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High Throughput Method for the Determination of PAHs in Seafood by QuEChERS-SBSE-GC-MS

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Scope

Applicable for the determination of naphthalene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo[a]pyrene in seafood tissues including finfish and shellfish. The method is expected to be applicable to alkylated PAHs and related compounds in similar matrices. Limits of quantitation were shown to be < 1ng/g.

Principle

This method uses a QuEChERS (quick, easy, cheap, effective, rugged, and safe) single-step acetonitrile (ACN) extraction and salting out liquid-liquid partitioning to extract PAHs from seafood tissue. Stir Bar Sorptive Extraction (SBSE) is then used as a combined cleanup and concentration step, eliminating organic acids and other polar and high molecular weight matrix components and providing a substantial concentration factor to easily meet regulatory limits of detection and requirements established for precision and accuracy for determination of PAHs in seafood tissue.

In brief, 3g of a homogenized seafood tissue sample in water is extracted with ACN in a 50 mL centrifuge tube followed by addition of 6.0 g $MgSO_4$ and 1.5 g sodium acetate which is shaken and centrifuged. A portion of the ACN extract (upper layer) is added to a 10 mL vial with

4 mL 0.1 M $NaHCO_3$ and a GERSTEL Twister™ stir bar and stirred to extract and concentrate the PAHs. The Twister stir bar is transferred to a thermal desorption tube in an autosampler tray for analysis by gas chromatography/mass spectrometry (GC/MS).

Introduction

The current method for testing seafood for contamination following petroleum spills focuses on the determination of polycyclic aromatic hydrocarbons (PAHs) in tissue using NOAA Method NMFS-NWFSC-59 2004. This method is time consuming and may not meet throughput needs when a major oil spill contaminates a large fishery. Recent developments have brought additional focus on laboratory throughput since a large number of seafood samples needed to be analyzed in order to ensure that such food supplies can safely be consumed. Stir Bar Sorptive Extraction (SBSE) has been established as an effective and fast technique for trace PAH determination in water and was therefore investigated as an alternative method to replace or complement the methods specified in current official methods for the analysis of seafood.

QuEChERS extraction [1] was first developed to extract a broad spectrum of pesticides from fruit and vegetables and has been shown to yield high recovery of apolar compounds from a variety of plant materials. The technique has since been extended to other analyte types including PAHs in fish tissue with recoveries of up to 90 % reported [2, 3]. The second step of the standard QuEChERS procedure uses dispersive Solid Phase Extraction (dSPE) with a Primary Secondary Amine (PSA) absorbent to remove organic acids. The QuEChERS cleanup step provides no additional concentration factor making it difficult to achieve detection limits meeting the current requirements for PAH analysis.

In 1999 Baltussen et al [4] developed a micro extraction technique, referred to as Stir Bar Sorptive Extraction (SBSE), based on a stir bar coated with a thick film of polydimethylsiloxane (PDMS) phase

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that had significantly higher capacity than PDMS solid phase microextraction (SPME) fibers. SBSE has been commercially available as the GERSTEL Twister™ since 2000, and the technique has gained popularity for extraction of aqueous samples based on the ease of use and extremely low detection limits possible for compounds with high octanol:water partition coefficients (log $K_{o/w}$ > 2). SBSE has been successfully applied for qualitative identifications of natural products in complex matrices and quantitative applications at trace levels in a wide array of application areas including food, environmental, and forensics as shown in a recent review [5]. Several reports [6, 7, 8, 9] demonstrate that apolar persistent pollutants such as PAHs, PCBs, PBDE flame retardants and bisphenol A are efficiently extracted from aqueous solutions or biological tissues and determined at parts per billion (ng/mL) levels using SBSE. The US EPA Region 7 lab has presented a summary [10] of a 90 day study performed to test SBSE for extraction of semivolatile organic pollutants in surface water and concluded that all 18 PAHs in the study met EPA Method 625 performance criteria.

