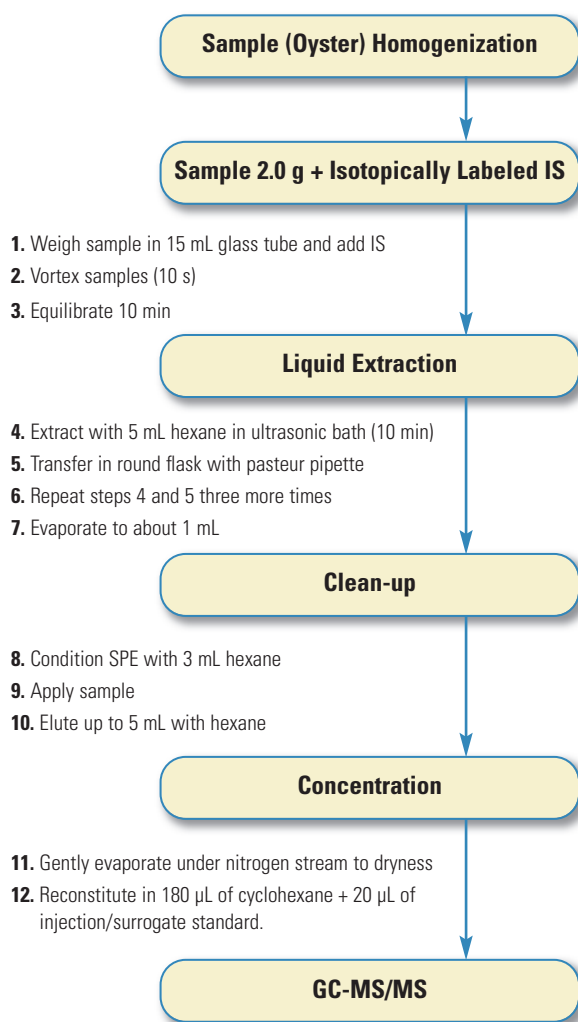


Determination of Polycyclic Aromatic Hydrocarbons (PAHs) and Aliphatic Hydrocarbons in Oysters by GC-MS/MS

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1. Schematic of Method



2. Scope

This method can be applied to oysters to detect the presence of aliphatic hydrocarbons and PAH contamination from crude oil found in the Gulf of Mexico in late May 2010. From the profile using GC-MS/MS, the method can be used to characterize the source of contamination. The method can give a semi-quantitative indication of whether levels of PAHs exceed safety limits for human consumption of oysters.

3. Principle

The method uses a liquid extraction of oysters with hexane, followed by a clean-up on a silica-SPE-cartridge. The sample is fortified with appropriate labeled internal standards and analyzed by simultaneous GC-MS/MS using a



Thermo Scientific TSQ Quantum XLS triple quadrupole mass spectrometer system. Aliphatic hydrocarbons and PAHs of food safety significance are measured and compared with the profile from crude oil collected from the Gulf of Mexico in late May 2010.

4. Reagent List

		Fisher Scientific USA Part Number
4.1	Acetone	A9491
4.2	Cyclohexane	C6201
4.3	Hexane	H3021
4.4	SPE Hypersep SI, 200 mg/3 mL	03251270
4.5	Toluene	AC176850010

5. Calibration Standards

5.1 PAHs

Acenaphthene – Ace (Sigma)
Acenaphthylene – Acy (Sigma)
Anthracene – Ant (Sigma)
Benz[a]anthracene – B(a)A (Sigma)
Benzo[a]pyrene – B(a)P (Sigma)
Benzo[b]fluoranthene – B(b)F (Sigma)
Benzo[g,h,i]perylene – B(g,h,i)P (Sigma)
Benzo[k]fluoranthene – B(k)F (Sigma)
Chrysene – Chr (Sigma)
Dibenz[a,h]anthracene – D(a,h)A (Sigma)
Fluoranthene – Flu (Sigma)
Fluorene – Fln (Sigma)
Indeno[1,2,3-cd]pyrene – I(1,2,3-c,d)P (Sigma)
Naphthalene – Naph (Sigma)
Phenanthrene – Phe (Sigma)
Pyrene – Pyr (Sigma)

Key Words

- TSQ Quantum XLS
- Aliphatic Hydrocarbons
- Gulf Oil Spill
- Oil Contamination
- Oyster Extraction
- PAHs

5.2 Injection Standard

5-methylchrysene – 5-MChr (Dr. Ehrenstorfer)

5.3 Internal Standards

Anthracene-D10 – Ant-D10 (Sigma)

Benzo[a]pyrene-D12 – B(a)P-D12 (Sigma)

Benzo[ghi]perylene-D12 – B(g,h,i)P-D12 (LGC Standards)

Chrysene-D12 – Chr-D12 (Sigma)

