Determination of PAHs in Edible Oils by DACC-HPLC with Fluorescence Detection

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Key Words
Polycyclic Aromatic Hydrocarbons, Solid Phase Extraction (SPE), On-Line Sample Enrichment

Goal
To develop an on-line donor-acceptor complex chromatography–high-performance liquid chromatography (DACC-HPLC) method to determine polycyclic aromatic hydrocarbons (PAHs) in edible oils

Introduction
Numerous PAHs are carcinogenic, making their presence in foods and the environment a health concern. Regulations around the world limit levels of a variety of PAHs in drinking water, food additives, cosmetics, workplaces, and factory emissions. PAHs also occur in charbroiled and dried foods, and may form in edible oils by pyrolytic processes, such as incomplete combustion of organic substances. PAHs in foods can also result from petrogenic contamination. The European Commission regulates the amounts of PAHs in foods, and has imposed a limit of 2.0 µg/kg for benzo[a]pyrene (BaP) in edible oils, as BaP was determined to be a good indicator of PAH contamination.[1]

PAHs have traditionally been separated using HPLC and determined using UV,[2] fluorescence,[3,4] electrochemical,[5] and mass spectrometry (using atmospheric-pressure photoionization)[6] detection methods. After an oxygenation reaction, PAHs can also be determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).[7] These methods of determining PAHs in edible oils require multiple manual sample preparation steps. One study of PAHs in over a dozen edible oils used a dimethyl sulfoxide (DMSO) extraction followed by three extractions with cyclohexanone and cleanup with a silica column.[8] Another study of six edible oils used SPE, but required solvent extraction steps before SPE and evaporation afterward.[9] These manual steps consume solvent, resources, and time.

In recent years, DACC has gained popularity for PAH analysis.[10–12] DACC stationary phases can be used for SPE, retaining PAHs while matrix components are flushed to waste. After elution of the analytes, solvent exchange is used to prepare the sample for HPLC analysis. Compared to traditional methods, this cleanup technique uses less solvent, is less labor intensive, and saves considerable time.[11] However, this approach still involves several manual sample-handling steps; therefore, it still requires labor and is prone to errors.

In 1996, van Stijn et al. developed an automated process for oil sample preparation and analysis.[12] The preparation consists of coupling a DACC cleanup column with an HPLC analytical column. This solution does not require manual cleanup and solves the previously described challenges. However, adopting the method for routine operation is difficult and requires advanced technical expertise to optimize the system configuration. This optimization can be time consuming. Furthermore, the described solution uses the autosampler software for system control and different software for data collection, instead of using an integrated chromatography data system for system control and monitoring. This leaves room for improvement in ease of operation, process monitoring and documentation, validation, reporting, and automated diagnosis.

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The method described here adapted van Stijn's solution to create a method for automated on-line determination of PAHs in edible oils that addresses the remaining challenges. This solution was performed on an HPLC system equipped with a dual-gradient HPLC pump and two switching valves, allowing on-line sample enrichment on a DACC column with HPLC analysis. On-line coupling of sample preparation and analysis eliminates the complex manual pretreatment required by traditional methods. This automation reduces unintentional errors and increases reproducibility. The analysis time per sample is approximately 80 min with the dual-gradient HPLC system, compared to 8–10 h with traditional methods. Moreover, this automated system can run 24 h a day, significantly increasing sample throughput and making this complex analysis routine.

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 Standard Dual System, including:
  - DPG-3600A Pump with SRD-3600 Air Solvent Rack
  - WPS-3000TSL Autosampler
  - TCC-3200 Thermostatted Column Compartment with two 2p-6p valves
  - RF-2000 Fluorescence Detector
- Thermo Scientific™ Dionex™ Chromeleon™ 6.80 SP1 Chromatography Workstation
- Chromeleon Chromatography Data System (CDS) software

Device configurations for the on-line DACC cleanup with analytical HPLC are shown in Figures 1–3. In these figures, the upper valve is the right valve and lower valve is the left valve in the TCC-3200.