Sandra et al [11] has developed a multiresidue pesticide method which first described a concentration procedure using a water miscible organic solvent to extract pesticides from plant materials followed by a dilution step. The organic extract is diluted in water to reduce the organic content to 10 % to allow concentration of the target analytes by SBSE, achieving ng/g detection limits. This technique was further described by Kende et al [12] who used spectral deconvolution techniques to minimize background interference from extracted matrix components. Llorca-Porcel et al [13] determined chlorophenols, bisphenol-A, 4-t-octylphenol and 4-nonylphenols in soil using ultrasonic extraction with water miscible solvents followed by dilution in water and SBSE concentration. They showed ng/g detection limits from one gram of soil. Crifasi et al [14] has shown that SBSE can be performed on direct biological tissue homogenates prepared in pH 9.7 buffer to identify a variety of drugs of forensic interest.

The work presented here demonstrates that SBSE can be used to eliminate matrix interference and concentrate PAHs from QuEChERS extracts of fish and shellfish tissue while reducing the sample preparation time and dramatically improving sample throughput. Furthermore, the combined QuEChERS-SBSE-GC/MS method meets the regulatory limits of detection and the requirements established for precision and accuracy for determination of PAHs in seafood tissue.

Experimental

Reagents and Standards

- Dichloromethane, A.C.S. spectrophotometric grade >99.5 % (Sigma-Aldrich, Cat.No. 154792-1L)
- Methanol, A.C.S. reagent >99.8 %, (Sigma-Aldrich, Cat. No,179337-1L)
- Acetonitrile, LC-MS Chromasolv 99.9 %, (Sigma-Aldrich, Cat. No. 34967)
- Sodium hydrogen carbonate, 99.99+% (Sigma-Aldrich, Cat. No. 43,144-3)
- PAH standards (MA-EPH, P/N 31458, Restek)
- SV Internal Standard Mix (P/N 31006, Restek)
- QuEChERS AOAC extraction kits (P/N 5982-5755, Agilent)
- Water (Milli-Q)

Equipment and Supplies

- Waring variable speed lab blender (LB10S) with steel bowl
- ATR Rotamix (Model RKVSD)
- Sorvall Evolution RC centrifuge (SH 3000 rotor)
- Vortex Genie
- Analytical Balance
- 500 mL Volumetric flask
- Adjustable Pipettor, 100-1000 µL (VWR, Cat.No. 83009-736)
- Adjustable Pipettor, 10-100 µL (VWR, Cat.No. 83009-730)
- Adjustable Pipettor, 0.5-5 mL (VWR, Cat.No. 53495-322)
- Pipette Tips, 5 mL (VWR, Cat.No. 89087-530)
- Pipette Tips, 1000 µL (VWR, Cat.No. 83007-378)
- Pipette Tips, 100 µL (VWR, Cat.No. 53503-769)
- 50 mL BD Falcon Tubes
- Twister™ stir bars (P/N 011222-001-00, GERSTEL)
- TC 2 tube conditioner (P/N 011222-001-00, GERSTEL)

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- 10 mL headspace screw neck vials (P/N 093640-038-00, GERSTEL)
- 20 mL headspace screw neck vials (P/N 093640-036-00, GERSTEL)
- Screw caps (P/N 093640-040-00, GERSTEL)
- 20 position magnetic stir plate (GERSTEL)
- 2 mL vials with screw caps (Supelco, Cat.No. 27531)
- MPS 2 autosampler with Maestro software control (GERSTEL)
- TDU Thermal Desorption Unit (GERSTEL)
- CIS 4 Programmed Temperature Vaporizing inlet (GERSTEL)
- 7890 GC with 5975 MSD (Agilent)
- Rxi-5Sil MS 30 m x 0.25 mm x 0.25 µm (Restek, P/N 13623)

Calibration and Internal Standard Solutions

The stock PAH standard (MA-EPH) is supplied as a 1000 µg/mL solution in dichloromethane. Note that PAH standards supplied in mixed dichloromethane/isooctane exhibited miscibility problems when diluted into acetonitrile. Table 1 shows the dilution scheme used to prepare the PAH spiking solutions.

Table 1: Dilution Scheme for PAH Spiking Solutions.

