



**U.S. FOOD & DRUG
ADMINISTRATION**

Compendium of Analytical Laboratory Methods for Food and Feed Safety

METHOD NUMBER: C3-001

VERSION: 1.0

EFFECTIVE DATE: Nov. 1, 2017

EXPIRATION DATE: Nov. 1, 2020

METHOD TITLE: Screening and Determination of Polycyclic Aromatic Hydrocarbons in Seafoods Using QuEChERS-Based Extraction and High-Performance Liquid Chromatography with Fluorescence Detection

AUTHORS: Samuel Gratz, Angela Mohrhaus Bryan Gamble, Jill Gracie, David Jackson, John Roetting, Laura Ciolino, Heather McCauley, Gerry Schneider, David Crockett, Douglas Heitkemper, and Fred Fricke

AUTHOR AFFILIATION: ORA/ORS/MPTSLO/Forensic Chemistry Center

VALIDATION STATUS: grandfathered method determined to be equivalent to a Level 3 multi-laboratory validated method

METHOD SUMMARY: The method provides a procedure to screen for fifteen targeted parent polycyclic aromatic hydrocarbons (PAHs) and provides an estimate of total PAH concentration including alkylated homologs in oysters, shrimp, crabs, and finfish. PAHs are extracted from seafood matrices using a modified QuEChERS sample preparation procedure. The method utilizes High-Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) for the determination step. This procedure is applicable to screen a variety of seafood matrices including oysters, shrimp, finfish and crab for the presence of parent PAHs and the common alkylated homologs due to oil contamination. This method was originally developed and validated in response to the 2010 Gulf of Mexico oil spill.

Screening and Determination of Polycyclic Aromatic Hydrocarbons in Seafoods Using QuEChERS-Based Extraction and High-Performance Liquid Chromatography with Fluorescence Detection

Version: 1.0 (May 2017)

Authors: Samuel Gratz, Angela Mohrhaus Bryan Gamble, Jill Gracie, David Jackson, John Roetting, Laura Ciolino, Heather McCauley, Gerry Schneider, David Crockett, Douglas Heitkemper, and Fred Fricke

Authors Affiliation: ORA/ORS/OMPTSLO/ Forensic Chemistry Center

Chemistry Research Coordination Group Posting Concurrence: 10/5/2017

Table of Contents

2017.1	METHOD TITLE
2017.2	SCOPE OF APPLICATION
2017.3	PRINCIPLE
2017.4	SAFETY CONSIDERATIONS
2017.5	REAGENTS
2017.6	STANDARDS
2017.7	PREPARATION OF SAMPLES OR TEST PORTIONS
2017.8	APPARATUS/INSTRUMENTATION
2017.9	METHOD
2017.10	CALCULATIONS AND REPORTING
2017.11	VALIDATION INFORMATION/STATUS
2017.12	REFERENCES

2017.1 METHOD TITLE

Screening and Determination of Polycyclic Aromatic Hydrocarbons in Seafoods Using QuEChERS-Based Extraction and High-Performance Liquid Chromatography with Fluorescence Detection

2017.2 SCOPE OF APPLICATION

The method provides a procedure to screen for the fifteen targeted parent polycyclic aromatic hydrocarbons (PAHs) listed in Table 1 at concentrations below the established FDA levels of concern in oysters, shrimp, crabs, and finfish. The analytical limits listed in Table 1 are presented as an example of results achievable for seafood matrices when using the method and equipment specified herein. Analytical limits will vary depending on instrumentation and actual operating conditions used.

Additionally, the method provides an estimate of total PAH concentration including alkylated homologs in the sample. This procedure is applicable to screen a variety of seafood matrices including oysters, shrimp, finfish and crab for the presence of parent PAHs and the common alkylated homologs due to oil contamination. This method was originally developed and validated in response to the 2010 Gulf of Mexico oil spill.

2017.3 PRINCIPLE

Polycyclic aromatic hydrocarbons are extracted from seafood matrices including oysters, shrimp, crab and finfish using a modification of the quick, easy, cheap, effective, rugged, and safe (QuEChERS) sample preparation procedure, employing acetonitrile as the solvent followed by the addition of salts to induce water partitioning. The extracts are centrifuged and filtered using 0.2 micron syringe filters, but require no post-extraction sample cleanup prior to analysis. The method utilizes High-Performance Liquid Chromatography with Fluorescence Detection (HPLC-FLD) for the determination step to screen for fifteen targeted parent PAHs and to provide an estimate of total PAH concentration including alkylated homologs in the sample. The chromatographic method employs a polymeric C18 stationary phase designed for PAH analysis with gradient elution to resolve the analytes using a 35 minute run time. This method is based on the work of Ranalhosa, *et al.* and Pule, *et al.* (1,2).

2017.4 SAFETY CONSIDERATIONS

Use appropriate personal protective equipment including safety glasses, gloves and lab coat. Analysts should consult and must be familiar with their lab's chemical hygiene and safety plan and Material Safety Data Sheets for all reagents and standards listed. Refer to the instrument manuals for safety precautions regarding use. All waste generated must be handled appropriately.

