

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

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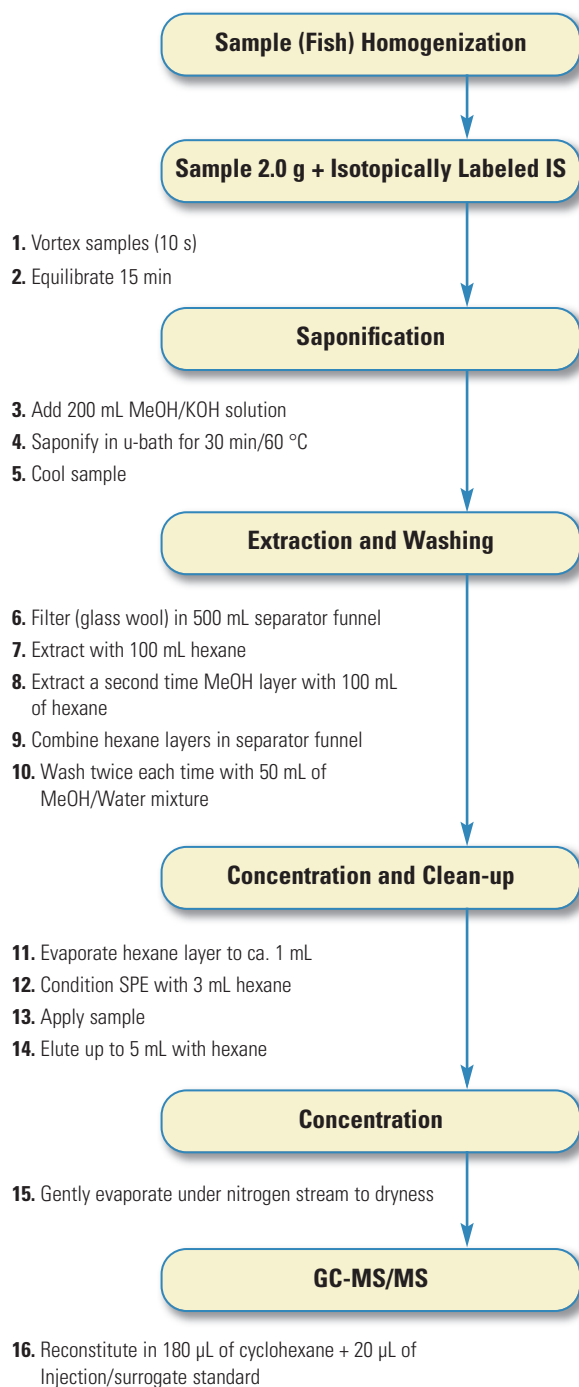
## Key Words

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

## 1. Sample Analysis Time

Sample preparation	3 hours
Instrument analysis	35 minutes/sample

## 2. Schematic of Method



## 3. Scope

This method can be applied to fish and other fatty seafood samples to detect simultaneously the presence of aliphatic hydrocarbons and PAH contamination from crude oil found in the Gulf of Mexico. From the profile using GC-MS/MS, the method can be used to characterize the source of contamination. The method gives a quantitative indication as to whether levels of PAHs exceed safety limits for human consumption.

## 4. Principle

The homogenized fish sample is fortified with appropriate labeled internal standards and saponified with methanolic KOH. After repeated extraction into hexane, further clean-up is carried out on a silica-SPE-cartridge. The concentrated extract is analyzed by GC-MS/MS using a Thermo Scientific TSQ Quantum XLS gas chromatography triple quadrupole mass spectrometer system. PAHs of food safety significance are quantified and compared with the profile from crude oil collected from the Gulf of Mexico in May 2010. Additionally the profile of aliphatic hydrocarbons can be examined.