Standard Concentration	µL of Standard	µL of ACN	Resulting Concentration
1000 µg/mL	100	900	100 µg/mL
100 µg/mL	50	950	5.0 µg/mL
100 µg/mL	20	980	2.0 µg/mL
100 µg/mL	10	990	1.0 µg/mL
2 µg/mL	100	900	0.20 µg/mL
2 µg/mL	10	990	0.020 µg/mL

The stock Internal Standard mix is supplied as a 4000 µg/mL solution in dichloromethane. Table 2 shows the dilution scheme used to prepare IS spiking solutions.

Table 2: Dilution Scheme for Internal Standard Solution.

Standard Concentration	µL of Standard	µL of ACN	Resulting Concentration
4000 µg/mL	25	1975	50 µg/mL
50 µg/mL	150	850	7.5 µg/mL
50 µg/mL	10	990	0.50 µg/mL

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Preparation of Calibration Standards

The sample size for this assay is 3.0 grams of tissue extracted with 15 mL acetonitrile, or 0.2 g/mL. Calibration standards are prepared such that a 10 µL spike into 1.0 mL of acetonitrile will be equivalent to 1, 10, 50, 100, and 250 ng/g of analytes, in a tissue sample. The internal standard is spiked at a level equivalent to 25 ng/g in tissue. Table 3 lists the spike levels and required concentration of standard. The spike solutions are prepared in acetonitrile.

Table 3: Calibration Spiking Solution Concentrations.

Spike Level in Tissue [ng/g]	Concentration of Spike Solution [µg/mL]
1	0.020
10	0.20
50	1.0
100	2.0
250	5.0
25 (Internal Standard)	0.50

Four deuterated PAH internal standards are used to calculate relative response factors for the nine target PAHs in this method. An average response factor, relative to the designated internal standard, for the five calibration levels is calculated for each analyte.

Spiking Samples with Internal Standard

A mixture of four deuterated internal standards is added to the tissue sample at a level of 25 ng/g before extraction. Two strategies were tested and found to be equivalent. Strategy 1: Add 150 µL of 0.50 µg/mL IS solution directly to the tissue sample in the 50 mL Falcon tube. Strategy 2: Add 10 µL of 7.5 µg/mL IS solution directly to the tissue sample in the 50 mL Falcon tube.

Other Solutions

In a volumetric flask, prepare 500 mL of a 0.1 M solution of sodium hydrogen carbonate in Milli-Q water by accurately weighing 4.2 g of $NaHCO_3$ into the flask and diluting to the mark.

Sample Preparation

Preparation of Homogenates

For method optimization and QC, sample homogenates were prepared by weighing 20-100 g of the edible portion of the tissue (fresh or partially frozen) into a lab blender or homogenizer. High water content samples such as oysters can be homogenized directly. Some samples such as shrimp or finfish are easier to homogenize by adding an equal weight of Milli-Q water to the blender bowl. Homogenize for 2-3 minutes. Weigh 3.0 +/- 0.1 g homogenized tissue (or 6.0 +/- 0.1 g of tissue homogenized 1:1 with water, equivalent to 3.0 g tissue), into a 50 mL BD Falcon centrifuge tube. The matrices used in this validation study included uncooked frozen shrimp, fresh oysters harvested from Maryland, and finfish (Croakers) purchased at a local grocery store. Also, NIST SRM 1974b "Organics in Mussel Tissue" was obtained from the National Institute of Standards and Technology.

For incurred samples, edible portions of the seafood tissue should be frozen, composited and homogenized by blending in a lab blender or equivalent for 2-3 minutes. The minimum sample size for this analysis is 3 g.

QuEChERS Extraction

Add 150 µL of the 0.50 µg/mL internal standard solution directly onto the tissue puree in the tube. Add Milli-Q water to bring the total weight to 15 g. Vortex the sample 30 seconds. Add 15.0 mL of acetonitrile, vortex the sample for an additional minute. Add the contents of the QuEChERS salt packet (6.0 g $MgSO_4$ and 1.5 g sodium acetate) to the sample. Shake manually for 1 minute. Place the samples on a rotator and agitate for 10 minutes. After agitation, centrifuge the samples for 5 minutes at 5 °C and 4700 rpm. The sample will separate into an upper organic layer and lower aqueous layer with a compact and stable tissue layer at the interface. The acetonitrile layer is transferred to a clean, labeled, 20 mL vial which can be stored refrigerated 24-48 hours before analysis.

