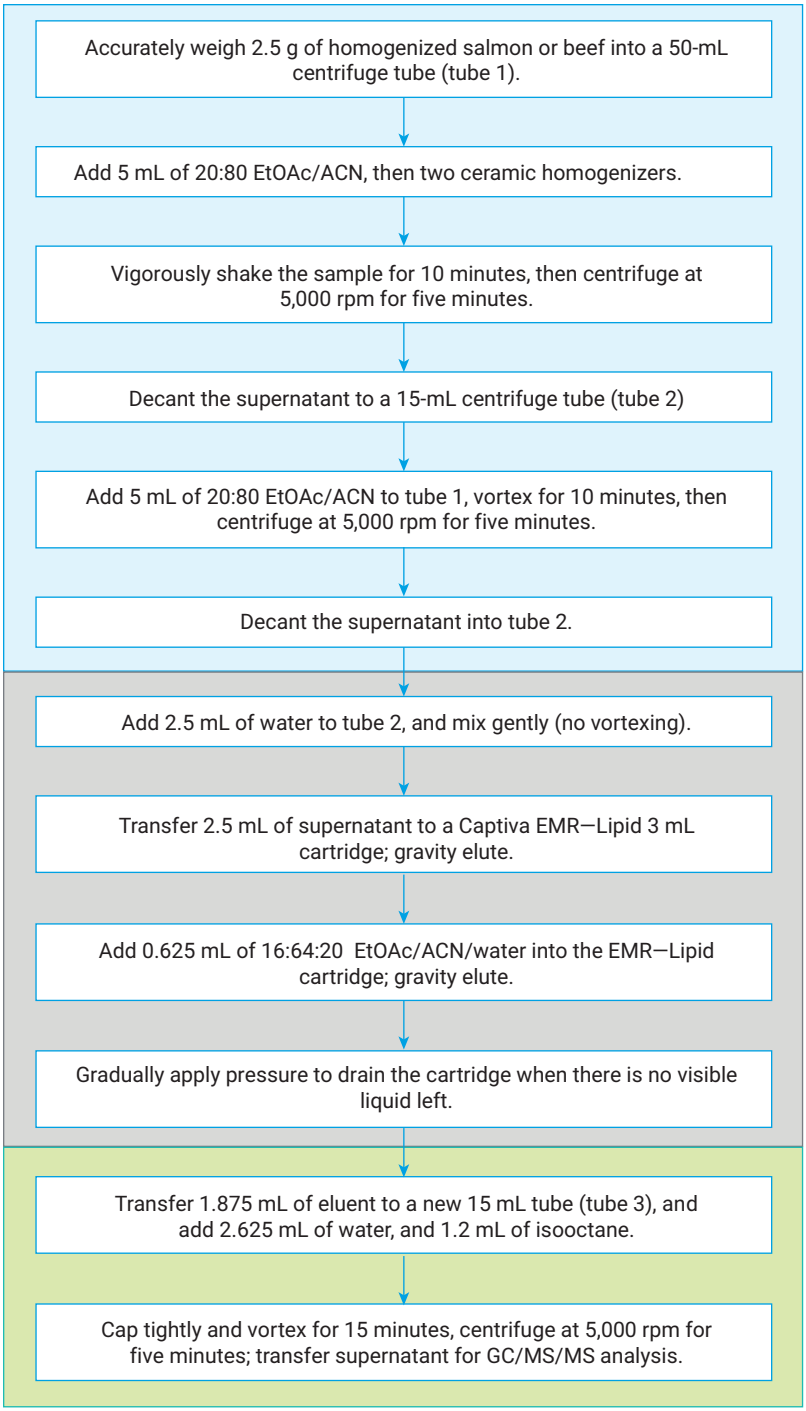

Figure 2. Schematic salmon or beef preparation procedure using solid/liquid extraction followed with Captiva EMR—Lipid cleanup.