Reagents and Standards

- Deionized water
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific)
- Isopropanol, HPLC grade, (Fisher Scientific)
- Charcoal, activated granular (activated carbon), chemical pure grade
- Mix of PAHs, EPA Sample for Method 610, 200 µg/mL for each component, including 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
- Benzo[b]chrysene, 50 µg/mL, used as an internal standard (I.S.)

Samples

- Two brands of olive oil (Olive Oils 1 and 2 from Italy and Spain, respectively)
- One brand of sesame oil (from China)

Preparation of Standards and Samples

Purification of Olive Oil Used as Blank and Matrix

Add 1 g activated carbon to 20 g olive oil, heat for 2 h at 60 °C while stirring, then filter through a pleated filter. Pass filtrate through a membrane filter (0.45 µm, PTFE) and store the resulting purified oil sample at 4 °C.

Preparation of Olive Oil Containing I.S. Used as Matrix

To prepare a 0.25 µg/mL stock I.S. solution, add 995 µL isopropanol using a 1 mL pipette to a 2 mL vial, and add 5 µL of 50 µg/mL I.S. oil using a 10 µL syringe.

Add 40 µL of the 0.25 µg/mL stock I.S. solution, using a 10 µL syringe, to ~10 g of the purified olive oil used as blank and matrix. The concentration of I.S. in the oil matrix is ~1 µg/kg. In the work presented in this study, the I.S. working standard was added to 10.0786 g of the purified olive oil sample. The resulting I.S concentration in the matrix was 0.992 µg/kg.

Preparation of Working Standards (Olive Oil as Matrix)

To prepare a 1 µg/mL stock standard solution, add 995 µL isopropanol, using a 1 mL pipette, and 5 µL of the 200 µg/mL standard solution, using a 10 µL pipette, to a 2 mL vial. Use the stock standard solution to prepare working standards as described in Table 5.

Edible Oil Sample Preparation

Prior to injection, filter oil through a 0.45 µm membrane (PTFE).

Precautions

Contaminants in solvents, reagents, glassware, and other sample processing hardware may cause method interferences, so glassware must be scrupulously cleaned. Use high-purity reagents and solvents to minimize interference problems.

Conditions	
Analytical Columns:	Two PAH columns (4.6 × 250 mm)
On-Line SPE Column:	DACC (3.0 × 80 mm)
Mobile Phases:	A. Water B. Acetonitrile for both loading and analysis pumps C. Isopropanol for loading pump
Flow Rate:	1 mL/min
Injection Volume:	80 µL (100 µL injection loop)
Column Temperature:	30 °C
Autosampler Temperature:	40 °C
Detection:	Fluorescence (Table 4)

Table 1 shows the gradient for on-line SPE using the loading pump, and Table 2 shows the gradient for separation using the analysis pump. Table 3 shows the valve-switching timing.

Because the maximum fluorescent responses of PAHs occur at different emission wavelengths, it is necessary to change the excitation and emission wavelengths based on individual PAH retention times. Table 4 shows the program for wavelength changes.

Table 1. Gradient program for on-line SPE.

Time (min)	Flow Rate (mL/min)	Solvent A (% vol)	Solvent B (% vol)	Solvent C (% vol)	Curve (%)
0.00	0.35	0	0	100	—
12	0.35	0	0	100	5
12.1	0.35	20	80	0	5
20.9	0.35	20	80	0	5
20.91	0.35	0	100	0	5
50.9	0.35	0	100	0	5
51.5	0.35	0	0	100	5
66.5	0.35	0	0	100	5

Table 2. Gradient program for separation.

Time (min)	Flow Rate (mL/min)	Solvent A (% vol)	Solvent B (% vol)	Curve (%)
0.00	0.4	20	80	—
14.6	0.4	20	80	5
16	1	20	80	5
30	1	0	100	6
58	1	0	100	5
58.1	1	20	80	5
65	1	20	80	5
65.5	0.4	20	80	5
70	0.4	20	80	5

Table 3. Valve-switching programs for the left and right valves.