## 5. Reagent List

		Fisher Scientific USA Part Number
5.1	Acetone	A9491
5.2	Cyclohexane	C6201
5.3	Hexane	H3021
5.4	Methanol	M/4058/17
5.5	Potassium hydroxide	P/5600/53
5.6	Toluene	AC176850010

## 6. Calibration Standards

### 6.1 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)

### 6.2 Injection Standard

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

### 6.3 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)

### 6.4 Quality Control Materials

FAPAS, smoked fish (T0642)  
Petroleum Crude oil (NIST Standard Reference Material®, 1582)

## 7. Standards and Reagent Preparation

- 7.1 MeOH/KOH solution, weigh 120 g KOH, add 60 mL of water and dilute in 900 mL Methanol
- 7.2 MeOH/H<sub>2</sub>O mixture: 400 mL of Methanol plus 100 mL of Water
- 7.3 Stock solutions of 2 µg/mL of PAH standards in toluene
- 7.4 Internal PAHs standard (IS) concentration: is 2 µg/mL (Benzo(g,h,i)perylene-d12, Anthracene-d10, Chrysene-d12) in toluene and 200µg/mL Benzo(a)pyrene-d12 in cyclohexane
- 7.5 Working standard solution mixture of 16 PAHs in toluene (100 ng/mL)
- 7.6 Working internal standard mixture of IS PAHs in toluene (200 ng/mL)
- 7.7 Syringe standard, 5-methyl-chrysene (200 ng/mL) in toluene
- 7.8 Spike solution of Petroleum crude oil (NIST 1582): 100 mg/mL in cyclohexane

## 8. Apparatus

Fisher Scientific USA  
Part Number

8.1	Centrifuge, Heraeus™ Multifuge™ X3	75-004-500
8.2	Thermo Scientific 16 port SPE vacuum manifold	03-251-252
8.3	Evaporator EVTm-130-32-16 (Fisher Scientific Germany)	3106395
8.4	Fisher precision balance	01918306
8.5	Vacuum pump	05-402-100
8.6	Rotavapor® R-210	05-024-21
8.7	Sartorius analytical balance	01-910-3224
8.8	Thermo Scientific Barnstead EASYpure™ II water	0905050
8.9	Ultrasonic bath Elmsonic S40H	154606Q
8.10	ULTRA-TURRAX® – dispergation tool	1425980
8.11	ULTRA-TURRAX – Plug-in coupling	14259023
8.12	ULTRA-TURRAX	142259301
8.13	Vortex shaker	14505141
8.14	Vortex standard cap	14-505-140
8.15	TSQ Quantum XLS Triple Quadrupole Mass Spectrometer	

## 9. Consumables

Part Number

9.1	GC vials	03393F
9.2	Pipette Finnpiptette 100-1000 µL	14386320
9.3	Pipette Finnpiptette 10-100 µL	14386318
9.4	Pipette Finnpiptette 500-5000 µL	14386321
9.5	Pipette holder	14245160
9.6	Pipette Pasteur soda lime glass 150 mm	136786A
9.7	Pipette suction device	03-692-350
9.8	Pipette tips 0.5 – 250 µL, 500/box	21377144
9.9	Pipette tips 1 – 5 mL, 75/box	2137750
9.10	Pipette tips 100 – 1000 µL, 200/box	2137746
9.11	Spatula, 18/10 steel	14356C
9.12	Spatula, nylon	NC9319088
9.13	SPE HyperSep SI, 200 mg/3 mL, 50 pc.	03251270
9.14	Tube holder	03840233
9.15	Wash bottle, PTFE	0340911A
9.16	Glass wool	386062500
9.17	GC column TR-50MS 30m, 0.25 mm ID, 0.25 µm film	260R142P

## Glassware (9. Consumables continued)

9.18	Beaker, 50 mL	FB100050
9.19	Fisherbrand laboratory bottle, 250 mL	9653630
9.20	Erlenmeyer Flask, 100 mL	9653520
9.21	Fisherbrand test tubes	14-958D
9.22	Funnel, 55mm	14353D
9.23	Glass tubes	14957E
9.24	Measuring cylinder, 100 mL	FB56449
9.25	Measuring cylinder, 1000 mL	FB 56453
9.26	Pasteur pipette	136786A
9.27	Round flask 50 mL, NS 29/32 Fisher Scientific Germany	9011835
9.28	Separator funnel, 250 mL	9203325
9.29	Separator funnel, 500 mL	9203328
9.30	Volumetric flask, 10 mL	FB40110
9.31	Volumetric flask, 25 mL	10200A

## 10. Procedure

### 10.1 Saponification

- 10.1.1 Accurately weigh the homogenized sample (ca. 2 g) into a 250 mL Duran Bottle
- 10.1.2 Add 50  $\mu$ L of PAH internal standard solution to the sample
- 10.1.3 Vortex the mixture for 10 s and wait 10 min for equilibration
- 10.1.4 Add 200 mL of MeOH/KOH solution
- 10.1.5 Put samples into an ultrasonic bath for 30 min at 60 °C for saponification
- 10.1.6 Cool sample

