

Determination of Polycyclic Aromatic Hydrocarbons (PAHs) in Edible Oils by Donor-Acceptor Complex Chromatography (DACC)-HPLC with Fluorescence Detection

INTRODUCTION

Numerous polycyclic aromatic hydrocarbons (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 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 DMSO extraction followed by three extractions with cyclohexanone and cleanup with a silica column.⁸ Another study of six edible oils used solid-phase extraction, but required solvent extraction steps before SPE and evaporation afterward.⁹ These manual steps consume solvent, resources, and time.

In recent years, donor-acceptor complex chromatography (DACC) has gained popularity for PAH analysis.¹⁰⁻¹³ DACC stationary phases can be used for solid phase extraction (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, and therefore still requires labor and is prone to errors.

In 1996, Van Stijn et al.¹² developed an automated process for oil sample preparation and analysis. The setup 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 know-how 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.

Maio et al.¹³ 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.

This application note describes the setup and method for determining PAHs in edible oils on-line using a Dionex UltiMate® 3000 ×2 Dual-Gradient HPLC platform, based on the solution described by Maio,¹³ and evaluates the method performance (i.e. linearities, detection limits, reproducibilities, recoveries, and carry over of autosampler).

EQUIPMENT

UltiMate 3000 ×2 Dual system consisting of:

DPG-3600A pump with SRD-3600 Air Solvent Rack

WPS-3000TSL autosampler

TCC-3200 thermostatted column compartment with two 2p-6p valves

RF2000 fluorescence detector

Chromeleon® 6.80 SP1 Chromatography Workstation

Device configurations for the online DACC cleanup with analytical HPLC are as shown in Figures 1 to 3. In these figures, the upper valve is the right valve and lower valve is the left valve in the TCC-3200.¹³

REAGENT AND STANDARDS

Deionized water from a Milli-Q® Gradient A10

Acetonitrile (CH₃CN), HPLC grade (Fisher Scientific)

Isopropanol, HPLC grade, (Fisher Scientific)

Charcoal, activated granular (activated carbon), chemical pure grade (Shanghai Chemical Reagent Company)

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 (Restek)

Benzo[b]chrysene, 50 µg/mL, used as an internal standard (I.S.) (AccuStandard)

SAMPLES

Two brands of olive oil (olive oil 1 and 2 from Italy and Spain, respectively), and one brand of sesame oil (from China)

CONDITIONS

Analytical

Columns: Two SUPELCOSIL™ LC-PAH columns, 4.6 × 250 mm (Supelco Cat.# 58229)

On-Line SPE

Column: ChromSpher Pi, 3.0 × 80 mm (Varian P/N: CP28159)

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 solid-phase extraction (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	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	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 RF2000 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

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, Millex™-LCR, Millipore) 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 about 10 g of the purified olive oil used as blank and matrix. The concentration of I.S. in the oil matrix is about 1 µg/kg. In the work presented in this application note, 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. The stock standard solution is used to prepare working standards as described in Table 5.

Table 5. Preparation of the Working Standards (Oil as Matrix)				
Vial # (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 # (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

Edible Oil Sample Preparation

Prior to injection, filter oil through a 0.45- μm membrane (PTFE, Millex-LCR, Millipore).

RESULTS AND DISCUSSION

Description of the On-Line DACC-HPLC Method

The flow scheme, shown in 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, the right valve switches to flush out the oils and IPA, 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).

Reproducibility, Detection Limits, and Linearity

Method reproducibility was estimated by making seven replicate injections of olive oil sample 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 internal standard 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 software. PAH method detection limits (MDLs) are also listed in Table 7.

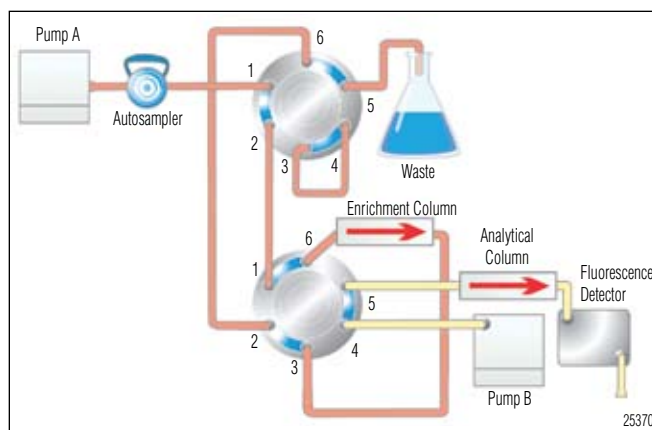


Figure 1. Flow scheme for on-line sample preparation and analysis. The valves are positioned for injection of the sample on the enrichment column.