Time (min)	Left Valve	Right Valve
0.00	6_1	1_2
12.1	No Movement	6_1
14.5	1_2	No Movement
17	6_1	No Movement
61.5	No Movement	1_2

Table 4. Wavelength changes for RF-2000 Fluorescence Detector.

Time (min)	Excitation Wavelength (nm)	Emission Wavelength (nm)
0.00	256	370
27.05	256	390
29.5	240	420
33.5	270	385
37.5	290	430
51.5	305	480
53.5	290	430

Results and Discussion

Description of the On-Line DACC-HPLC Method

The flow scheme (Figure 1) couples the DACC cleanup directly with the analytical HPLC run, using a second gradient pump and two column-switching valves. Figure 1 shows the valve positions at the time of the injection. The filtered and undiluted oil is injected directly, using isopropanol (IPA) to transfer the sample onto the enrichment column (DACC column). The analytical separation column is equilibrated with the second pump at the same time. After the analytes are bound to the DACC column and most of the oil has been sent to waste, the right valve switches to flush out the IPA and remaining oil in a backflow mode with acetonitrile/water (Figure 2). When all IPA and oils have been removed, the system switches the enrichment column into the analytical flow path (Figure 3).

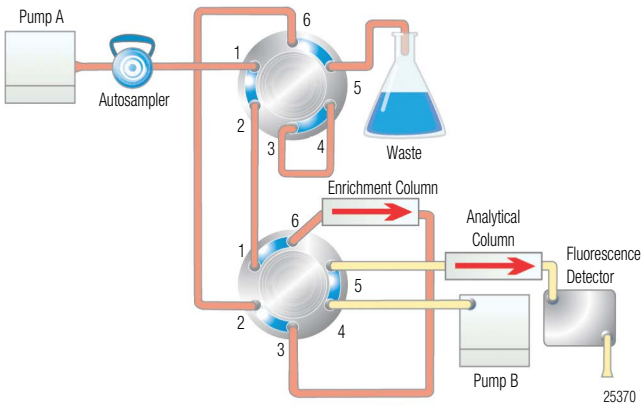

Figure 1. Flow scheme for on-line sample preparation and analysis. The valves are positioned for injection of the sample on the enrichment column.

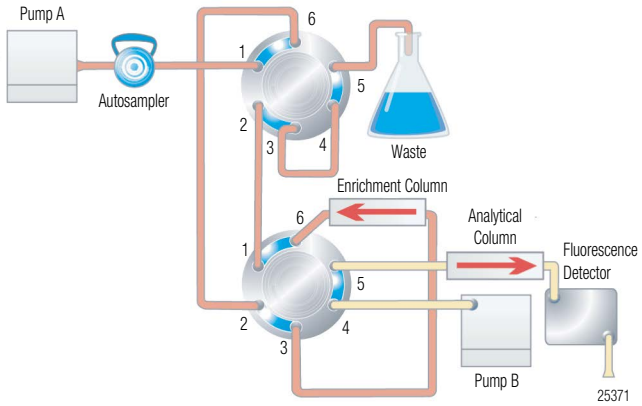

Figure 2. Isopropanol is flushed out of the enrichment column in backflow mode.

Reproducibility, Detection Limits, and Linearity

Method reproducibility was estimated by making seven replicate injections of Olive Oil 1 spiked with the PAHs standard mix (Vial 6 in Table 5) (Figure 4). Table 6 summarizes the retention time and peak area precision data. Calibration linearity for the determination of PAHs was investigated by making five replicate injections of a mixed standard of PAHs prepared at four different concentrations. The I.S. method was used to calculate the calibration curve and for real sample analysis. Table 7 reports the data from this determination as calculated by Chromeleon CDS software. PAH method detection limits (MDLs) are also listed in Table 7.

Table 5. Preparation of the working standards (oil as matrix).