### 10.2 Extraction

- 10.2.1 Filter saponified sample through glass wool into a 500 mL separating funnel
- 10.2.2 Add 100 mL of hexane to the sample and shake for 3 min
- 10.2.3 Transfer the hexane layer into a 100 mL Erlenmeyer flask
- 10.2.4 Repeat the extraction (10.2.2) one more time
- 10.2.5 Combine hexane layers in a separator funnel
- 10.2.6 Wash hexane layer by shaking with 50 mL of MeOH/H<sub>2</sub>O solution for 1 min
- 10.2.7 Repeat washing step (10.2.6) two more times
- 10.2.8 Evaporate to 1 mL under vacuum (220 mbar/50 °C)

### 10.3 Clean-up

- 10.3.1 Condition the SPE-Cartridge with 3 mL of hexane
- 10.3.2 Apply the extract to the cartridge and elute into an evaporator glass tube with 5 mL of hexane
- 10.3.3 Evaporate at 40 °C to dryness using a blow-down apparatus under a gentle stream of nitrogen
- 10.3.4 Reconstitute in 180  $\mu$ L of cyclohexane plus 20  $\mu$ L of injection standard

## 10.4 Analysis

### 10.4.1 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 Scientific TR-50MS 30 m

Column ID: 0.25 mm, 0.25  $\mu$ m 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

### 10.4.2 Mass spectrometric conditions

MS analysis is carried out using a Thermo Scientific 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 (69, 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 were as follows:

Ionization mode: EI positive ion

Ion volume: closed EI

Emission current: 50  $\mu$ A

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 two 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 of 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

## 11. Calculation of Results

### 11.1 Aliphatic Hydrocarbons

Any detectable aliphatic hydrocarbon peaks in Fish can be identified based on their retention times which are given in Table 4. This is illustrated in Figure 4. Measure the specific peak area ratios to characterize the source of hydrocarbon contamination.

### 11.2 Identification of 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.

### 11.3 Quantification of PAHs

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

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

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

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

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

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

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

**Calculations for each sample the absolute amount of PAH that was extracted from the sample:**

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

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

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

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

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

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

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

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

$m$  – sample weight in g

## 12. Interpretation of Results

The analytical data generated in the method requires careful interpretation to collect convincing evidence of aliphatic hydrocarbon contamination of fish originating from actual crude oil sample from Gulf of Mexico and consequent PAH contamination. The method provides a hydrocarbon profile and quantification of PAHs which can be matched against that of crude oil sample from Gulf of Mexico. Although the method provides a PAH profile and simultaneous screening of aliphatic hydrocarbons, it should be noted that the composition of any crude oil contamination may change with time through biodegradation and there may be preferential uptake by fish of individual PAHs and aliphatic hydrocarbon eventually giving a different profile in fish from that of the crude oil.

Subject to satisfactorily meeting the requirements for identification of PAHs the method can be used to quantify levels of PAHs in fish.

## 13. Method Performance

The method performance was established by spiking experiments with blank oily fish with a mixture of 16 PAH standards. The method accuracy was demonstrated first by analysis of surplus proficiency test material samples (FAPAS smoked fish - T0642) with defined PAH values and second by using NIST 1582 petroleum crude oil containing certified levels of PAHs.

### 13.1 Recovery

**Aliphatic hydrocarbons** – The method was shown to be unsuitable for recovery of aliphatic hydrocarbons below *n*-hexadecane due to losses during concentration of the sample extract.

**PAHs** – Average recoveries of the 16 PAHs of food safety significance ranged from 58-113%

### 13.2 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** – Using Selected Reaction Monitoring (SRM) the 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 qualifier/quantifier ion were in close agreement with the ion ratios of the standards as indicated in Table 1.

### 13.3 Limits of Detection

The limits of detection in fish were found to be in the range 1-7 ng/g depending on the individual PAH.