Matrix co-extractives removal evaluation

The matrix removal was investigated by gravimetric determination of sample co-extractive residue. The amount of co-extractive residue was determined by gravimetric measurement[9] to study matrix removal after the sample extraction and cleanup procedure. The co-extractive residue weight was collected based on 1 mL of final sample extract (n = 2) with correction for the dilution factor when applicable, and the average weight was used to determine the matrix removal %.

The cleanup efficiency of Captiva EMR can also be observed visually based on the amount of residue left over after drying 1 mL of sample.

Method validation

The optimized sample preparation method was validated in terms of analyte recoveries, quantitation accuracy and precision, limits of quantitation (LOQs), and calibration curve linearity in salmon and beef. The calibration standards included 1, 2, 5, 10, 20, 50, 100, 250, 400, and 500 ng/g in salmon and beef. Three concentrations of QC samples were quantified against calibration curves at n = 6 for low-level (1 ng/g), mid-level (10 ng/g), and high-level 100 ng/g in salmon or beef. Analyte identification and quantitation were determined from retention times and MRM transitions.

Results and discussion

EMR—Lipid sorbent and product

EMR—Lipid sorbent uses a novel chemistry that combines size exclusion and hydrophobic interactions, providing high lipid removal selectivity and efficiency. Only lipid-like molecules containing unbranched hydrocarbon chains can enter the EMR—Lipid sorbent pores and be retained by hydrophobic interactions. Target analytes that do not have lipid-like structures are unable to enter the sorbent pores, remaining in solution for subsequent analysis. As a result, EMR—Lipid sorbent can differentiate lipids from other target analytes, and deliver high analyte recovery and lipid removal efficiency.

Sample preparation optimization

Sample preparation method optimization included three stages:

1. SoLE
2. Captiva EMR—Lipid cleanup
3. Post treatment for water removal

The extraction step is critical for achieving high recovery of hydrophobic PAH compounds in fatty matrix. The challenge for sample extraction is due to the high hydrophobicity of both PAH analytes and fatty food matrices. Given the success of a previous application for hydrophobic pesticides in oil matrix[9], the SoLE using 20:80 EtOAc/ACN extraction solvent was used directly as the preliminary protocol. The extraction time and multiple SoLE were optimized for analyte recovery, and Figure 3A shows the results. The results show that longer extraction time increased PAH extraction recovery. A two-step SoLE also improved extraction efficiency over one-step SoLE. As a result, the two-step SoLE with 5 mL of extraction solvent with 10 minutes of shaking for each step was used as the optimal extraction method.

The analyte recovery for the EMR—Lipid cartridge cleanup step was then studied. As PAH compounds are very lipophilic, especially heavy PAHs, the use of a second elution is important to achieve good elution recoveries. The results (Figure 3B) showed that the second elution can increase the elution recovery by approximately 20–25 % on average. In addition, the use of stronger solvent 16:64:20 EtOAc/ACN/water provided the best elution for heavy PAH elution.

After the optimization of sample extraction and EMR—Lipid cleanup, the post treatment step for water removal was investigated. There are three major methods for EMR—Lipid post treatment to remove water residue prior to GC/MS/MS analysis:

- Salt partition using anhydrous $MgSO_4$
- drying and reconstitution
- hydrophobic solvent back extraction (BE)

Table 3 contains a list of general methodology, pros and cons, and suitability for each of the three post treatment procedures. Since PAHs are a class of compounds with high hydrophobicity, this application fits well for using hydrophobic solvent back-extraction, which provided solvent switching and partial concentration, as well as feasibility for both light and heavy PAH compounds. Thus, solvent BE using isooctane was used for water removal after Captiva EMR—Lipid cleanup. Figure 3C shows the isooctane BE step recoveries.

Using this optimized method, the analyte recovery for the entire method was collected at three spiking levels: 1, 10, and 100 ng/g in both salmon and beef (n = 6). Even with variations in different matrices, all PAH analyte recoveries were within the acceptable limits (50–120 %) at different spiking levels, in two matrices.

Figure 3. Sample preparation method optimization based on PAH recovery for the solid/liquid extraction step (A), EMR—Lipid cleanup step (B), and isooctane back extraction (C).