Vial Number (1.5 mL)	Vial 1	Vial 2		
Volume of 1 µg/mL PAH stock standard solution (µL)	50	100		
Volume of isopropanol (µL)	450	400		
Concentration of PAHs (µg/mL)	0.1	0.2		
Vial Number (1.5 mL)	**Vial 3**	**Vial 4**	**Vial 5**	**Vial 6**
Volume of diluted standard (Vial 1 or Vial 2) or stock standard (µL)	10 µL, Vial 1	10 µL, Vial 2	10 µL, stock standard	20 µL, stock standard
Added weight of the cleaned olive oil used as matrix (containing I.S.) (g)	1.0355	1.0376	1.0389	1.0358
Final concentration of PAHs (µg/kg)	0.956	1.909	9.534	18.943
Final concentration of I.S. (µg/kg)	0.983	0.983	0.983	0.973

Table 6. Reproducibility of retention times (RTs) and peak areas.[a]

PAH	RT RSD	Area RSD
Phenanthrene	0.064	6.733
Anthracene	0.055	4.350
Fluoranthene	0.072	4.491
Pyrene	0.044	4.965
Benzo[a]anthracene	0.031	4.628
Chrysene	0.026	4.469
Benzo[b]fluoranthene	0.027	4.325
Benzo[k]fluoranthene	0.027	4.173
Benzo[a]pyrene	0.031	4.399
Dibenzo[a,h]anthracene	0.041	4.383
Benzo[g,h,i]perylene	0.042	5.038
Indeno[1,2,3-cd]pyrene	0.048	4.484

[a] Seven injections of Olive Oil 1 spiked with 20 µg/kg mixed PAHs standard.

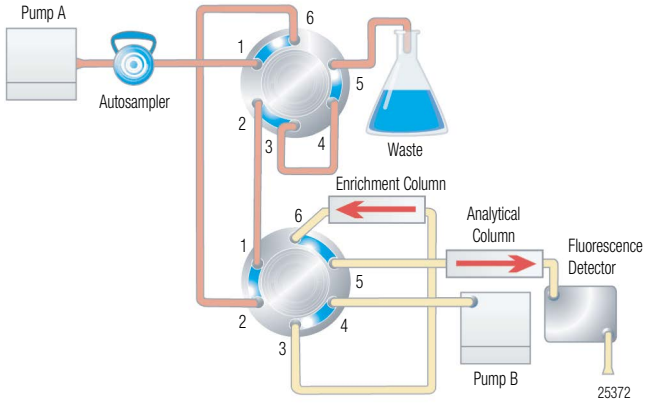

Figure 3. The enrichment column is switched into the analytical flow path, eluting the PAHs onto the analytical column for gradient separation followed by fluorescence detection.

On-Line SPE
Column: DACC (3.0 × 80 mm)
Analytical Columns: Two PAH columns (4.6 × 250 mm) for separation
Eluent: A) water, B) acetonitrile for both loading and analysis pumps, C) isopropanol for loading pump
Flow Rate: 1 mL/min
Inj. Volume: 80 µL
Temperature: 30 °C for column, 40 °C for autosampler

Detection: Fluorescence
Sample: Mixture of PAHs standard

Peaks:	µg/kg
1. Phenanthrene	20
2. Anthracene	20
3. Fluoranthene	20
4. Pyrene	20
5. Benzo[a]anthracene	20
6. Chrysene	20
7. Benzo[b]fluoranthene	20
8. Benzo[k]fluoranthene	20
9. Benzo[a]pyrene	20
10. Dibenzo[a,h]anthracene	20
11. Benzo[g,h,i]perylene	20
12. Indeno[1,2,3-cd]pyrene	20
13. Benzo[b]crysene (added as I.S.)	20