### 13.4 Accuracy

Accuracy was demonstrated by analysis of a smoked fish proficiency test material (FAPAS® T0642) which had assigned values for the significant PAHs. After following the full extraction and cleanup procedure, the FAPAS® sample was analyzed by GC-MS/MS and the results are shown in Table 2. Average recoveries of B(a)A, B(a)F, B(a)P, I(1,2,3-c,d)P and B(g,h,i)P were 101, 96, 97, 116 and 94 % respectively. The accuracy of this method for these critical PAHs in fish was thus demonstrated.

Accuracy was demonstrated by analysis of blank fish spiked with a solution of NIST crude oil containing certified levels of PAHs. After following the full extraction and cleanup procedure, samples were analyzed by GC-MS/MS and the results are shown in Table 3. Average recoveries of B(a)A, B(a)P, I(1,2,3-c,d)P and B(g,h,i)P were 107, 112, 82 and 112 % respectively. The accuracy of this method for determining these critical PAHs in fish in the presence of crude oil was thus demonstrated.

Segment	Duration (min)	PAH and IS	Retention Time (min)	Precursor Ion	Quantifier Ion (m/z)	Qualifier Ion (m/z)	Ion Ratio	Collision Energy (V)
1	10.50	Naph	8.65	127.9	102.0	77.8	0.38	15
2	2.50	Acy	12.12	152.0	151.1	126.0	0.11	10
		Ace	12.34	154.0	153.0	152.0	0.12	10
3	1.50	Flu	13.36	165.9	165.0	162.9	0.03	10
4	3.00	Ant	15.84	178.0	176.0	152.0	0.70	30
		Ant-D10	15.86	188.1	160.2	158.2	0.38	30
		Phe	15.91	178.0	176.0	152.0	0.65	30
5	4.50	Flu	19.08	202.0	201.1	200.1	0.18	10
		Pyr	19.93	202.0	201.0	200.1	0.17	10
6	3.70	B(a)A	23.47	228.1	226.0	202.1	0.15	20
		Chr-D12	23.62	240.2	238.1	215.1	0.11	30
		Chr	23.70	228.1	226.2	202.2	0.15	20
		5MChr	24.94	242.1	241.1	227.5	0.15	30
7	3.80	B(b)F	26.72	252.1	250.1	226.1	0.18	30
		B(k)F	26.80	252.1	250.1	226.1	0.18	30
		B(a)P-D12	27.85	264.1	260.1	236.0	0.38	30
		B(a)P	27.93	252.1	250.1	226.1	0.18	30
8	5.50	I(1,2,3-c,d)P	30.91	276.1	274.0	250.0	0.05	35
		D(a,h)A	30.93	278.0	276.0	226.1	0.05	35
		B(g,h,i)P-D12	31.84	288.2	286.1	125.1	0.06	35
		B(g,h,i)P	31.95	276.1	274.0	250.0	0.05	35

Table 1: Parameters for SRM analysis of PAHs grouped according to Figure 1

PAH	Assigned Value [ng/g]	Satisfactory Range	Measured Value [ng/g]	Recovery [%]
B(a)A	6.35	3.56 - 9.14	6.43	101
B(b)F	1.31	0.73 - 1.89	1.26	96
B(a)P	3.41	1.91 - 4.90	3.32	97
I(1,2,3-cd)P	2.53	1.42 - 3.64	2.38	94
B(g,h,i)P	4.37	2.45 - 6.30	5.11	116

Table 2: Analysis of FAPAS® smoked fish T0642 proficiency test material (values given in ng/g, n=4)

PAH	Assigned Value [ng/g]	Measured Value [ng/g]	Recovery [%]
B(a)A	28.12	30.20	107
B(a)P	11.05	12.40	112
I(1,2,3-c,d)P	1.71	1.40	82
B(g,h,i)P	17.07	17.40	102

Table 3: Analysis of spiked fish with NIST 1582 crude oil (values given in ng/g, n=4)