Table 1. Typical Method detection limits (MDL) and limits of quantitation (LOQ) determined for parent PAHs in edible seafood matrices.^a Units are µg/kg.

PAH Compound	Oysters		Shrimp		Crab ^b		Finfish	
	MDL	LOQ	MDL	LOQ	MDL	LOQ	MDL	LOQ
naphthalene	8.1	18	3.9	8.4	6.9	15	8.1	18
acenaphthene	0.80	1.7	0.76	1.7	1.6	3.4	0.80	1.7
fluorene	0.56	1.2	0.22	0.48	0.70	1.5	0.56	1.2
phenanthrene	0.31	0.67	0.59	1.3	0.79	1.7	0.31	0.67
anthracene	0.12	0.27	0.09	0.19	0.73	1.6	0.12	0.27
fluoranthene	2.2	4.8	1.6	3.5	0.86	1.9	2.2	4.8
Pyrene	1.7	3.7	0.65	1.4	1.0	2.2	1.7	3.7
benzo[a]anthracene	0.65	1.4	0.46	0.99	0.56	1.2	0.65	1.4
chrysene	0.70	1.5	0.42	0.92	0.60	1.3	0.70	1.5
benzo[b]fluoranthene	0.28	0.61	0.53	1.2	0.54	1.2	0.28	0.61
benzo[k]fluoranthene	0.25	0.53	0.09	0.20	0.38	0.81	0.25	0.53
benzo[a]pyrene	0.27	0.59	0.10	0.22	0.50	1.1	0.27	0.59
dibenzo[a,h]anthracene	2.5	5.5	1.7	3.8	1.7	3.6	2.5	5.5
benzo[g,h,i]perylene	1.6	3.5	1.2	2.6	1.1	2.4	1.6	3.5
indeno[1,2,3-cd]pyrene	3.1	6.7	2.9	6.4	7.6	17	3.1	6.7

^aDetermination based on five replicates from each of the three laboratories (n=15).

^bCrab data was obtained under wavelength switching conditions.

2017.5 REAGENTS

- Acetonitrile (CH₃CN), HPLC grade (Fisher p/n A998, or equivalent)
- Isopropyl alcohol (IPA), HPLC grade (Fisher p/n A451, or equivalent)
- Water, 18.2 MΩ water from a Millipore Milli-Q Gradient A-10 water source (or equivalent) referred to as DIW (de-ionized water)
- NIST Standard Reference Material 1974b, Organics in Mussel Tissue (*Mytilus edulis*)

2017.6 STANDARDS

- Parent PAH Stock Standard mix: 16 parent PAH compounds (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene,

benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene) each at 2000 µg/mL in methylene chloride. Sources include CLP SemiVolatiles PAH standard from Absolute Standards, Inc.

Note: Although the mix contains 16 PAH compounds, acenaphthylene does not display any appreciable fluorescence under the method conditions.

- Alkylated PAH Homologs Stock Standard Mix: A 20-component PAH compounds stock standard mix (Chiron NPD Cocktail) with components ranging from 100 – 500 µg/mL in isooctane, was obtained from Chiron. Alkylated naphthalenes and alkylated phenanthrenes account for 13 of the 20 components, with concentrations in the range 250 – 500 µg/mL. A listing of the components follows: 1- methyl-naphthalene, 2- methyl-naphthalene, 1,3-dimethyl-naphthalene, 1,4- dimethyl-naphthalene, 1,5- dimethyl-naphthalene, 1,6-dimethyl-naphthalene, 1,7-dimethyl-naphthalene, 2,6- dimethyl-naphthalene, 2,7-dimethyl-naphthalene, biphenyl; phenanthrene, 1- methyl-phenanthrene, 2-methyl-phenanthrene, 3-methyl-phenanthrene, 9- methyl-phenanthrene, dibenzothiophene, 1-methyldibenzothiophene, 2- methyldibenzothiophene, 3-methyldibenzothiophene, and 4-methyldibenzothiophene.
- Parent PAH Individual Solution Standards (200 µg/mL in methanol or methylene chloride) of naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, and indeno[1,2,3-cd]pyrene) were obtained from Supelco Analytical (Bellefonte, PA). Solid state standards of biphenyl and dibenzothiophene were obtained from Aldrich (Milwaukee, WI), and benzo[e]pyrene was obtained from Supelco. Individual standards were used to establish retention times.
- PAH Alkylated Homologs Individual Standards of C1-C4 naphthalenes, C1-C3 fluorenes, and C1-C4 phenanthrenes were obtained as follows: 1-methyl-naphthalene, 2- methyl-naphthalene, 1,3-dimethyl-naphthalene, and 2,7-dimethyl-naphthalene were obtained from Acros Organics (Morris Plains, NJ). 1,6-dimethyl-naphthalene, 2,6- dimethyl-naphthalene, 1-methylfluorene, and 1-methylphenanthrene were obtained from Ultra Scientific (North Kingstown, RI). 1,4-dimethyl-naphthalene, 1,5- dimethyl-naphthalene, and 2,3,5-trimethyl-naphthalene were obtained from MP Biomedicals (Santa Ana, CA). Stock solutions (500 – 1000 µg/mL) of 1,2,5,6- tetramethyl-naphthalene, 1,7-dimethylfluorene, 9-n-propylfluorene, 2- methylphenanthrene, 1,3-dimethylphenanthrene, 1,2,6-trimethylphenanthrene, and 1,2,6,9-tetramethylphenanthrene were obtained from Chiron AS (Emeryville, CA).