5.4 Quality Control Materials

Petroleum Crude oil (NIST Standard Reference Material®, 1582)

Aliphatic Hydrocarbons in 2,2,4-Trimethylpentane (NIST Standard Reference Material, 1494)

6. Standards and Reagent Preparation

- 6.1 Stock solutions of 2 µg/mL of PAH standards in toluene
- 6.2 Internal PAHs standard (IS) concentration: 2 µg/mL (Benzo[ghi]perylene-d₁₂, Anthracene-d₁₀, Chrysene-d₁₂) in toluene and 200 µg/mL Benzo[a]pyrene-d₁₂ in cyclohexane
- 6.3 Working standard solution mixture of 16 PAHs in toluene (100 ng/mL)
- 6.4 Working internal standard mixture of IS PAHs in toluene (200 ng/mL)
- 6.5 Syringe standard, 5-methyl-chrysene (200 ng/mL) in toluene.
- 6.6 Spiked solution of Petroleum crude oil (NIST 1582): 100 mg/mL in cyclohexane

7. Apparatus

	Fisher Scientific USA Part Number
7.1 Centrifuge, Heraeus™ Multifuge™ X3	75-004-500
7.2 Thermo Scientific 16 port SPE vacuum manifold	03-251-252
7.3 Evaporator EVTm-130-32-16 (Fisher Scientific Germany)	3106395
7.4 Fisher precision balance	01918306
7.5 Vacuum pump	05-402-100
7.6 Rotavapor® R-210	05-024-21
7.7 Sartorius analytical balance	01-910-3224
7.8 Thermo sci. Barnstead EASYpure™ II water	0905050
7.9 Ultrasonic bath Elmsonic S40H	154606Q
7.10 ULTRA-TURRAX® – dispergation tool	1425980
7.11 ULTRA-TURRAX – Plug-in coupling	14259023
7.12 ULTRA-TURRAX	142259301
7.13 Vortex shaker	14505141
7.14 Vortex standard cap	14-505-140
7.15 GC column TR-50MS 30 m, 0.25 mm ID, 0.25 µm film	260R142P
7.16 TSQ Quantum XLS™ Triple Quadrupole Mass Spectrometer	

8. Consumables

	Part Number
8.1 GC vials	03393F
8.2 Pipette Finnpiquette 100-1000 µL	14386320
8.3 Pipette Finnpiquette 10-100 µL	14386318
8.4 Pipette Finnpiquette 500-5000 µL	14386321
8.5 Pipette holder	14245160
8.6 Pipette Pasteur soda lime glass 150 mm	136786A
8.7 Pipette suction device	03-692-350
8.8 Pipette tips 0.5 – 250 µL, 500/box	21377144
8.9 Pipette tips 1 – 5 mL, 75/box	2137750
8.10 Pipette tips 100 – 1000 µL, 200/box	2137746
8.11 Spatula, 18/10 steel	14356C
8.12 Spatula, nylon	NC9319088
8.13 SPE Hypersep SI, 200 mg/3 mL, 50 pc.	03251270
8.14 Tube holder	03840233
8.15 Wash bottle, PTFE	0340911A
Glassware	
8.16 Beaker, 50 mL	FB10050
8.17 Fisherbrand test tubes	14-958D
8.18 Funnel, 55 mm	14353D
8.19 Glass tubes	14957E
8.20 Pasteur pipette	136786A
8.21 Round flask 50 mL, NS 29/32 (Fisher Scientific Germany)	9011835
8.22 Volumetric flask, 10 mL	FB40110
8.23 Volumetric flask, 25 mL	10200A

9. Procedure

9.1 Sample Preparation

Rinse the glassware with acetone before proceeding with the method to avoid cross contamination. Homogenize a suitable amount (e.g. 250 g) of oyster meat appropriately to give a slurry using a high speed blender, e.g. ULTRA-TURRAX.

9.2 Extraction

- 9.2.1 Accurately weigh the homogenized sample (ca. 2 g) into a glass tube.
- 9.2.2 Add 50 µL of PAH internal standard solution to the sample.
- 9.2.3 Vortex the mixture for 10 s and wait 10 min for equilibration.
- 9.2.4 Add 5 mL of hexane to the sample and put it into an ultrasonic bath for 10 min.
- 9.2.5 Transfer the supernatant hexane layer into a 50 mL round flask with a Pasteur pipette.
- 9.2.6 Repeat the extraction (9.2.4 and 9.2.5) three more times.
- 9.2.7 Centrifuge for 5 min at 4500 rpm and 5 °C and decant supernatant.
- 9.2.8 Evaporate to 1 mL under vacuum (220 mbar/50 °C).