Table 3. Sample post treatment for water removal after Captiva EMR—Lipid cleanup.

Method for Water Removal	General Methodology	Advantages	Disadvantages	Suitability
Salt partition by anhydrous $MgSO_4$	– Add 700 mg of anhydrous $MgSO_4$ per 1 mL of EMR—Lipid eluent. – Vortex vigorously and centrifuge.	– Usually no significant analyte loss	– Labor-intensive operation – Time-consuming – Inability to swap for a GC-amenable solvent	– Multiresidue analysis
Dry and reconstitute	– Use sample evaporation equipment to dry EMR eluent (TurboVap, CentriVap). – Reconstitute in a GC-amenable solvent. – Mix thoroughly.	– Relative easy operation – Feasible sample concentrating and solvent swapping	– Time-consuming – Volatile analyte loss – Possible degradation of labilecompounds	– Nonvolatile and stable analytes – Samples can be relatively low volume – Concentrating is required to reach low LOQ
Hydrophobic solvent back-extraction	– Add water to the EMR eluent to approximately 1:2 organic/water. – Add isooctane (equivalent to the organic volume or slightly less). – Vortex thoroughly for 10 minutes, and centrifuge.	– Relatively easy operation – Feasible solvent swapping and partial sample concentrating – Further removal of dissolved polar matrix co-extractives	– Loss of polar to medium-polar compounds – Possible leaking during sample mixing	– Hydrophobic compounds with log P ≥3

Method validation

The quantitation method validation includes limit of detection (LOD), calibration curve linearity, analyte accuracy, and precision at three spiking levels. Five internal standard (IS) compounds: naphthalene-D_8, acenaphthylene-D_{10}, phenanthrene-D_{10}, chrysene-d_{12}, and perylene-D_{12} were used for analyte quantitation. Table 4 summarizes the method validation results in salmon and beef.

The four heavy PAHs: benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, and chrysene, are important compounds being regulated with extremely low LOQ (0.9 ng/g) and LOD (0.3 ng/g)[2]. The chromatograms of these four compounds at the established LOQ (1 ng/g) in each matrix are shown in Figure 5. Given the validated LOQ of 1 ng/g in both matrices (Table 4) and signal-to-noise ratio (S/N) at LOQ for the four analytes (Figure 5), we are confident that this method can be validated for 0.9 ng/g LOQ, and may even go lower. Further study is planned to investigate the lower LOQ and LOD for these analytes.

Figure 4. PAH recovery in salmon (A) and beef (B) using the optimal sample preparation method.

Table 4. Quantitative validation results for the analysis of PAHs in salmon and beef, using the optimized method.