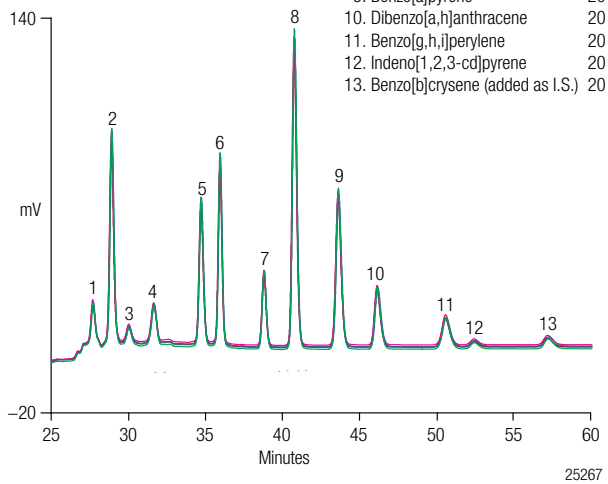

Figure 4. Overlay of chromatograms of seven serial injections of Olive Oil 1 spiked with the PAHs standard mixture (20 µg/kg).

Carryover Performance

Carryover performance for the WPS-3000TSL autosampler was investigated by serial injections of 500 µg/kg of benzo[b]crysene (I.S.) and a purified olive oil sample prepared as a blank. Figure 5 shows exceptional carryover performance with external needle wash by acetonitrile both before and after the injection. There was no cross contamination observed when using the WPS-3000TSL autosampler for this application.

Effect of the Purified Olive Oil Used as Blank and Matrix

One brand of olive oil was prepared as a blank and to serve as a matrix according to the procedure specified above. Figure 6 is an overlay of chromatograms of the original and purified olive oils; this shows that many ingredients were eliminated from the original olive oil. However, impurities persisted in the prepared olive oil used as a blank and matrix, which may have affected determination of some PAHs. To overcome this effect, the baseline of the purified olive oil blank was subtracted during data processing with Chromeleon CDS software.

Table 7. Calibration data for the 12 PAHs.

Phenols	Equations	r (%)	MDL (µg/kg)
Phenanthrene	A = 12.0911 C + 7.4235	99.5173	0.42
Anthracene	A = 53.2837 C + 49.1644	99.1062	0.26
Fluoranthene	A = 4.6993 C + 2.8308	98.0798	1.19
Pyrene	A = 11.0580 C + 11.0016	99.0524	0.69
Benzo[a]anthracene	A = 35.6167 C + 68.1072	98.5246	0.68
Chrysene	A = 44.2503 C + 51.2535	98.6398	0.34
Benzo[b]fluoranthene	A = 19.8706 C + 19.8867	99.0712	0.21
Benzo[k]fluoranthene	A = 89.5111 C + 86.5361	99.0725	0.39
Benzo[a]pyrene	A = 53.4937 C + 48.0755	99.1057	0.75
Dibenzo[a,h]anthracene	A = 22.5211 C + 21.6513	99.1431	0.41
Benzo[g,h,i]perylene	A = 14.7151 C + 13.0643	99.1995	0.58
Indeno[1,2,3-cd]pyrene	A = 2.9058 C + 1.8162	99.4115	0.59

The single-sided Student's *t* test method (at the 99% confidence limit) was used for estimating MDL, where the standard deviation of the peak area of seven injections of Olive Oil 1 spiked with 2 µg/kg mixed PAHs standard is multiplied by 3.14 (at n = 7) to yield the MDL.

On-Line SPE
Column: DACC (3.0 × 80 mm)
Analytical Columns: Two PAH columns (4.6 × 250 mm) for separation
Eluent: A) water
B) acetonitrile for both loading and analysis pumps
C) isopropanol for loading pump

Flow Rate: 1 mL/min
Inj. Volume: 80 µL
Temperature: 30 °C for column, 40 °C for autosampler
Detection: Fluorescence

Peaks:
1. Benzo[b]crysene (added as I.S.)