9.3 Clean-up

- 9.3.1 Condition the SPE-Cartridge with 3 mL of hexane.
- 9.3.2 Apply the extract to the cartridge and elute into an evaporator tube with 5 mL of hexane.
- 9.3.3 Evaporate at 40 °C to dryness using a blow-down apparatus under a gentle stream of nitrogen.
- 9.3.4 Reconstitute in 180 µL of cyclohexane plus 20 µL of injection standard.

9.4 Analysis

9.4.1 GC operating conditions

GC analysis was performed on a Thermo Scientific TRACE GC Ultra™ system (Thermo Fisher Scientific, Waltham, MA USA). The GC conditions were as follows:
Column: Thermo TR-50MS 30 m, I.D.: 0.25 mm, 0.25 µm film capillary column
Injection mode: splitless with a 5 mm injection port liner
Injection port temperature: 270 °C
Flow rate: 1.2 mL/min
Split flow: “On”, flow: 25 mL/min
Splitless time: 1 min
SSL carrier method mode: constant flow
Initial value: “On” with 1.2 mL/min
Initial time: 1 min
Gas saver flow: 15 mL/min
Gas saver time: 3 min
Vacuum compensation: “On”
Transfer line temperature: 270 °C
Oven Temperature: 60 °C for 1 min, then programmed at 12 °C/min to 210 °C, then 8 °C/min to 340 °C with 5 min hold time

9.4.2 Mass Spectrometric Conditions

MS analysis is carried out using a TSQ Quantum XLS triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA USA). A satisfactory tune of the mass spectrometer is achieved when the detector is set at m/z 300 or less and the three FC 43 (calibration gas) ions (68, 219, and 502) are at least half the height of their respective windows and the ions at 502 and 503 are resolved.

The MS conditions for PAHs are as follows:

Ionization mode: EI positive ion
Ion volume: closed EI
Emission current: 50 uA
Ion source temperature: 250 °C
Scan type: Full scan in range m/z 45-650 and SRM
Scan width: 0.15 for SRM
Scan time 0.2 s for full scan and 0.05 for SRM
Peak width: Q1, 0.7 Da; Q3, 0.7 Da FWHM
Collision gas (Ar) pressure: 0.5 mTorr

The mass spectrometer is programmed to be able to simultaneously monitor the hydrocarbon profile in scanning Full Scan (FS) GC-MS and quantify the presence of PAHs by MS/MS within a single chromatographic run. Eight segments are programmed each with 2 simultaneous scan events. One scan event is used to monitor the aliphatic

hydrocarbon profile throughout the whole chromatographic run (i.e. in all segments), while SRM traces are set up for the target PAHs in the other scan event. The program of segments for SRM events (#1) is shown in Table 1.

Setting of scan event #2 for hydrocarbon profiling was kept constant in all segments:

- Scan type: FS in range 45-650 m/z
- Scan time: 0.2 s
- FWHM: 0.7 Da
- Collision gas pressure: 0.5

10. Calculation of Results

10.1 Aliphatic Hydrocarbons

From the scanned GC-MS data, print a reconstructed ion chromatogram (extracted ion chromatogram) for m/z 57 and plot this alongside a similar m/z 57 extracted chromatogram for the standard mixture of hydrocarbons. Any detectable aliphatic hydrocarbon peaks in oysters can be identified based on their retention times which are given in Table 2. This is illustrated in Figure 1. Measure the specific peak area ratios to characterize the source of hydrocarbon contamination.

10.2 PAHs

The occurrence of one or more of any of the 16 PAHs of food safety concern is indicated by the presence of transition ions (quantifier and qualifier) as indicated in Table 1 at retention times corresponding to those of the respective standards shown in Table 1. This is illustrated in Figure 1. Careful visual inspection of the SRM chromatograms should be carried out to check for interferences. The measured peak area ratios of precursor to quantifier ion should be in close agreement with those of the standards as shown in Table 1. If the presence of any of the 16 PAHs is confirmed based on retention times and ion ratios then quantification should be carried out as indicated below.

Calibration by the internal standardization is applied for the quantification of PAHs. This calibration requires the determination of response factors R_f defined by the equation below.

Calculation of the response factor:

$$R_f = \frac{A_{St} \times C_{[IS]}}{A_{[IS]} \times C_{St}}$$

R_f – the response factor determined by the analysis of standards PAH and internal standard

A_{St} – the area of the PAH peak in the calibration standard

$A_{[IS]}$ – the area of the internal standard peak for the calibration standard

c_{St} – PAH concentration for the calibration standard solution

$c_{[IS]}$ – the internal standard concentration for the calibration standard solution

Calculations for each sample the absolute amount of PAH that

was extracted from the sample:

$$X_{\text{PAH}} = \frac{A_{\text{PAH}} \times X_{[\text{IS}]}}{A_{[\text{IS}]} \times R_f}$$