Twister Extraction

Standard Solutions

Add 1.00 mL of acetonitrile to a 10 mL screw neck vial. Spike 10 µL of the appropriate concentration standard (See Table 3) into the vial to generate standards equivalent to 1-250 ng/g in a tissue sample. Spike 10 µL of the 0.50 µg/mL internal standard solution into the vial. Add 4.00 mL of 0.1 M $NaHCO_3$ solution. Add a conditioned Twister to the vial. Cap the vial. Extract the standard for 90 minutes at room temperature and 1200 rpm.

Samples

Add 1.00 mL of acetonitrile extract, from Step 1.5, to a 10 mL screw neck vial. Add 4.00 mL of 0.1 M $NaHCO_3$ solution. Add a conditioned Twister to the vial. Cap the vial. Extract the standard for 90 minutes at room temperature and 1200 rpm. After extraction, Twisters are rinsed briefly in Milli-Q water, blotted dry with a lint-free tissue, placed into a glass thermal desorption tube with transport adapter and sealed in a sample tray on the MPS 2 autosampler.

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Thermal Desorption Gas Chromatography with Mass Spectrometric Detection

The samples and standards are analyzed by thermal desorption gas chromatography with mass spectrometric detection. Table 4 lists the SIM parameters for the mass spectrometer.

Analysis conditions

TDU	
Tube type	Empty Glass
Mode	Splitless
Sample Mode	Sample Remove
Temperature	40 °C (0.20 min); 720 °C/min to 300 °C (5 min)
Transfer Line	300 °C
CIS	
Liner Type	Quartz Wool
Mode	Solvent Venting
Vent Flow	100 mL/min
Vent Pressure	8.23 psi until 0.00 min
Split Flow	10 mL/min at 0.01 min
Temperature	-120 °C (0.20 min); 12 °C/s to 300 °C (3 min)
GC	
Oven	60 °C (1.0 min); 15 °C/min to 325 °C (3 min)
Constant Flow	1.0 mL/min Helium
Column	30 m Rxi-5Sil MS (Restek)
	d_i=0.25 mm, d_f=0.25 µm
MSD	
MS Source	*230 °C*
MS Quad	*150 °C*

Table 4: MS Acquisition Groups.

Group (Start Time)	Mass (Dwell)
1 (2.5 min)	78, 102, 108, 115, 126, 128, 134, 136, 150 (10)
2 (7.6 min)	115, 141, 142 (50)
3 (9.0 min)	151, 152, 153, 154, 160, 162, 164 (10)
4 (10.0 min)	165, 166, 167 (50)
5 (11.5 min)	160, 176, 178, 179, 184, 188 (20)
6 (13.5 min)	200, 202, 203 (50)
7 (15.5 min)	120, 226, 228, 229, 236, 240 (20)
8 (17.4 min)	126, 132, 252, 253, 260, 264 (20)
9 (19.2 min)	138, 139, 276, 277, 278, 279 (20)

Twister Reconditioning

After use, the Twisters must be re-conditioned. Place the Twisters in a 20 mL screw cap vial. Add a 50/50 mixture of methanol and CH2Cl2 to just cover the Twisters in the vial. Let them soak for at least 2 hours. Remove the Twisters to a clean watch glass and let air dry in a fume hood for 1 hour. After drying, place the Twister in an empty TDS tube (up to 5 can be placed in each tube). Place the tubes in the TC 2 conditioner. Set the temperature to 300 °C. Thermally condition the Twisters for 2 hours under a flow of nitrogen, 50-80 mL/min. After conditioning, allow the Twisters to cool to room temperature under a flow of nitrogen. Remove the Twisters from the TDS tubes and place them in clean 2 mL autosampler vials for storage until needed.

Results and Discussion

Calculation of Relative Response Factors

Relative response factors are used when calculating the final concentration of analytes in the tissue samples. These relate the response of the analyte to the reference compound. Standards diluted in acetonitrile are used for these calculations. Peak areas for each of the analytes and deuterated internal standards listed in Table 5 are obtained using the appropriate quant ion for the standard solutions prepared for Twister extractions.