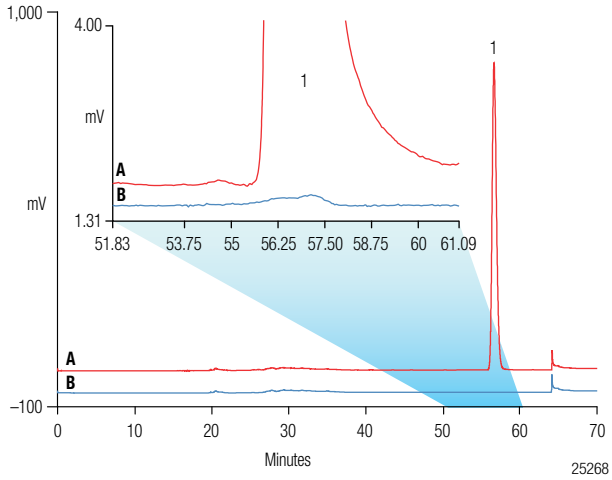

Figure 5. Carryover test on the WPS-3000TSL autosampler. A) Purified olive oil spiked with 500 µg/kg of benzo[b]crysene (I.S.). B) Purified olive oil prepared as a blank, analyzed immediately after A).

On-Line SPE
Column: DACC (3.0 × 80 mm)
Analytical Columns: Two PAH columns (4.6 × 250 mm) for separation
Eluent: A) water
B) acetonitrile for both loading and analysis pumps
C) isopropanol for loading pump

Flow Rate: 1 mL/min
Inj. Volume: 80 µL
Temperature: 30 °C for column, 40 °C for autosampler
Detection: Fluorescence

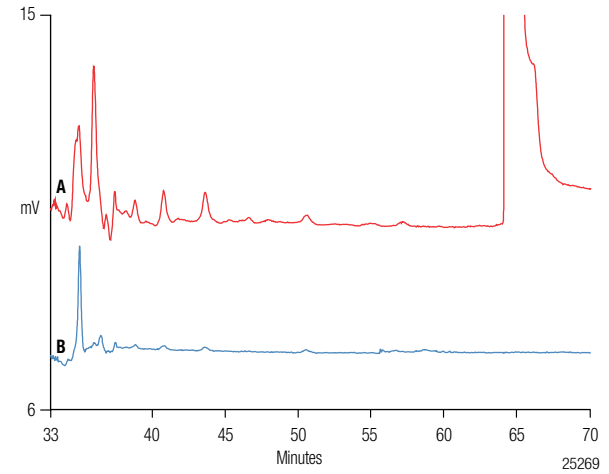

Figure 6. Overlay of chromatograms of A) untreated olive oil and B) purified olive oil used as a blank.

Table 8. Analytical results for Olive Oil 1, Olive Oil 2, and sesame oil.

PAH	Olive Oil 1			Olive Oil 2	Sesame Oil
	Detected (µg/kg)	Added (µg/kg)	Recovery (%)	Detected (µg/kg)	Detected (µg/kg)
Phenanthrene	37	5	120	13.2	52
Anthracene	4.5	5	109	3.2	6.1
Fluoranthene	1.0	5	112	ND	ND
Pyrene	2.2	5	131	1.3	ND
Benzo[a]anthracene	2.8	5	108	2.1	18
Chrysene	4.4	5	110	3.2	5.3
Benzo[b]fluoranthene	ND	5	90	ND	ND
Benzo[k]fluoranthene	ND	5	84	ND	ND
Benzo[a]pyrene	2.7	5	106	2.5	3.9
Dibenzo[a,h]anthracene	ND	5	84	ND	ND
Benzo[g,h,i]perylene	ND	5	70	ND	1.2
Indeno[1,2,3-cd]pyrene	ND	5	82	ND	ND

One sample and one spiked sample were prepared, and two injections of each were made.

Sample Analysis

Two olive oil samples and one sesame oil sample were analyzed. The results are summarized in Table 8. Figure 7 shows chromatograms of the oil samples. Spike recoveries for these PAHs were in the range from 70 to 131%. Some PAHs were found in the edible oil samples. Five PAHs—phenanthrene, anthracene, benzo[a]anthracene, chrysene, and benzo[a]pyrene—existed in all of the three samples and phenanthrene was obviously the most abundant PAH.