Analyte abbr.	IS	Salmon						Beef					
		Calibration curve			Mean accuracy% (RSD%), n = 6			Calibration curve			Mean accuracy% (RSD%), n = 6		
		LOQ (ng/g)	HLOQ (ng/g)	R^2	Low QC (1 ng/g)	Mid QC (10 ng/g)	High QC (100 ng/g)	LOQ (ng/g)	HLOQ (ng/g)	R^2	Low QC (1 ng/g)	Mid QC (10 ng/g)	High QC (100 ng/g)
Na*	Naphthalene-D_8	1	500	0.9960	103(5.7)	93(4.5)	103(2.1)	NA	NA	NA	NA	NA	NA
Ac	Acenaphthylene-D_{10}	1	500	0.9958	86(13.9)	91(3.4)	96(3.7)	1	500	0.9953	97(5.5)	99(3.3)	102(3.9)
F		1	500	0.9940	90(11.1)	86(6.8)	93(5.9)	1	500	0.9935	100(6.0)	100(6.1)	103(4.9)
Pa		1	500	0.9962	94(11.6)	93(2.2)	98(2.7	1	500	0.9982	90(8.9)	96(2.3)	93(6.0)
A**	Phenanthrene-D_{10}	2	500	0.9968	75(7.2)	86(3.3)	93(1.5)	1	500	0.9963	80(10.2)	92(3.2)	99(3.1)
P**		1	500	0.9970	66(10.9)	87(3.7)	96(2.5)	2	500	0.9951	90(6.0)	104(6.3)	105(6.8)
BcF		1	500	0.9976	98(8.9)	89(2.0)	96(3.3)	1	500	0.9970	89(7.6)	105(5.7)	104(9.1)
BaA		1	500	0.9963	83(4.0)	89(1.0)	96(1.5)	1	500	0.9990	87(6.4)	91(1.7)	99(2.6)
Ch	Chrysene-D_{12}	1	500	0.9986	91(6.2)	88(3.2)	98(1.5)	1	500	0.9994	93(10.0)	93(1.3)	100(2.2)
5MeCh		1	500	0.9977	80(3.8)	86(1.4)	93(2.0)	1	500	0.9960	95(7.0)	104(8.7)	107(2.9)
BbF		1	500	0.9949	80(3.6)	83(4.6)	89(3.2)	1	500	0.9937	100(3.8)	95(8.5)	99(2.9)
BkF		1	500	0.9984	75(6.7)	80(1.5)	85(3.0)	1	500	0.9984	88(9.0)	89(10.3)	96(4.2)
BjF		1	500	0.9977	84(6.7)	87(3.0)	90(4.5)	1	500	0.9958	83(6.8)	102(8.2)	101(3.8)
BeP	Perylene-D_{12}	1	500	0.9964	93(4.8)	91(2.9)	99(1.5)	1	500	0.9968	102(7.8)	95(4.0)	99(2.2)
BaP		1	500	0.9970	68(6.4)	82(1.2)	91(1.1)	1	500	0.9982	90(9.7)	84(3.2)	86(2.9)
Perylene		1	500	0.9978	89(3.0)	87(2.2)	95(1.1)	1	500	0.9986	84(7.9)	91(5.8)	96(2.3)
DBahA		1	500	0.9974	81(7.3)	80(2.1)	91(5.9)	1	500	0.9957	87(7.7)	78(8.5)	91(11.1)
IP		1	500	0.9957	57(7.2)	69(3.5)	79(6.3)	1	500	0.9972	72(7.1)	65(7.4)	73(10.2)
BghIP		1	500	0.9979	69(7.5)	73(1.5)	82(5.9)	1	500	0.9967	71(5.2)	64(8.2)	70(4.0)

* Naphthalene is not quantifiable in beef due to highly incurred level.

** Raised LOQ due to slightly incurred level detected sample blank.

IS = Internal standard; LOQ = Limit of quantification (low end); HLOQ = High limit of quantification; QC = quality control; PAH abbreviations refer to Table 1.