Table 5: Analytes and Ions Used for Quantification.

Compound	Quant Ion
Naphthalene-d8	136
Naphthalene	128
Fluorene	166
Phenanthrene-d10	188
Phenanthrene	178
Anthracene	178
Chrysene-d12	240
Fluoranthene	202
Pyrene	202
Benz[a]anthracene	228
Chrysene	228
Perylene-d12	264
Benzo[a]pyrene	252

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The Relative Response Factor is calculated from the equation:

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

A_{std} = Peak Area of the Standard

A_{IS} = Peak Area of the Internal Standard

[IS] = Concentration of the Internal Standard, as spiked into tissue, ng/g

[Std] = Concentration of the Standard, as spiked into tissue, ng/g

In Table 5, the deuterated internal standards are highlighted. The analytes which are related to each internal standard are listed below them. Table 6 shows typical data for pyrene, with an internal standard concentration of 25 ng/g, as spiked into tissue.

Table 6: Pyrene Relative Response Data.

Standard spiked into tissue [ng/g]	Pyrene Area	Chrysene-d12 Area	Rf
1	25644	475747	1.35
10	309338	593758	1.30
50	1368996	423635	1.62
100	2053334	407897	1.26
250	5069292	410566	1.23
		Average Rf	*1.35*

Calculation of Final Analyte Concentration

Once the average relative response factors have been calculated and the samples are analyzed, the analyte concentration can be calculated. The concentration of the analyte in the tissue is calculated from:

$$[Analyte] = \frac{[IS] \times A_{analyte}}{A_{IS} \times R_f}$$

$A_{analyte}$ = Peak Area of the Analyte

A_{IS} = Peak Area of the Internal Standard

[IS] = Concentration of the Internal Standard, as spiked into tissue, ng/g

[Analyte] = Concentration of the Analyte in the tissue, ng/g

R_f = Relative Response Factor for the analyte

Table 7 lists example data for pyrene in a sample at a known level of 18.0 ng/g. Three replicates of the sample were prepared.

Table 7: Example Pyrene Sample Data.

Standard spiked into tissue [ng/g]	Pyrene Area	Chrysene-d12 Area	Pyrene calculated [ng/g]
25	345900	343531	18.6
25	331837	341328	18.0
25	370653	341094	20.1
		Average [Pyrene]	*18.9*

Croaker, Shrimp, and Oyster

Samples of frozen gulf shrimp, fresh oysters, and Atlantic Croaker were obtained from local markets. The samples were prepared and extracted using the above procedure with the exception that they were spiked with standard solutions prior to addition of the internal standard, to obtain levels of 2.5, 50, and 250 ng/g PAHs in the tissue. Each matrix was spiked in triplicate. The croaker sample was spiked only at the 50 ng/g level. The results for these spikes are found in Table 8.

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Table 8: Spike and Recoveries from Croaker, Oyster and Shrimp Matrices.

	Croaker	Shrimp			Oyster		
Spike Level (ng/g)	50	2.5	50	250	2.5	50	250
	Percent Recovery						
Naphthalene	70	125	71	85	126	72	82
Fluorene	63	104	79	90	93	75	84
Phenanthrene	71	101	67	80	118	70	82
Anthracene	69	83	68	82	96	67	80
Fluoranthene	94	101	78	94	138	89	101
Pyrene	86	114	76	91	131	86	98
Benz[a]anthracene	64	84	69	85	91	69	84
Chrysene	65	84	67	83	104	66	81
Benzo[a]pyrene	64	74	65	83	72	65	84
Average %Recovery	*72*	*97*	*71*	*86*	*108*	*73*	*86*

The linearity of the method for all three matrices was tested by spiking each matrix with PAHs at levels of 1, 10, 50, 100, and 250 ppb. The internal standards were spiked at a level of 25 ppb. The peak area for the analyte divided by the peak area for the internal standard was plotted versus the concentration of analyte divided by the concentration of internal standard. Figure 1 shows calibration curves for pyrene in the three different matrices. The linear regression data for all analytes is shown in Table 9.