Parent PAH Stock Standards Preparation

The CLP SemiVolatiles PAH Stock Standard (*or equivalent*) is a solution that contains a mixture of sixteen PAHs in methylene chloride, each at a concentration of 2000 µg/mL (ppm). Additional stock solutions are prepared via serial dilution for fortification studies or other uses as follows:

- 250 µg/mL (ppm) stock standard: 10 mL of this stock spiking solution is prepared by combining 1.25 mL of the CLP SemiVolatiles PAH Stock Standard, followed by dilution to 10 mL with CH₃CN.

- 5.0 µg/mL (ppm) stock standard: 25 mL of a 5 µg/mL spiking solution is prepared by adding 500 microliters of the 250 µg/mL stock standard to a 25 mL volumetric flask, and diluting to volume with CH₃CN.
- 0.5 µg/mL (ppm) stock standard: 25 mL of a 0.5 µg/mL spiking solution is prepared by adding 2.5 mL of the 5 µg/mL stock standard to a 25 mL volumetric flask and diluting to volume with CH₃CN.

Calibration Standards Preparation

Calibration standards are prepared at concentrations of 2.5, 25, and 50 ng/mL (ppb) as follows:

- 50 ng/mL (ppb) standard: prepare a 1:10 dilution of the 0.5 µg/mL (ppm) stock standard with CH₃CN.
- 25 ng/mL (ppb) standard: prepare a 1:20 dilution of the 0.5 µg/mL (ppm) stock standard with CH₃CN.
- 2.5 ng/mL (ppb) standard: prepare a further 1:10 dilution of the 25 ng/mL calibration standard with CH₃CN.

Check Standards/Continuing Calibration Verification (CCV) Standards

A check standard made to the same final concentration as the extracts from the spiked matrix samples should be used. Parent PAH check standards were prepared by serial dilution of the nominally 250 µg/mL (ppm) stock standard spiking solution. Refer to Table 2 for preparation of typical check standards at three nominal concentrations. All dilutions are prepared in acetonitrile.

For preparation of the alkylated PAH homologs check standard, a 30,000-fold dilution of the alkylated PAH homologs stock mix was made by initial dilution in IPA (25 µL stock mix + 975 µL IPA), followed by serial dilution in CH₃CN (25 µL 1st dilution + 975 µL CH₃CN, then 53 µL 2nd dilution + 947 µL CH₃CN).

2017.7 PREPARATION OF SAMPLES OR TEST PORTIONS

Seafood samples should be prepared by first obtaining the edible portion as described in Table 3. Samples are then composited and homogenized by blending in Robot Coupe food processor or equivalent for 2-3 minutes. Seafood samples were stored frozen, but partially thawed prior to preparation for analysis. Compositing of multiple individuals from the same site may be appropriate. The minimum sample size for this analysis is 5 grams.

Table 2. Dilution scheme for preparation of typical parent PAH Check/CCV standards (*equivalent dilution schemes may be substituted*).

Spiking Level	Spiked Matrix Sample Solution Final Concentration(ng/mL)	Check Standard Preparation
High and Mid	33.3	First Dilution: Prepare a 1000-fold dilution of the 250 µg/mL stock standard spiking solution by taking a 100 µl aliquot into a 100 ml volumetric flask, and diluting to volume.
		Second Dilution: Prepare a 7.5-fold dilution of the first dilution by taking 133 µl first dilution plus 867 µl CH ₃ CN. Use the second dilution as the check standard for the high and mid level spiked samples.
Low (oysters, crab, shrimp)	16.7	Third Dilution (oysters, crab, shrimp): Prepare a 2-fold dilution of the second dilution by taking 500 µl second dilution plus 500 µl CH ₃ CN. Use the third dilution as the check standard for the low level spiked samples of oysters, crab, and shrimp.
Low (finfish)	8.31	Third Dilution (finfish): Prepare a 4-fold dilution of the second dilution by taking 250 µl second dilution plus 750 µl CH ₃ CN. Use the third dilution as the check standard for the low level spiked samples of finfish.

Table 3. Directions for obtaining edible tissue portion of selected matrices.