X_{PAH} – the absolute amount of PAH that was extracted from the sample

A_{PAH} – the area of PAH peak of the sample

$A_{[\text{IS}]}$ – the area of the internal standard peak of the sample

$X_{[\text{IS}]}$ – the absolute amount of internal standard added to the sample

The concentration of PAH in the sample (ng/g):

$$c \text{ (ng/g)} = \frac{X_{\text{PAH}}}{m}$$

c – the concentration of PAH in the sample (ng/g)

m – the sample weight in g

11. Interpretation of Results

The analytical data generated in the method requires careful interpretation to collect convincing evidence of aliphatic hydrocarbon contamination of oysters originating from an actual crude oil sample from Gulf of Mexico and consequent PAH contamination. The method provides a hydrocarbon profile and PAH profile which can be matched against that of crude oil sample from the Gulf of Mexico. The composition of crude oil from the Gulf of Mexico is given in Table 4 indicating relatively high levels of n-hexadecane, n-heptadecane and pristane which are characteristic. Characteristic pristane/C-17 ratio (0.7) phytane/C-18 ratio (0.35) were observed. The relative amounts of any combination of individual aliphatic hydrocarbons can be measured and matched against the crude oil sample from the Gulf of Mexico composition. As illustrated in Figure 4 which shows both direct analysis of crude oil from the Gulf of Mexico as well as analysis after cleanup from oysters. However, it should be noted that the composition of the oil changes with time and the uptake by oysters eventually may have a different profile from the crude oil. The composition of other samples of crude oils is illustrated in Figure 5 again indicating differences in profile.

Similarly the pattern of PAHs found in crude oil is very characteristic as shown in Table 4 with levels of Ant, Phe, Flu and Chr being 100 times higher than levels of B(a)P. Subject to satisfactorily meeting requirements for identification of PAHs, the method gives semi-quantitative values for the higher mass PAHs which can be used as a good guide as to whether oysters samples are above or below safety limits. Accurate results require confirmation using a more refined cleanup procedure.

12. Method Performance

Method performance was established by separate spiking experiments for blank oysters with firstly a mixture of aliphatic hydrocarbon standards (NIST1494 – C10-C34 hydrocarbons) and secondly a mixture of 16 PAH standards. To evaluate method performance with combined aliphatic hydrocarbons and PAHs, spiking was carried out with NIST 1582 petroleum crude oil.

12.1 Recovery

Aliphatic hydrocarbons – The method was shown to be unsuitable for recovery of aliphatic hydrocarbons below n-pentadecane due to losses during concentration of the sample extract. Average recoveries of n-hexadecane (C-16) to n-tetratricontane (C-34) ranged from 52-108%.

PAHs – Background contamination and lack of availability of a real blank sample made it impossible to make an accurate estimate of the recoveries of the lower mass PAHs (Naph, Ace, Acy, Flu, Ant, Phe, Fln and Pyr). However average recoveries of the remaining higher mass PAHs [(B(a)P, Chr, B(b)F, B(k)F, B(k)F, B(a)P, B(g,h,i)P, and D(a,h)A] ranged from 65-126%.

12.2 Specificity

Aliphatic hydrocarbons – Full scan spectra were obtained in each case. Identification was confirmed by close agreement of retention times for standards and comparison with scanned spectra, particularly checking for evidence of interferences. Extracted ion chromatograms using m/z 57 were used for profiling but additional ions characteristic of aliphatic hydrocarbons (e.g. m/z 71) can be used as an additional check of specificity.

PAHs – By SRM, specificity was confirmed based on the presence of transition ions (quantifier and qualifier) at the correct retention times corresponding to those of the respective PAH standards. Furthermore, the measured peak area ratios of precursor to quantifier ion should be in close agreement with those of the standards.

12.3 Limits of Detection

Aliphatic hydrocarbons – LODs for aliphatic hydrocarbons were estimated to be between 0.2 and 1 ng (on-column injected) in full scan mode. For 1 μL of extract injected into the GC-MS this is equivalent to 20-100 ng/g (ppb) hydrocarbon contamination of the oysters.

PAHs – Background contamination made it impossible to make an accurate estimate of the LODs of the lower mass PAHs (Naph, Ace, Acy, Flu, Ant, Phe, Fln and Pyr). However, LODs of the remaining higher mass PAHs [(B(a)P, Chr, B(b)F, B(k)F, B(k)F, B(a)P, B(g,h,i)P, and D(a,h)A] were estimated to be between 0.01 and 0.07 ng (on-column injected) in SRM mode. For 1 μL of extract injected into the GC-MS/MS this is equivalent to 1-7 ng/g (ppb) PAH and oil contamination of oysters.