Table 9: Linear Regression Data for Matrix Spikes.

	Croaker			Shrimp			Oyster		
	r^2	m	b	r^2	m	b	r^2	m	b
Naphthalene	0.9943	1.215	-0.053	0.9905	1.35	-0.195	0.9916	1.331	-0.157
Fluorene	0.9912	1.345	-0.156	0.9930	1.67	-0.292	0.9912	1.621	-0.318
Phenanthrene	0.9948	1.339	-0.025	0.9932	1.43	-0.229	0.9957	1.331	-0.058
Anthracene	0.9951	1.454	-0.104	0.9810	1.55	-0.396	0.9960	1.398	-0.109
Fluoranthene	0.9919	1.166	-0.179	0.9940	1.21	-0.192	0.9937	1.330	-0.176
Pyrene	0.9918	1.203	-0.228	0.9920	1.28	-0.225	0.9937	1.402	-0.186
Benz[a]anthracene	0.9908	1.070	-0.251	0.9929	1.32	-0.248	0.9930	1.309	-0.220
Chrysene	0.9916	1.007	-0.200	0.9933	1.25	-0.216	0.9934	1.218	-0.170
Benzo[a]pyrene	0.9919	1.252	-0.203	0.9915	1.34	-0.300	0.9940	1.263	-0.195

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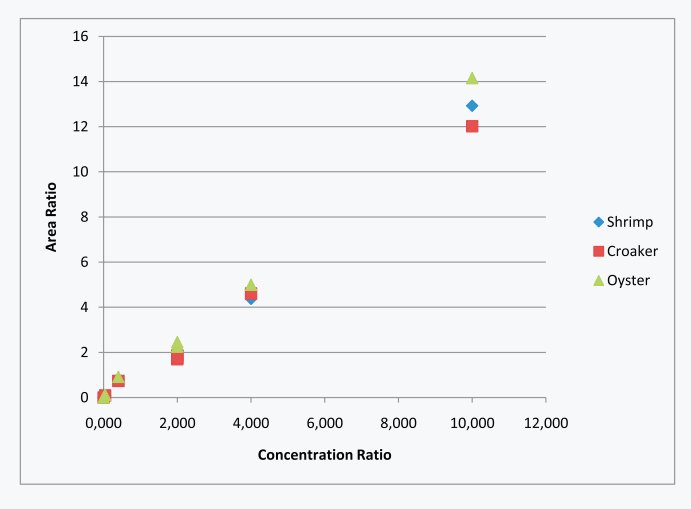

Figure 1: Pyrene Calibration Curves for Matrix Spikes.

SRM Material

A standard reference material, NIST SRM 1974b "Organics in Mussel Tissue" was obtained from the National Institute of Standards and Technology. The sample was processed as outlined in the QuEChERS- and Twister Extraction sections. The Twisters were analyzed by thermal desorption GC/MS using the method parameters listed under 1.7. The results are summarized in Table 10. A total ion chromatogram (SIM data) for the SRM material is shown in Figure 2.

Table 10: SRM Analysis Data.

Analyte	Acceptable range	SRM		
		Certificate of Analysis	QuEChERS SBSE	% RSD
Naphthalene	1.6 - 3.3	2.4	1.1	18.5
Fluorene	0.3 - 0.7	0.49	0.35**	14.7
Phenanthrene	1.7 - 3.5	2.6	1.9	11.1
Anthracene	0.3 - 0.8	0.53	2.4*	15.2
Fluoranthene	11.5 - 23.1	17	19	7.0
Pyrene	12.2 - 24.2	18	18	7.2
Benz[a]anthracene	2.9 - 6.9	4.7	3.5	7.2
Chrysene + Triphenylene	7.4 - 13.8	10.6	8.6	6.8
Benzo[a]pyrene	2.0 - 3.6	2.8	1.7	10.4
Total		59.12	58.05	

* Possible PCB coelution
**n=3 (Only detected using 60 m column)