Hydrocarbons	Empirical Formula	Molecular Ion	Retention Time
<i>n</i> -hexadecane	C <sub>16</sub> H <sub>34</sub>	226.2	10.48
<i>n</i> -heptadecane	C <sub>17</sub> H <sub>36</sub>	240.2	11.47
pristane	C <sub>19</sub> H <sub>40</sub>	268.3	11.26
<i>n</i> -octadecane	C <sub>18</sub> H <sub>38</sub>	254.3	12.39
phytane	C <sub>20</sub> H <sub>42</sub>	282.3	12.29
<i>n</i> -nonadecane	C <sub>19</sub> H <sub>40</sub>	268.3	13.29
<i>n</i> -eicosane	C <sub>20</sub> H <sub>42</sub>	282.3	14.15
<i>n</i> -heneicosane	C <sub>21</sub> H <sub>44</sub>	296.3	15.03
<i>n</i> -docosane	C <sub>22</sub> H <sub>46</sub>	310.3	16.00
<i>n</i> -tricosane	C <sub>23</sub> H <sub>48</sub>	324.3	16.83
<i>n</i> -tetracosane	C <sub>24</sub> H <sub>50</sub>	338.3	17.73
<i>n</i> -pentacosane	C <sub>25</sub> H <sub>52</sub>	352.4	18.80
<i>n</i> -hexacosane	C <sub>26</sub> H <sub>54</sub>	364.4	19.54
<i>n</i> -heptacosane	C <sub>27</sub> H <sub>56</sub>	378.4	20.45
<i>n</i> -octacosane	C <sub>28</sub> H <sub>58</sub>	394.4	21.30
<i>n</i> -nonacosane	C <sub>29</sub> H <sub>60</sub>	408.4	22.20
<i>n</i> -triacontane	C <sub>30</sub> H <sub>62</sub>	432.4	23.02

Table 4: Aliphatic hydrocarbons monitored in fish spiked with crude oil from Gulf of Mexico