12.4 Accuracy

The accuracy for measurement of PAHs was determined by spiking NIST crude oil standard into oysters and following the full extraction and cleanup procedure. Background contamination made it impossible to make an accurate estimate of the recoveries of the lower mass PAHs (Naph, Ace, Acy, Flu, Ant, Phe, Fln and Pyr). However average recoveries of (B(a)A, B(a)P, B(g,h,i)P, and I(1,2,3-c,d)P) were 124, 92, 81 and 86 % respectively as shown in Table 3. Bearing in mind that the method is intended as a semi-quantitative screen this accuracy was deemed to be satisfactory.

Segment	Duration (min)	PAH and IS	Retention Time (min)	Precursor Ion	Quantifier Ion	Qualifier Ion	Ion Ratio	Collision Energy
1	10.50	Naph	8.66	127.9	102.0	77.8	0.38	15
2	2.50	Acy	12.13	152.0	151.1	126.0	0.11	10
		Ace	12.35	154.0	153.0	152.0	0.12	10
3	1.50	Flu	13.37	165.9	165.0	162.9	0.05	10
4	3.00	Ant	15.87	178.0	176.0	152.0	0.70	30
		Phe	15.95	178.0	176.0	152.0	0.70	30
		Ant-D10	15.89	188.1	160.2	158.2	0.40	30
5	4.50	Flu	19.13	202.0	201.1	200.1	0.40	10
		Pyr	19.97	202.0	201.0	200.1	0.40	10
6	3.70	B(a)A	23.48	228.1	226.0	202.1	0.15	20
		Chr	23.71	228.1	226.2	202.2	0.15	20
		Chr-D12	23.65	240.2	238.1	215.1	0.11	30
		5MChr	24.98	242.1	241.1	227.5	0.15	30
7	3.80	B(b)F	26.75	252.1	250.1	226.1	0.18	30
		B(k)F	26.82	252.1	250.1	226.1	0.18	30
		B(a)P	27.96	252.1	250.1	226.1	0.18	30
		B(a)P-D12	27.87	264.1	260.1	236.0	0.38	30
8	5.50	B(g,h,i)P	31.99	276.1	274.0	250.0	0.05	35
		I(1,2,2-c,d)P	30.96	276.1	274.0	207.0	0.05	35
		BgP-D12	31.86	288.2	286.1	125.1	0.06	35
		D(a,h)A	30.97	278.0	276.0	226.1	0.05	35

Table 1: Parameters for SRM analysis of PAHs

Hydrocarbon	Empirical Formula	Molecular Ion	Retention Time
<i>n</i> -decane	C ₁₀ H ₂₂	142.1	3.99
<i>n</i> -undecane	C ₁₁ H ₂₄	156.2	4.97
<i>n</i> -dodecane	C ₁₂ H ₂₆	170.2	6.14
<i>n</i> -tridecane	C ₁₃ H ₂₈	184.2	7.30
<i>n</i> -tetradecane	C ₁₄ H ₃₀	198.2	8.42
<i>n</i> -pentadecane	C ₁₅ H ₃₂	212.2	9.50
<i>n</i> -hexadecane	C ₁₆ H ₃₄	226.2	10.51
<i>n</i> -heptadecane	C ₁₇ H ₃₆	240.2	11.45
pristane	C ₁₉ H ₄₀	268.3	11.24
<i>n</i> -octadecane	C ₁₈ H ₃₈	254.3	12.41
phytane	C ₂₀ H ₄₂	282.3	12.30
<i>n</i> -nonadecane	C ₁₉ H ₄₀	268.3	13.28
<i>n</i> -eicosane	C ₂₀ H ₄₂	282.3	14.14
<i>n</i> -docosane	C ₂₂ H ₄₆	310.3	15.90
<i>n</i> -tetracosane	C ₂₄ H ₅₀	338.3	17.73
<i>n</i> -hexacosane	C ₂₆ H ₅₄	366.4	19.56
<i>n</i> -octacosane	C ₂₈ H ₅₈	394.4	21.35
<i>n</i> -triacontane	C ₃₀ H ₆₂	422.4	23.08
<i>n</i> -dotriacontane	C ₃₂ H ₆₆	450.5	24.77
<i>n</i> -tetratricontane	C ₃₄ H ₇₀	478.5	26.45

Table 2: Aliphatic hydrocarbons monitored in oysters spiked with NIST 1494

PAH	Assigned Value [ng/g]	Measured Value [ng/g]	Recovery [%]
B(a)A	14.06 ± 1.00	17.39	124
B(a)P	5.52 ± 1.00	5.11	92
I(1,2,3-c,d)P	0.85 ± 0.50	0.69	81
B(g,h,i)P	8.54 ± 0.2	7.37	86