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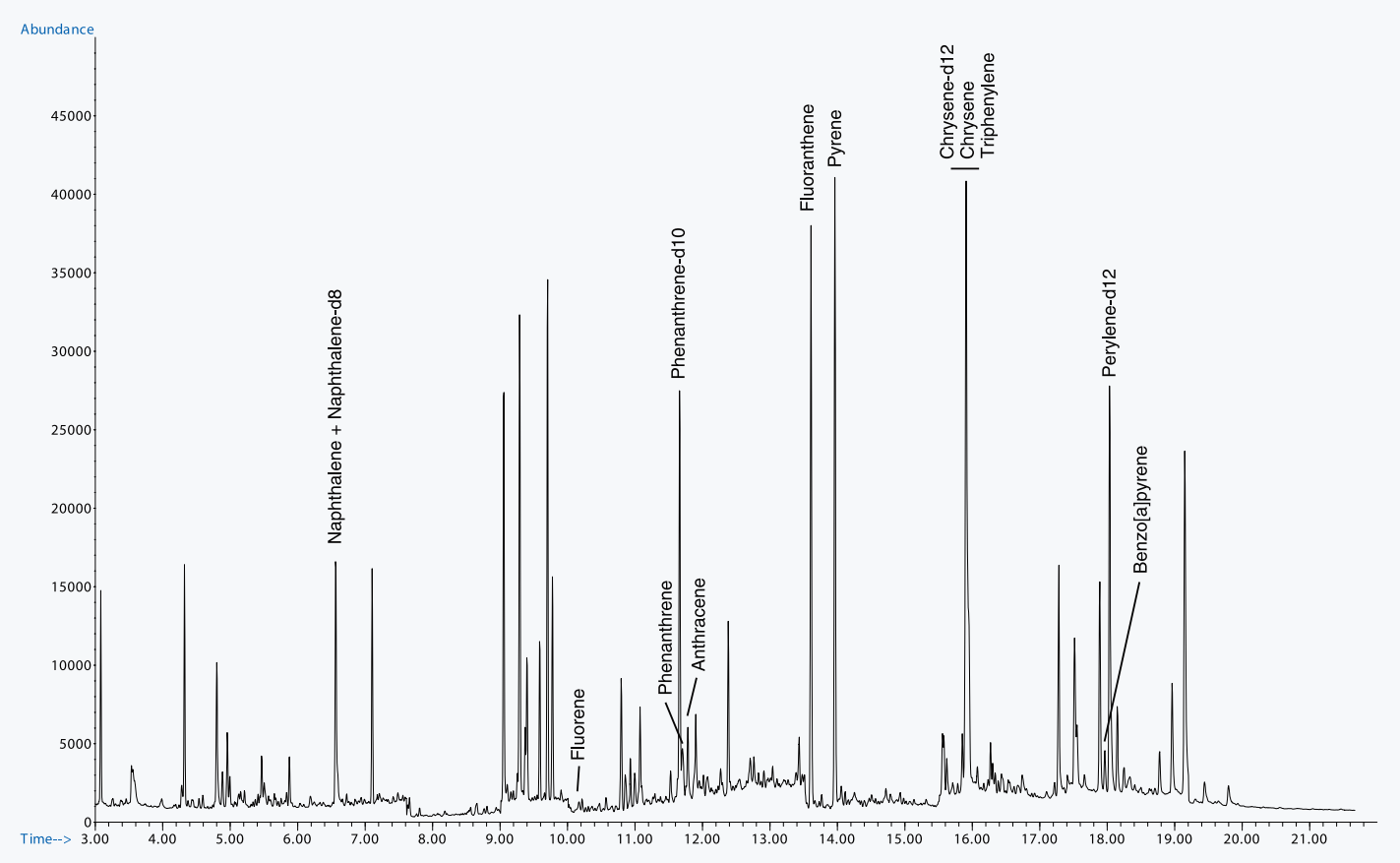

Figure 2: Total Ion Chromatogram, SIM Data, Standard Reference Material

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Conclusion

The described method shows good linearity over the range 1-250 ng/g. The analysis of the standard reference material shows good agreement with actual values. The combination of QuEChERS extraction with SBSE for concentration and matrix management allows accurate single digit ppb detection of the PAHs in marine tissues. The sample throughput is estimated at 20-40 samples/analyst/instrument/day.

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