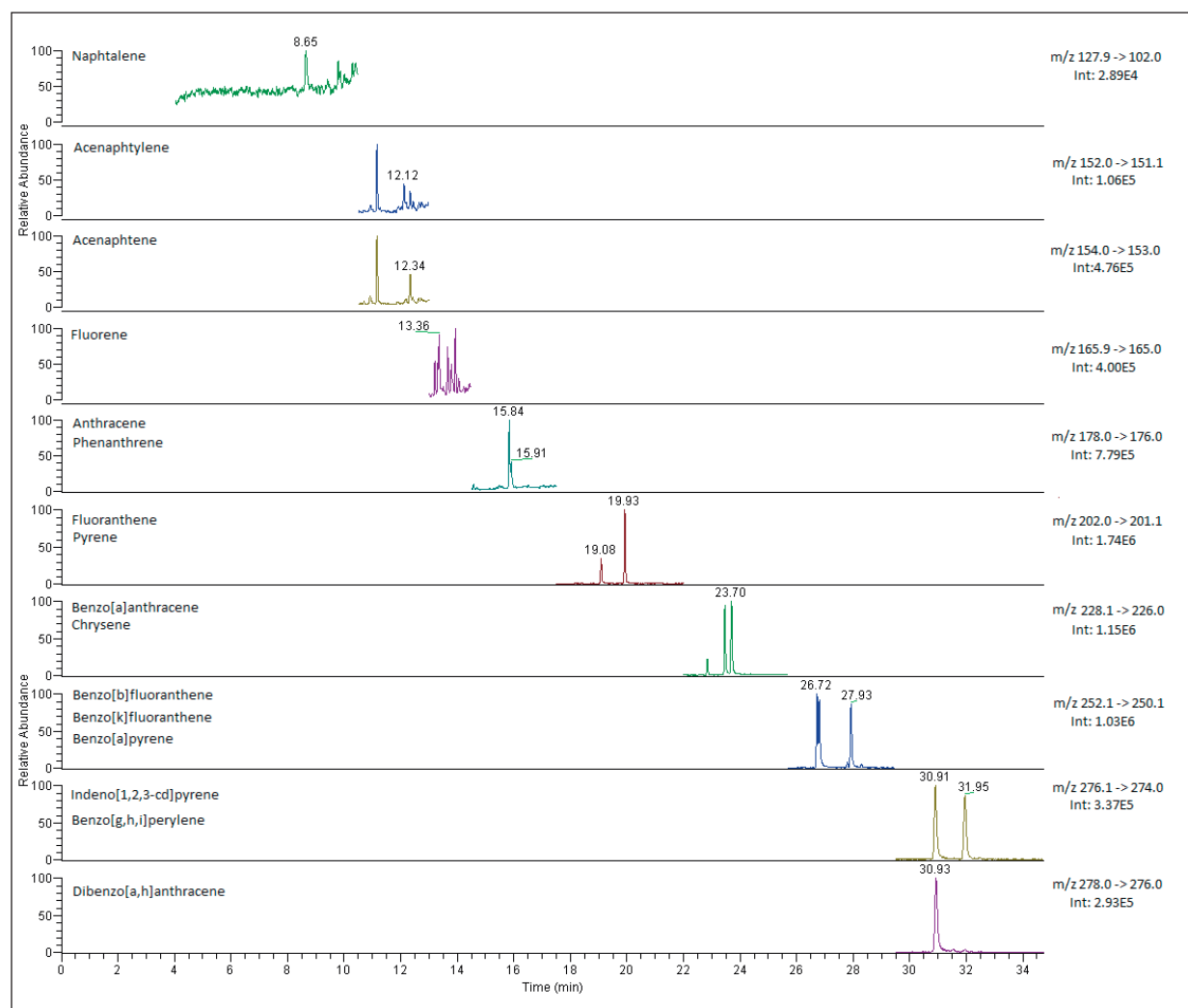


Figure 1: Chromatogram of red snapper fish sample spiked with 5 ng/g PAHs

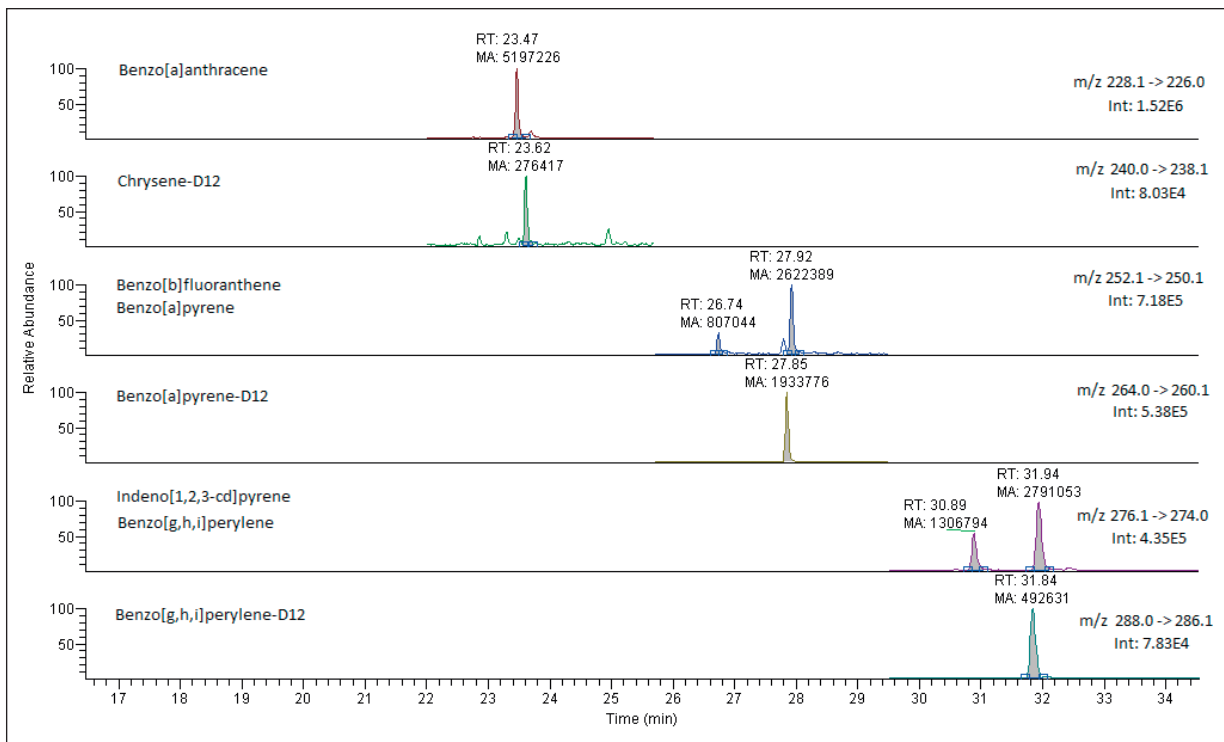


Figure 2: Chromatogram of FAPAS® T0642 smoked fish quality control sample showing peaks of the measured B(a)A, B(b)F, B(a)P, B(g,h,i)P, I(1,2,3-c,d)P PAHs and the respective internal standards