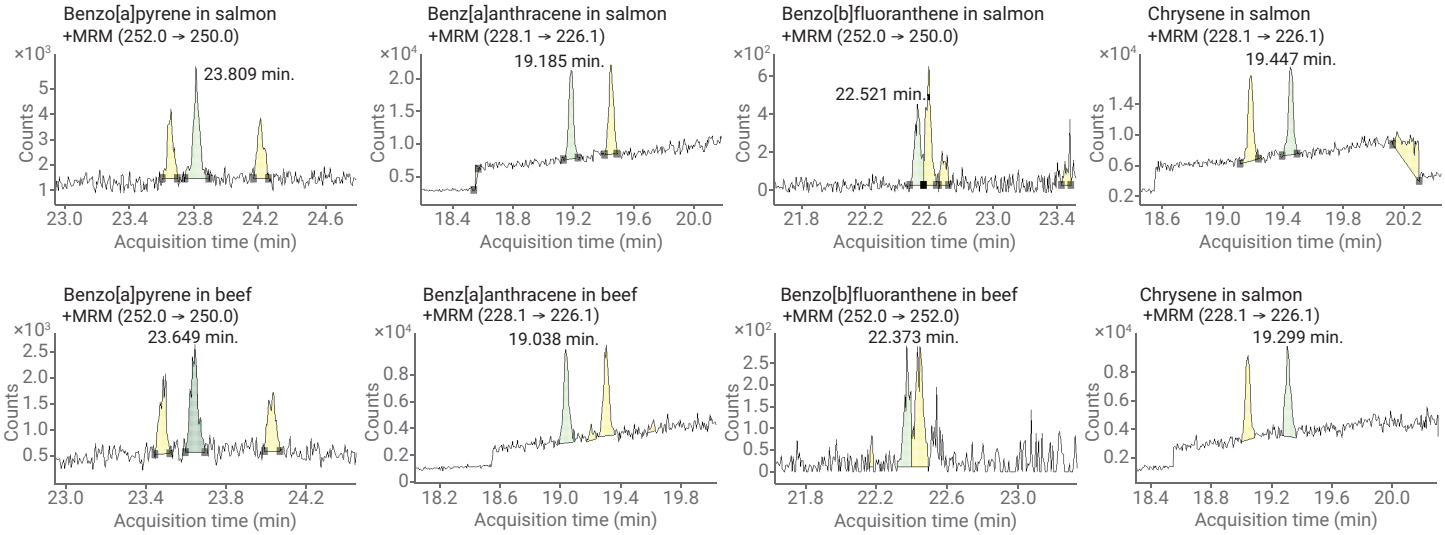

Figure 5. Critical PAH compound chromatograms at LOQ of 1 ng/g in salmon (top) and beef (bottom).

Matrix cleanliness assessment

The sample matrix residue in the final extract, and matrix residue removal by cleanup was investigated in both salmon and beef. Figure 6 shows the visual appearance of dried sample residue with the actual residue weight. Based on the difference of dried residue weight between sample without cleanup and with EMR—Lipid cleanup, the EMR—Lipid cleanup provided 60 % of matrix removal for salmon and 92 % matrix removal for beef.

Conclusion

A simple, rugged, and reliable method using solid-liquid extraction followed by Captiva EMR—Lipid cartridge cleanup was developed and validated for the multiresidue analysis of PAHs in salmon and beef. The method was optimized to improve the extraction efficiency and complete elution on a Captiva EMR—Lipid cartridge, followed by an isooctane back-extraction for water removal and solvent swamping. The quantitative analysis showed that all the tested PAHs provided acceptable average recoveries (50–120 %) and excellent reproducibility, with <20 % average RSD, which meets the EC acceptance criteria. The method also demonstrates the potential to achieve a lower LOQ for the four critical PAHs required by the EU Commission. The results demonstrate that the optimized method provides high matrix cleanup, excellent analyte recovery, and precision results for multiresidue analysis of PAHs in salmon and beef.

	Salmon	Beef
No cleanup (mg/mL crude extract, n = 2)		
	5.64	2.75
Captiva EMR—Lipid cleanup		
Residue (mg/mL final extract, n = 2)	2.24	0.21
Matrix residue removal (%)	60	92

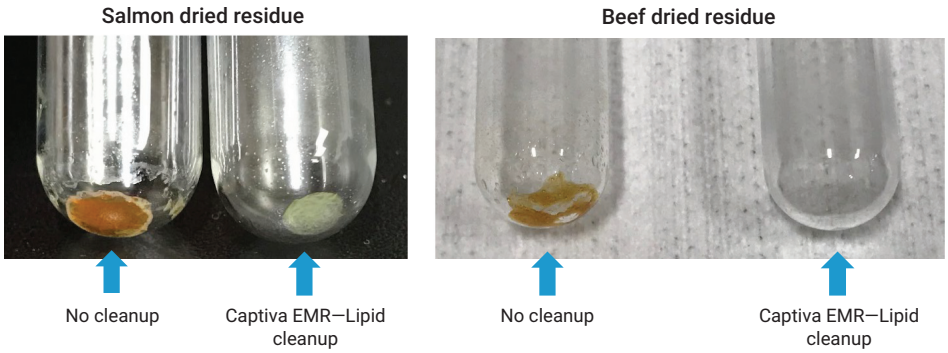

Figure 6. Matrix residue removal assessment by residue weight and appearance.

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