Finfish	Remove heads, tails, scales, fins, viscera and bones, save edible portion. If the skin is considered edible, collect it as well.
Crab	Remove the front claw (propus) and the next section of the claw (merus), break the pincher off by pulling down on it. Insert an oyster tool into the opening and break the exoskeleton so that the meat inside can be removed. With the crab head up pull off the top shell (carapace) and discard. Remove viscera and gills. Collect the meat that is around the outer edge of the bottom section of the crab. These are in cartilage sections; an oyster tool can be used to break through the cartilage to obtain the small portion of meat. Approximately 20 grams of meat was typically obtained from a 6 inch blue crab.
Shrimp	Remove the head, shell, legs, and tail. Save the remaining edible portion.
Oyster	Find an opening between the top and bottom shell of the oyster to wedge the oyster tool into. When the correct location is found, a small amount of liquid inside the oyster will seep out around the edge where the 2 halves of the oyster come together. Pry the 2 shells apart, then scrape and collect all of the insides including the liquor.

2017.8 APPARATUS/INSTRUMENTATION

- Liquid chromatograph (Agilent 1200 series) with binary pump, degasser, autosampler, thermostatted column compartment and multiwavelength fluorescence detector.
- Agilent Chemstation software for controlling LC and data analysis
- Zorbax Eclipse PAH analytical column, rapid resolution HT, 4.6 x 50 mm 1.8 μm (Agilent p/n 959941-918)
- Zorbax Eclipse analytical guard column, 4.6 x 12.5 mm 5 μm (Agilent p/n 820950-939)
- Guard column hardware kit, high pressure (Agilent p/n 820888-901)
- Centrifuge capable of 3000 x g for 50 mL centrifuge tubes
- Balance, sensitivity of 0.1 mg
- Adjustable pipettes (10 - 100 μL , 100 - 1000 μL , and 1 - 10 mL) and disposable tips
- Centrifuge tube racks for 50 mL (30 mm) tubes
- Robot Coupe processor with stainless steel bowl (Robot Coupe p/n R301UB)
- Buffered QuEChERS extraction tubes with foil packet containing 6 g of magnesium sulfate and 1.5 g of sodium acetate (AOAC Method 2007.01, Agilent p/n 5982-5755)
- Ceramic homogenizers for 50 mL tubes (100/pk), (Agilent p/n 5982-9313)
- Syringes (without needles, nonsterile, BD Luer-Lok Tip, 5 mL capacity), (Fisher p/n 14-823-16D)
- PTFE syringe filters (0.20 μm pore size, 25 mm dia.), (Fisher p/n SLFG 025 NK) *Note: to minimize interferences, it is critical that PTFE not be substituted with nylon or other materials.*
- 4 mL amber glass vials with PTFE lined caps, (Fisher p/n B7800-2A)

All equipment and supplies listed may be substituted with equivalent.

Disclaimer: The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration. Equivalent performance may be achievable using apparatus and materials other than those cited here.

2017.9 **METHOD**

Sample Extraction

Finfish, Shrimp and Crab

For analysis of finfish, shrimp and crab, 5 grams of homogenized sample composite and a ceramic homogenizer are transferred to a QuEChERS extraction tube. Five grams of DIW water are then added to the extraction tube followed by vortex mixing or shaking for 1 minute. A 15 mL volume of CH₃CN is added to the extraction tube followed by a second one minute vortex or shaking step. Next, the contents of the QuEChERS extraction foil packet (6 g of magnesium sulfate and 1.5 g of sodium acetate) are added to the mixture. The mixture is shaken vigorously for 1 minute; and the extract is centrifuged at 3000 x g for 10 minutes, allowing for removal of the CH₃CN (upper) layer. Approximately 6-8 mL of the 15 mL total volume of CH₃CN readily separates into the upper layer. A portion (approximately 4 mL) of the supernatant extract is filtered through a 0.2 µm PTFE syringe filter into an amber glass vial and analyzed without further dilution using HPLC-FLD.

Oysters

For analysis of oysters, the extraction differs only in that no water is added to the sample, thereby eliminating one mixing step described above for the other matrices. The addition of water to homogenized oyster samples was determined to be unnecessary due to the amount of water present in the native tissue.

Quality Control Samples

Fortification / Spike Recovery Samples

The analysis of one fortified sample matrix with each batch of 20 or fewer samples is required. Five grams of homogenized composite is fortified with 50 µL of the 5.0 µg/mL stock solution. This provides a fortification level of 0.05 µg/g of sample.

SRM 1974b Organics in Mussel Tissue

The analysis of SRM 1974b in triplicate is required as an initial demonstration of accuracy. For SRM 1974b, the extraction procedure is identical to that described for oysters. Due to the low levels of PAHs in the SRM, a ten-fold concentration step of the filtered extract is necessary. This is accomplished by evaporating 1 mL of extract to dryness under a stream of dry air without heating followed by reconstitution with 100 µL of acetonitrile.

Method and Solvent Blanks

A method blank must be analyzed with each batch of 20 or fewer samples to monitor for contamination from laboratory sources. Method blanks and fortified method blanks are prepared by substituting 5g of DIW water in place of sample composite and performing the extraction procedure as for oysters described above.