Ruggedness of the SPE Column

The tolerance of the SPE column used in this on-line analysis of PAHs in edible oils was investigated by comparing the separation of PAHs using two different SPE columns. One of these columns already had extracted over 600 injections of an edible oil sample; the other was nearly new. Figure 8 shows an overlay of chromatograms of PAHs using these two SPE columns. Final results of the PAH analyses are very similar, despite the different exposure levels of the two columns.

On-Line SPE
Column: DACC (3.0 × 80 mm)
Analytical Columns: Two PAH columns (4.6 × 250 mm) for separation
Eluent: A) water
B) acetonitrile for both loading and analysis pumps
C) isopropanol for loading pump
Flow Rate: 1 mL/min
Inj. Volume: 80 µL
Temperature: 30 °C for column, 40 °C for autosampler
Detection: Fluorescence

Peaks:
1. Phenanthrene
4. Pyrene
5. Benzo[a]anthracene
6. Chrysene
7. Benzo[b]fluoranthene
8. Benzo[k]fluoranthene
9. Benzo[a]pyrene
11. Benzo[g,h,i]perylene
12. Indeno[1,2,3-cd]pyrene

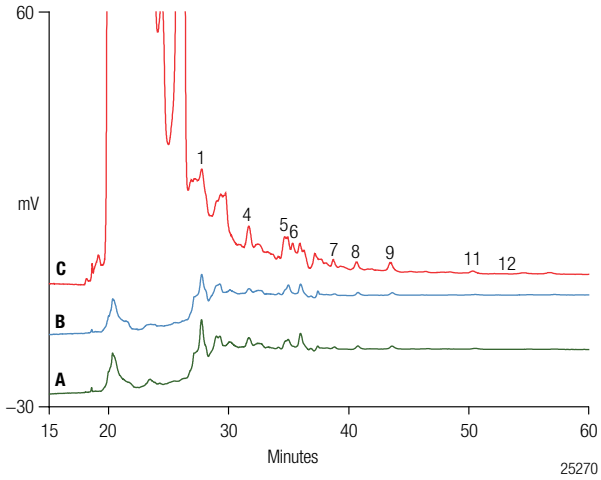

Figure 7. Overlay of chromatograms of A) Olive Oil 1, B) Olive Oil 2, and C) sesame oil samples.

On-Line SPE
Column: DACC (3.0 × 80 mm)
Analytical Columns: Two PAH columns (4.6 × 250 mm) for separation
Eluent: A) water
B) acetonitrile for both loading and analysis pumps
C) isopropanol for loading pump
Flow Rate: 1 mL/min
Inj. Volume: 80 µL
Temperature: 30 °C for column, 40 °C for autosampler
Detection: Fluorescence
Sample: Mixture of PAHs standard

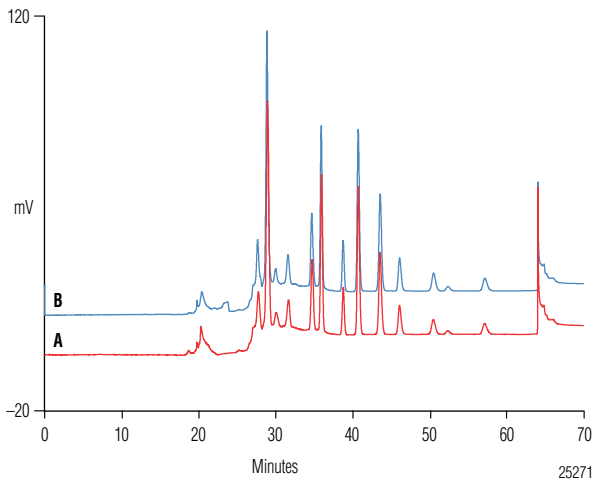

Figure 8. Separation of PAHs in olive oil using different SPE columns. A) SPE column with 600 prior injections; B) new SPE column.

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