Table 3: Analysis of spiked oysters with NIST 1582 crude oil

Hydrocarbon	Average amount [µg/g] (n=2)	PAH	Average amount [µg/g] (n=2)
<i>n</i> -pentadecane	407	Naph	19
<i>n</i> -hexadecane	1484	Acy	436
<i>n</i> -heptadecane	1329	Ace	96
Pristane	928	Flu	144
<i>n</i> -octadecane	337	Ant	11857
Phytane	118	Phe	11287
<i>n</i> -nonadecane	330	Flu	958
<i>n</i> -eicosane	289	Pyr	547
<i>n</i> -docosane	188	B(a)A	29
<i>n</i> -tetracosane	146	CHR	804
<i>n</i> -hexacosane	82	B(b)F	428
<i>n</i> -octacosane	43	B(k)F	40
<i>n</i> -triacontane	31	B(a)P	2
<i>n</i> -dotriacontane	23	B(g,h,i)P	7
<i>n</i> -tetratricontane	10	I(1,2,3-c,d)P	2
		D(h)A	3

Table 4: Composition of Crude oil from Gulf of Mexico. Characteristic pristane/C-17 ratio (0.7) phytane/C-18 ratio (0.35) were observed.

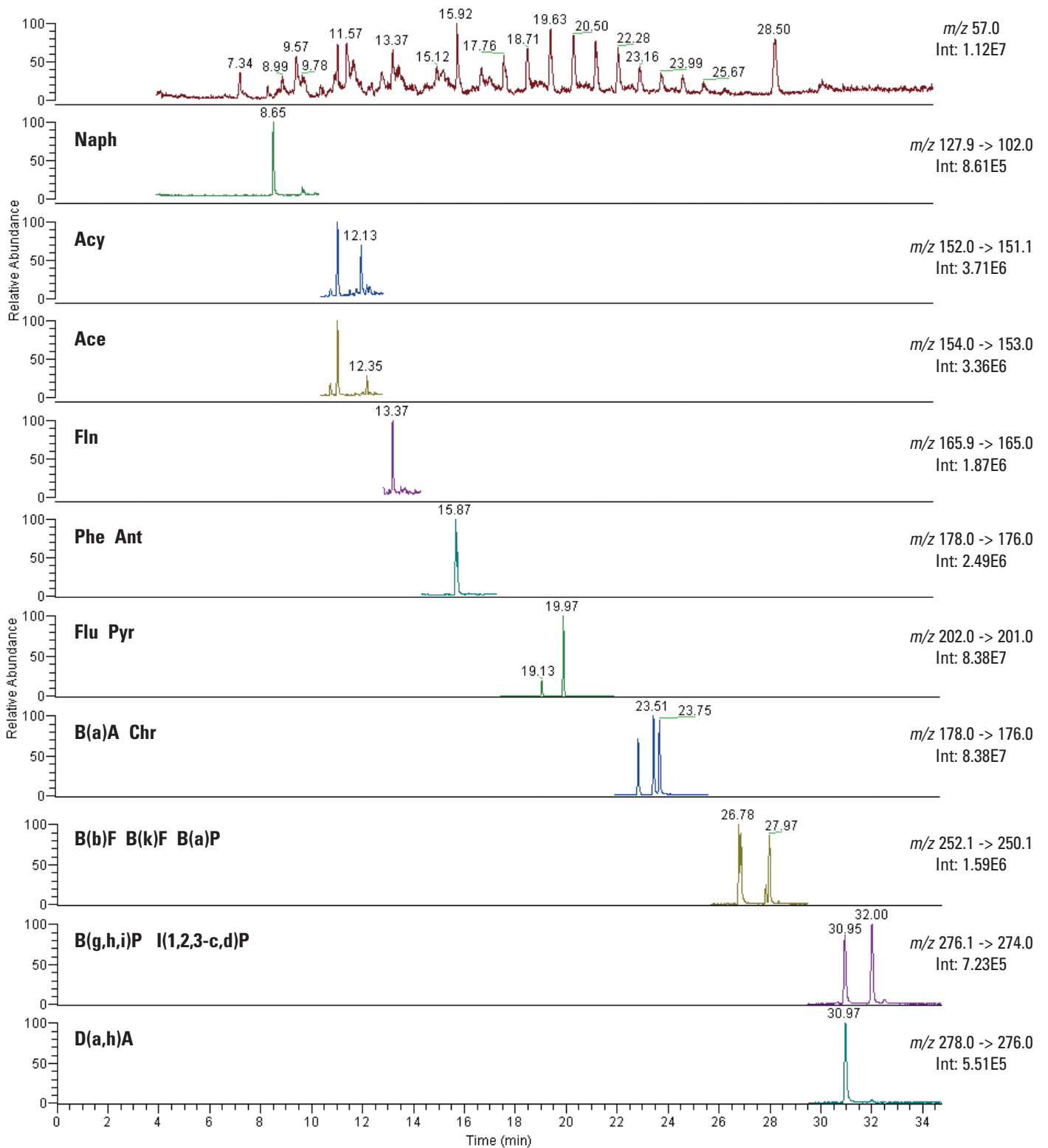


Figure 1: Chromatogram of oyster sample spiked with aliphatic hydrocarbons plus 10 ng/g PAH mixture. Top chromatogram shows m/z 57 for hydrocarbon profiling, while lower chromatograms are SRM traces for 16 individual PAHs.