Additionally, a solvent blank (CH₃CN) should be analyzed between one or more samples or sample types to demonstrate lack of carryover from run to run.

Liquid Chromatography with FLD Analysis

Samples, fortified samples, standards and blanks are analyzed using an Agilent 1200 Series liquid chromatograph equipped with a binary pump, degasser, autosampler, thermostatted column compartment and a fluorescence detector, all operated under the control of Chemstation software.

Separation of PAHs was accomplished at a flow rate of 0.8 mL/min on a Zorbax Eclipse PAH Rapid Resolution HT (4.6 x 50 mm, 1.8 µm) column with a Zorbax Eclipse Analytical Guard Column (4.6 x 12.5 mm, 5 µm). The mobile phase consisted of water and acetonitrile run as a gradient with conditions described in Table 4. The column thermostat was set to 18 °C and all injections were 10 µL.

Thorough mobile phase degassing is required to minimize oxygen quenching of PAH fluorescence, with benzo[a]pyrene demonstrating the most susceptibility. Daily purging of the solvent reservoir intake channels, and continuous degassing of mobile phase solvents is required. The use of a helium sparge, especially for startup purging of the solvent reservoir intake channels, has been found to help significantly with quenching issues even when in-line vacuum degassing is used.

Table 4. HPLC Mobile Phase Gradient Program

time	volume % acetonitrile	volume % water	Comment
0	60	40	Analysis (from 0 to 30 min.)
1.5	60	40	
7.0	90	10	
13.0	100	0	
30.00	100	0	
30.01	60	40	Re-equilibration to initial conditions (from 30 – 35 min.)
35.00	60	40	

Fluorescence detection is used with the following parameters: excitation wavelength 260 nm; multiwavelength emission detection (352, 420 and 460 nm); PMT gain setting of 13 for general screening and PMT gain setting 15 for analysis of SRM 1974b only. The three emission wavelengths are necessary for detection of all 15 parent PAH compounds (see Table 5 below). Alkylated homologs of the parent PAHs are generally detected at the same emission wavelength as the parent compound. The PMT gain setting of 13 was chosen to maximize sensitivity for the 0 – 50 ng/mL (ppb) concentration range, and is suitable for this work. Lower PMT gain settings (11 or 12) can be substituted to increase the linear range upwards into the 300 - 500 ng/mL (ppb) range, as needed. An example HPLC-FLD chromatogram showing the separation of the fifteen parent PAH compounds is shown in Figure 1.

Initial work was conducted using programmable wavelength switching, in which the emission wavelength was changed during the chromatographic run. Programmable wavelength switching

may provide a minor increase in sensitivity for individual compounds compared to multiwavelength emission detection. Our laboratory observed an average of 16% decrease in signal slope across parent PAH compounds for multiwavelength detection compared to wavelength switching. However, wavelength switching should be limited to the targeted screening of the parent PAHs only, whereas multiwavelength detection may be applied to more general PAH screening, including both the parent PAHs and their alkylated homologs.

Table 5. Multiwavelength Emission Detection

Emission λ (nm)	Detected PAH Compounds
352	naphthalene, acenaphthene, fluorene, phenanthrene
420	anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[ghi]perylene
460	indeno[1,2,3-cd]pyrene

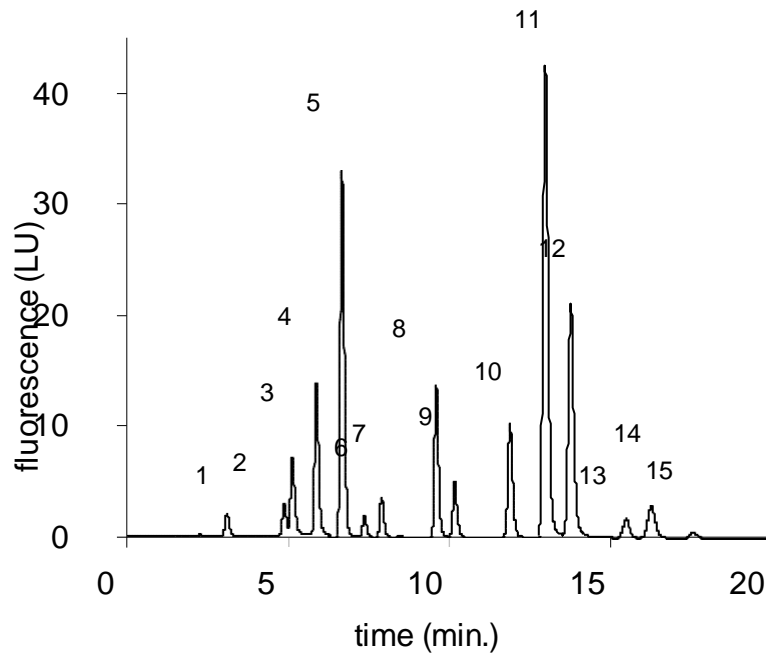


Figure 1. LC-FLD chromatogram of 15 PAH standard mix, 33 ng/mL each. Peak identifications as follows: 1 naphthalene, 2 acenaphthene, 3 fluorene, 4 phenanthrene, 5 anthracene, 6 fluoranthene, 7 pyrene, 8 benzo[a]anthracene, 9 chrysene, 10 benzo[b]fluoranthene, 11 benzo[k]fluoranthene, 12 benzo[a]pyrene, 13 dibenzo[a,h]anthracene, 14 benzo[g,h,i]perylene, 15 indeno[1,2,3-cd]pyrene.