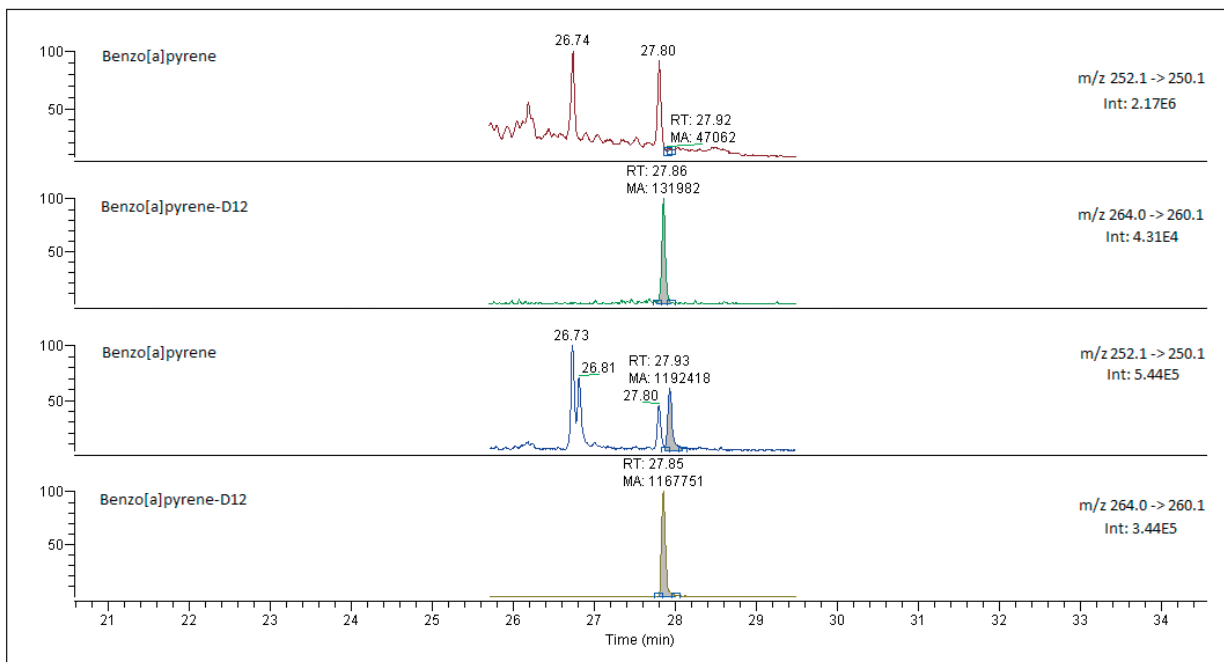


Figure 3: Chromatogram of m/z 252.1 -> 250.1 transition (for B(b)F, B(k)F and B(a)P- marked) in red snapper fish sample spiked with the actual oil spill sample from the Gulf of Mexico. Last two chromatograms representing the same sample (same transition and the relevant internal standard) spiked with standard addition at 5 ng/g PAHs concentration level.