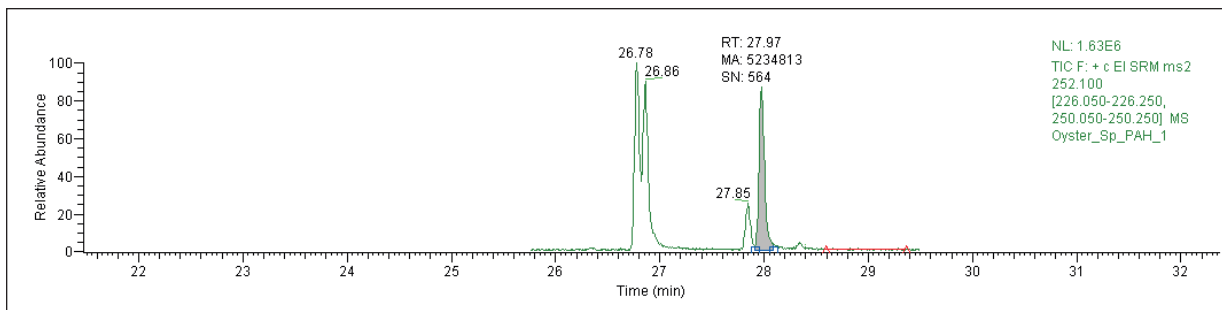


Figure 2: Chromatogram of oyster sample spiked with 10 ng/g B(a)P

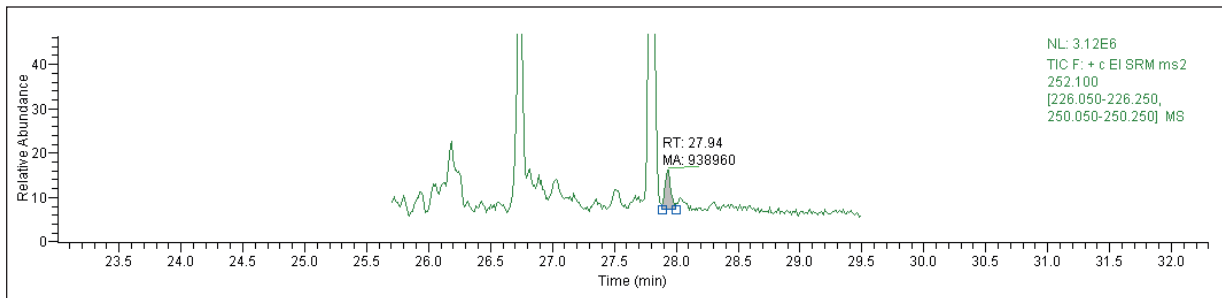


Figure 3: Chromatogram of oyster sample spiked with 5 µg/g crude oil sample taken from the Gulf of Mexico in late May 2010 and found to contain 5 ng/g B(a)P