Quality Control Elements

- A minimum of three calibration standard levels must be analyzed to demonstrate linearity with $r^2 \geq 0.99$ for all analytes.
- A single low level spike is required as an initial demonstration of recovery for each matrix type. Spike recoveries should be demonstrated at 50 ng/g for oysters, crab and shrimp and 25 ng/g for finfish.
- The analysis of SRM 1974b in triplicate is required as an initial demonstration of accuracy and precision. Analysis of SRM 1974b should fall within the acceptable range (see Table 7) for 8 or more of the PAHs screened.
- The Method Detection (MDL) for a given analyte should be determined using a minimum of five replicates of matrix recoveries fortified with approximately 5 ng/g for each of the PAHs identified in Table 5.
- The Limit of Quantitation (LOQ) for a given analyte should be determined according to 40 CFR Part 136⁵ using a minimum of 5 replicates of matrix recoveries fortified with approximately 5 ng/g for each of the PAHs identified in Table 5.
- HPLC/FLD retention times should be within 1% of the corresponding standard for peak identification in samples.
- Continuing calibration verification (CCV) standards should be analyzed at the beginning and end of each batch of 20 or fewer samples. If CCV results do not meet the following criteria, then the entire batch and calibration standards must be reanalyzed. The concentrations of the CCV standards run at the beginning and end of each batch should agree within $\leq 10\%$ of their known concentrations as determined using the external standard calibration curve (prepared from a minimum of 3 standards). Typically, this standard is at a concentration near the middle of the calibration range such as the 16.7 ng/mL standard (see also Table 1).
- A minimum of one fortified sample matrix with each batch of 20 or fewer samples is required. Recoveries of the 0.05 $\mu\text{g/g}$ PAH spike through the method must be in the range 60% - 130%. The retention times in the spiked samples should be within 1% of the RT of the corresponding standard.
- A minimum of one method blank made with 5 g DIW water in place of sample matrix must be analyzed with each batch of 20 or fewer samples. The PAH concentrations found in the method blank should be subtracted from the concentrations found in the samples. Some PAHs, such as naphthalene, are ubiquitous and may be difficult to eliminate. With the exception of benzo[a]pyrene, higher background levels may be acceptable. Concentrations in the method blank should not exceed 3 times the certified concentration for the PAH in SRM 1974b.
- A minimum of one sample replicate must be analyzed with each batch of 20 or fewer samples. For triplicate replicates, the precision is considered acceptable if the percent relative standard deviation (RSD) is ≤ 15 percent for all analytes detected above the

LOQ. For duplicate replicates, this translates to a relative percent difference of ≤ 30 percent for all analytes detected above the LOQ.

2017.10 CALCULATIONS AND REPORTING

Blank Run Subtraction for Acetonitrile Gradient

Given the gradient conditions, the chromatographic baseline is not flat, and may cause difficulty in obtaining accurate peak integrations, especially for the routine screening of uncontaminated or low level contaminated samples. The gradient baseline is much more pronounced for detection at 352 nm, but may also be problematic with detection at 420 or 460 nm. For accurate integration, the chromatogram for the solvent blank (CH₃CN) should be subtracted out prior to integration using the data system software (this feature is available on Agilent systems). When conducting subtraction of the solvent blank run, ensure that the detection emission wavelengths are matched between the sample and solvent blank chromatograms (*i.e.*, subtract 352 nm solvent blank run from the 352 nm sample run, *etc.*). Blank run subtraction is recommended for all standards, samples, and method blanks. Choose a mid to late solvent blank run from the sequence for subtraction, *i.e.*, do not use the first solvent blank for subtraction (typically the first injection in the sequence), as the system may not be fully equilibrated.

Method Blank Evaluation

Method blanks are used to monitor for background PAH levels associated with all solvents and materials (extraction tubes, reagent packs, *etc.*) used for sample preparation and extraction. Method blanks frequently show the presence of some naphthalene and phenanthrene, which should be subtracted from the corresponding sample peak areas when calculating the individual amounts. Method blank chromatograms may also be used to assess any unusually high background PAH levels associated with specific batches of solvents or materials.

Peak Identification

Individual chromatographic peaks were identified based on comparison of their retention times to those of known reference standards. Allowable variability of the LC/FLD retention times should be within 1% of the corresponding standard for peak identification in samples.