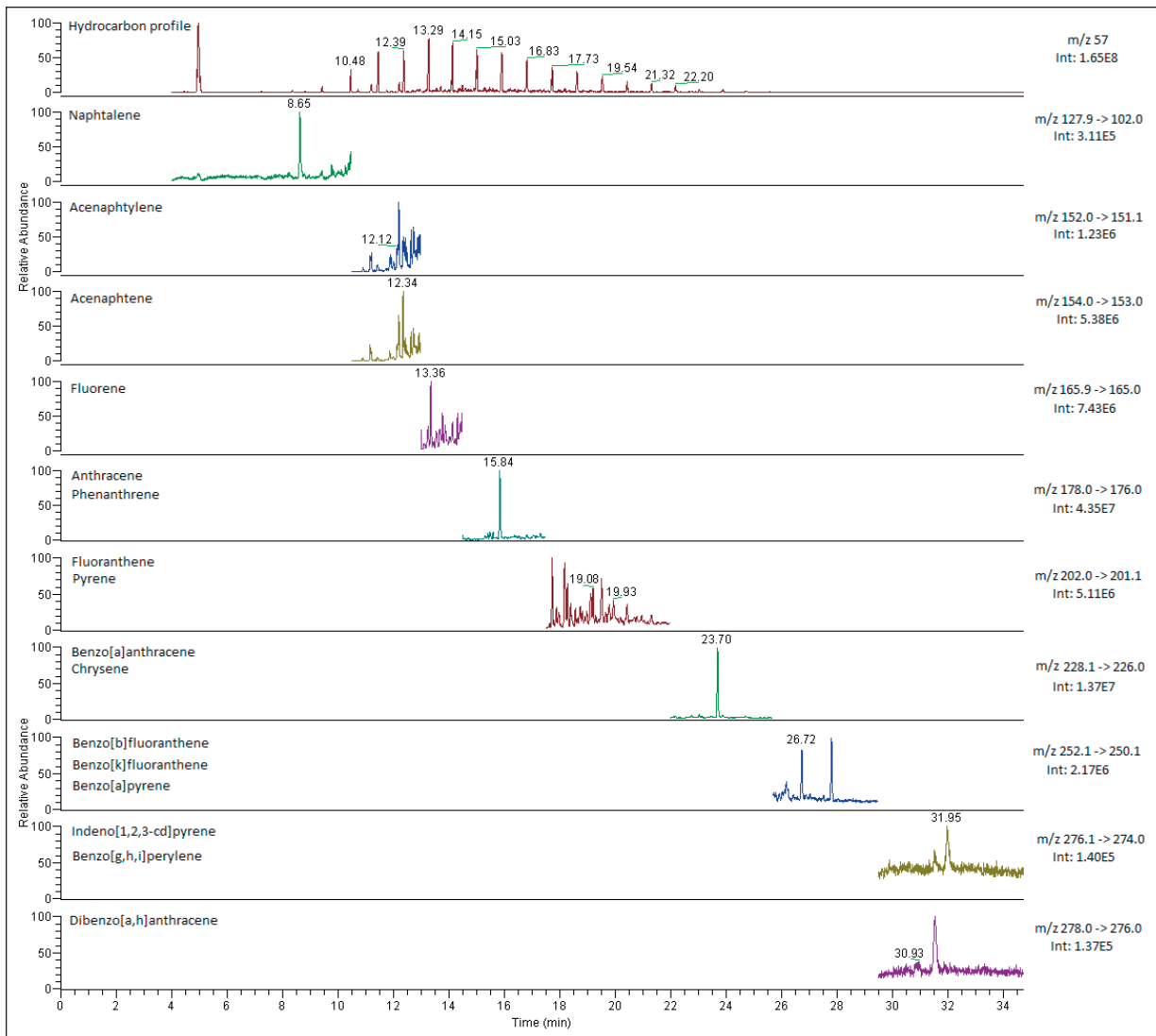


Figure 4: Chromatogram of red snapper fish sample spiked with actual oil spill sample (5 mg/g fish) from the Gulf of Mexico. Retention times indicate PAHs found in the sample.

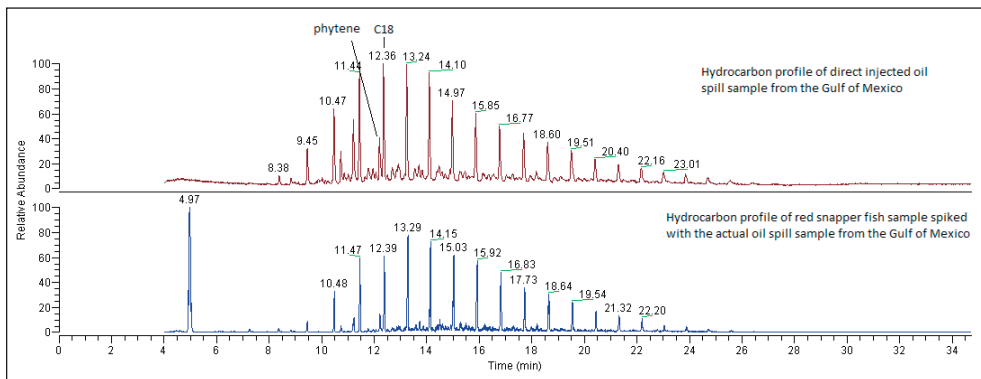


Figure 5: Hydrocarbon profile at  $m/z$  57 of actual oil spill sample taken from the Gulf of Mexico after direct injection (top) and after spiking it at 5 mg/g fish concentration level into red snapper fish sample (bottom).

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