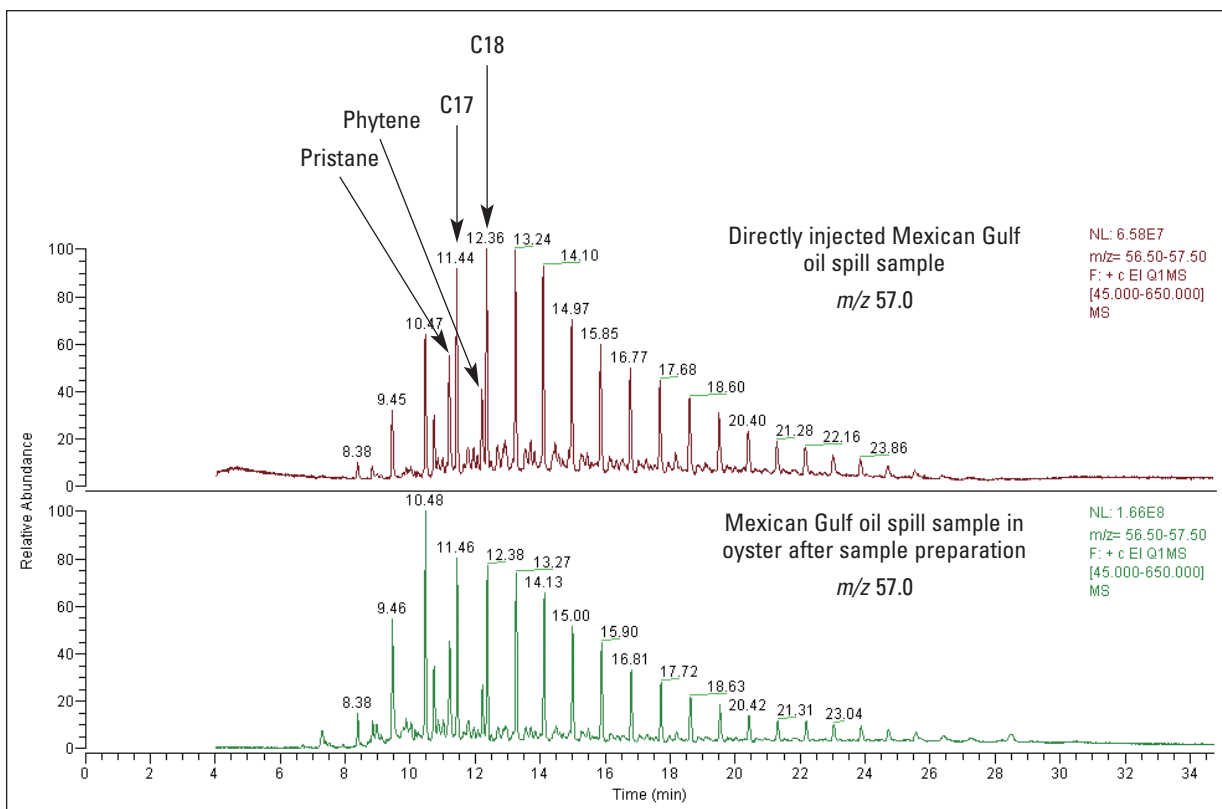


Figure 4: Hydrocarbon profile of crude oil sample taken from the Gulf of Mexico in late May 2010 by direct analysis (top) and after 5 mg/kg spiking into oyster sample (bottom) showing m/z 57

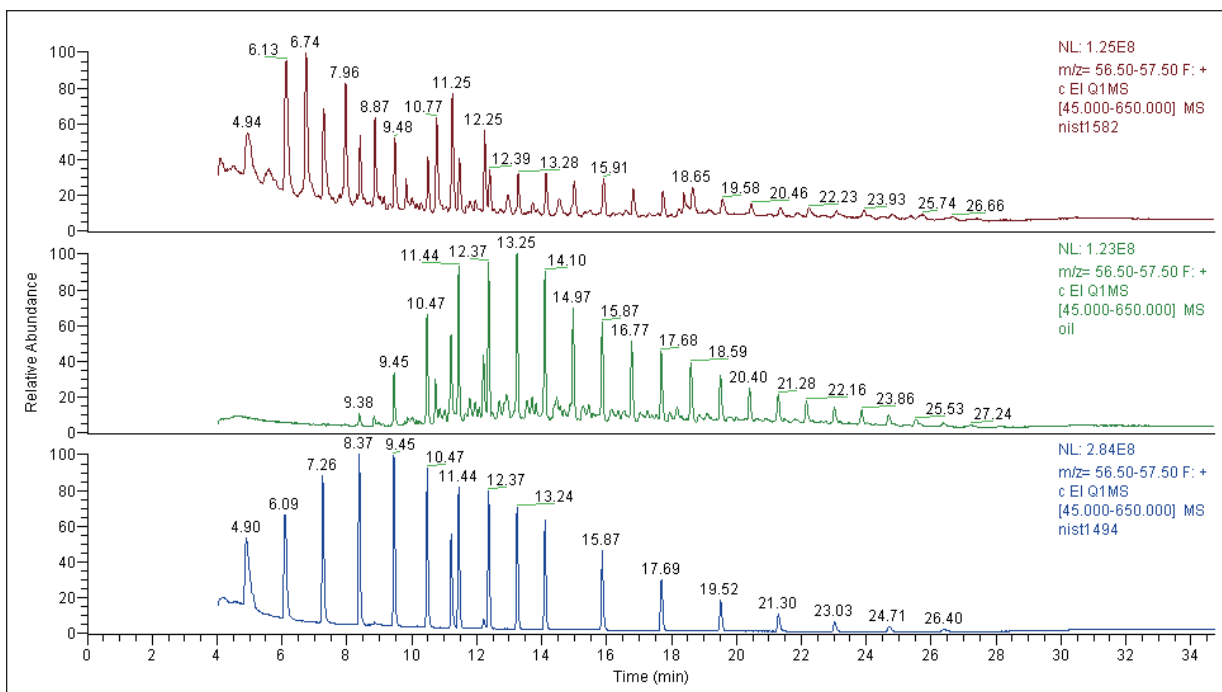


Figure 5: Comparison of hydrocarbon distribution of different type of oils showing m/z 57. Top: NIST1582 petroleum crude oil, middle: crude oil sample taken from the Gulf of Mexico in late May 2010, at the bottom: NIST1494 hydrocarbon standard.

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