Quantitation of Individual PAHs

Concentrations of individual PAHs in the sample extracts are determined using peak areas and external calibration. Calculations may be based on generated external calibration curves or CCV standards.

Concentrations of individual PAHs in the samples (w/w) are calculated as follows:

$$\text{PAH in sample (ng/g)} = \text{PAH in extract (ng/mL)} \times (15 \text{ mL} / 5 \text{ g sample})$$

Estimation of Total PAH Concentration

A conservative estimate of the total amount of PAHs in samples including alkyl homologues, which are often the major PAH components in weathered crude oil, is determined using the total peak area determined over the RT range of 2.5 – 20 minutes and the sensitivity (slope of the calibration curve) for the least sensitive parent compound which is typically naphthalene. The total area can be obtained as the summation of individually integrated peaks, or via integration of a single “peak” with the baseline drawn from 2.5 – 20 minutes.

Example using a naphthalene slope of 0.43: A sample is analyzed and found to contain 10 peaks in the RT range of 2.5 – 20 minutes. Peaks may or may not match RT for known parent compounds.

Estimated Total PAHs in extract (ng/mL) =

(total area of peaks integrated from 2.5-20 min after solvent blank run subtraction) / 0.43

Estimated Total PAHs in sample (ng/g) = Estimated total PAH in extract X (15 mL / 5g)

Total fractional amount (Carcinogenic PAH)

The total fractional amount is the sum of all percentages for carcinogenic analytes and should be less than 1. The carcinogenic analytes include benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, and indeno[1,2,3-cd]pyrene.

Total Fractional Amount = \sum level determined (mg/kg) / level of concern (mg/kg)

Reporting

Table 6 provides the FDA established levels of concern for parent PAHs in shrimp, crab, finfish, and oysters following the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (4). The HPLC-FLD method described in this document is considered to be a screening method for PAH contamination in seafood. In the past, any positive or indeterminate findings were confirmed using another method such as the NOAA method (3). Sample results from the HPLC-FLD method were evaluated for 1) individual parent PAH concentrations and 2) estimated total PAH concentration.

Individual parent PAH concentrations.

For the HPLC-FLD screening method, the levels requiring confirmatory analysis were set at 50% of the FDA established levels of concern as shown in Table 6. If any one or more parent PAH concentrations exceeded these levels, the sample was required to undergo confirmatory analysis.

Estimated total PAH concentration.

The estimated total PAH concentration in the sample is intended as a very conservative estimate that is only used to determine if a confirmatory analysis should be performed. A confirmatory analysis is needed if the total PAH estimate exceeds 50% of the FDA established level of concern for naphthalene, as shown in Table 6. Thus for shrimp and crab the estimated total PAH level requiring confirmatory analysis is 61.5 mg/kg, while in oysters and finfish, the estimated total PAH levels requiring confirmatory analysis are 66.5 and 16.3 mg/kg, respectively.

Total fractional amount (Carcinogenic PAH)

The total fractional amount of carcinogenic analytes should be less than 1. Total fractional amounts of carcinogenic PAHs greater than 1 required confirmatory analysis.

Table 6. FDA established levels of concern for PAHs in shrimp, crab, finfish and oysters and LC-FLD screen levels requiring confirmatory analysis.

Compound	FDA level of concern (mg/kg)*			LC-FLD screen levels requiring confirmation analysis (mg/kg)		
	Shrimp and Crab	Oysters	Finfish	Shrimp and Crab	Oysters	Finfish
naphthalene	123	133	32.7	61.5	66.5	16.3
acenaphthene	NA	NA	NA	NA	NA	NA
fluorene	246	267	65.3	123	133	32.6
phenanthrene*				923	1000	245
anthracene*	1846 ¹	2000 ¹	490 ¹	923	1000	245
fluoranthene	246	267	65.3	123	133	32.6
pyrene	185	200	49.0	92.5	100	24.5
benzo[a]anthracene	1.32	1.43	0.35	0.66	0.71	0.17
chrysene	132	143	35.0	66	71.5	17
benzo[b]fluoranthene	1.32	1.43	0.35	0.66	0.71	0.17
benzo[k]fluoranthene	13.2	14.3	3.5	6.6	7.1	1.7
benzo[a]pyrene	0.132	0.143	0.035	0.066	0.071	0.017
dibenzo[a,h]anthracene	0.132	0.143	0.035	0.066	0.071	0.017
benzo[g,h,i]perylene*	NA	NA	NA	NA	NA	NA
indeno[1,2,3-cd]pyrene	1.32	1.43	0.35	0.66	0.71	0.17

NA = not applicable

¹ Represents the sum of level of concern for phenanthrene and anthracene.

*For the FDA levels of concern, determination of the alkylated homologues should be included, e.g.

C-1, C-2, C-3, C-4 naphthalenes; C-1, C-2, C-3 fluorenes; C-1, C-2, C-3, C-4 anthracene/phenanthrene.

Table 7. Parent PAH certified and values and Control Limits for NIST SRM 19074b mussel tissue

<i>Compound</i>	<i>Certified Value^a</i> ($\mu\text{g}/\text{kg}$)	<i>Control Limits Range^b</i> ($\mu\text{g}/\text{kg}$)
Naphthalene ^c	2.43	1.6 - 3.3
Fluorine	0.494	0.3 - 0.7
Phenanthrene	2.58	1.7 - 3.5
Anthracene	0.527	0.3 - 0.8
Fluoranthene	17.1	11.5 - 23.1
Pyrene	18.04	12.2 - 24.2
Benzo[a]anthracene	4.74	2.9 - 6.9
Chrysene	6.3	3.7 - 9.5
Benzo[b]fluoranthene	6.46	4.1 - 9.2
Benzo[k]fluoranthene	3.16	2.1 - 4.3
Benzo[a]pyrene	2.80	1.7 - 4.1
Dibenzo[a,h]anthracene ^c	0.327	0.2 - 0.5
Benzo[g,h,i]perylene	3.12	2.0 - 4.5
Indeno[1,2,3-cd]pyrene ^c	2.14	1.4 - 2.9

^aWet mass basis as given in NIST certificate (5).

^bNOAA (3) defines the lower control limit as 0.7 x (certified value – uncertainty value), and the upper control limit as 1.3 x (certified value + uncertainty value).

^cThe certified value is below the method LOQ for these compounds, making them exempt in the NOAA criteria

2017.11 VALIDATION INFORMATION/STATUS

As previously stated, this method was originally developed and validated in response to the 2010 Gulf of Mexico oil spill. Method development and validation of this method is described elsewhere (6,7). The procedure was validated in three laboratories for the parent PAHs using spike recovery experiments at PAH fortification levels ranging from 25 to 10 000 $\mu\text{g}/\text{kg}$ in oysters, shrimp, crab, and finfish, with recoveries ranging from 78% to 99%. Additional validation was conducted for a series of alkylated homologs of naphthalene, dibenzothiophene, and phenanthrene, with recoveries ranging from 87 to 128%. Method accuracy was further assessed based on analysis of NIST SRM 1974b. The method provides method detection limits in the sub to low ppb ($\mu\text{g}/\text{kg}$) range, and practical LOQs in the low ppb ($\mu\text{g}/\text{kg}$) range for most of the PAH compounds studied.

In 2017, the method and validation package was submitted for acceptance into the Compendium of Analytical Laboratory Methods for Food and Feed Safety. The method was determined to be equivalent to a Level 3 multi-laboratory validated method (8).

2017.12 REFERENCES

- (1) Ramalhosa, M.J., Paiga, P., Morais, S., Delerue-Matos, C., & Oliveira, M.B.P.P. (2009) *J. Sep. Sci.* **32**, 3259-3538
- (2) Pule, B.O., Mmualefe, L.C., and Torto, N. (2010) Analysis of Polycyclic Aromatic Hydrocarbons in Fish with Agilent SampliQ QuEChERS AOAC Kit and HPLC-FLD, Agilent Technologies Application Note. 5990-5441EN.
- (3) Sloan, C.A., Brown, D.W., Pearce, R.W., Boyer, R.H., Bolton, J.L., Burrows, D.G., Herman, D.P., and Krahn, M.M. (2004). Extraction, Cleanup, and Gas Chromatography/Mass Spectrometry Analysis of Sediments and Tissues for Organic Contaminants. U.S. Dept. Commer., NOAA Technical Memorandum NMFS-NWFSC-59, 47.
- (4) Protocol for Interpretation and Use of Sensory Testing and Analytical Chemistry Results for Re-opening Oil-impacted Areas Closed to Seafood Harvesting, June 18, 2010, FDA/CFSAN, College Park, MD.
- (5) NIST Certificate of Analysis, Standard Reference Material 1974b, Organics in Mussel Tissue, 2003, Gaithersburg, MD.
- (6) Gratz, S., Mohrhaus, A., Gamble, B, Gracie, J., Jackson D., Roetting, J., Ciolino, L., McCauley, H., Schneider, G, Crockett, D., Krol, W., Arsenault, T., White, J., Flottmeyer, M., Johnson, Y., Heitkemper, D., and Fricke, F., "Screen for the Presence of Polycyclic Aromatic Hydrocarbons in Select Seafoods Using LC-Fluorescence," *Laboratory Information Bulletin*, (April 2011) LIB 4475.
- (7) Gratz, S., Ciolino, L., Mohrhaus, A., Gamble, B, Gracie, J., Jackson D., Roetting, J., McCauley, Heitkemper, D., Fricke, F., Krol, W., Arsenault, T., White, J., Flottmeyer, M., Johnson, Y., "Screening and Determination of Polycyclic Aromatic Hydrocarbons in Seafoods Using QuEChERS Based Extraction and High Performance Liquid Chromatography with Fluorescence Detection," *J. AOAC Int.* **94** (2011) 1601-1616
- (8) FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program; <http://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM298730.pdf>.