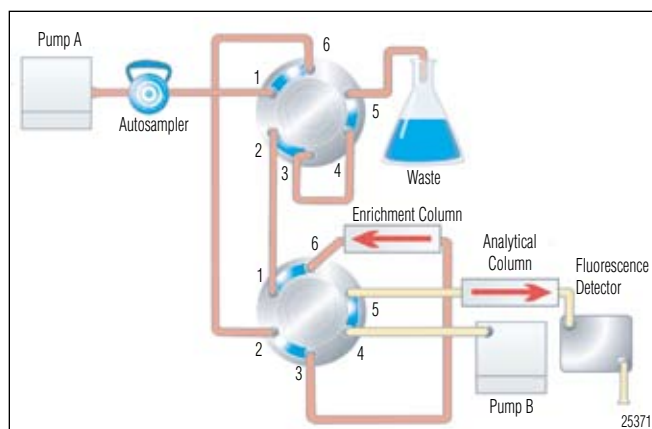


Figure 2. Isopropanol is flushed out of the enrichment column in backflow mode.

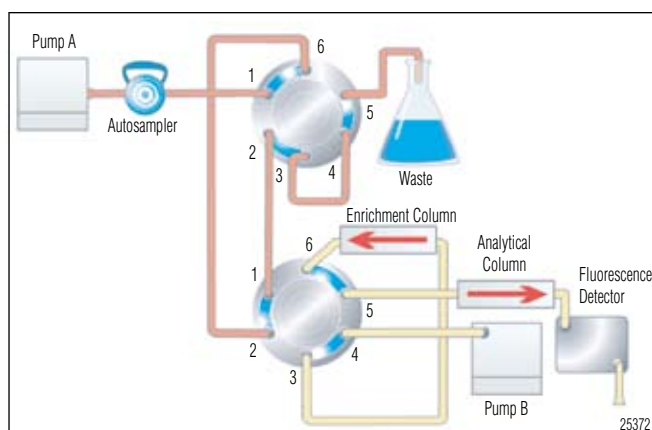


Figure 3. The enrichment column is switched into the analytical flow path, eluting the PAHs onto the analytical column for gradient separation followed by fluorescence detection.

Table 6. Reproducibility of Retention Times 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

^aSeven injections of olive oil sample 1 spiked with 20 µg/kg mixed PAH standards.

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 test method (at the 99% confidence limit) was used for estimating MDL, where the standard deviation (SD) of the peak area of seven injections of olive oil sample 1 spiked with 2 µg/kg mixed PAHs standard is multiplied by 3.14 (at n = 7) to yield the MDL.

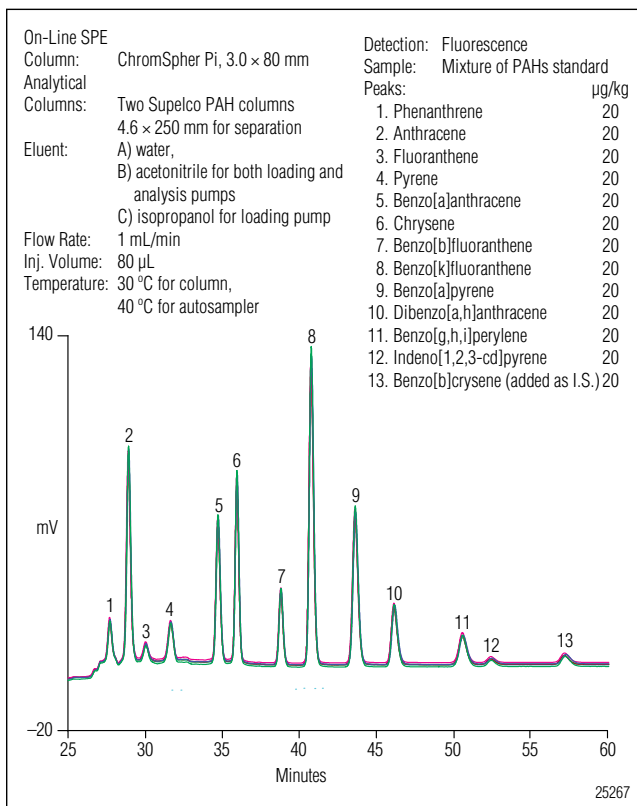


Figure 4. Overlay of chromatograms of seven serial injections of olive oil sample 1 spiked with a PAH standard mixture (20 µg/kg).

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.

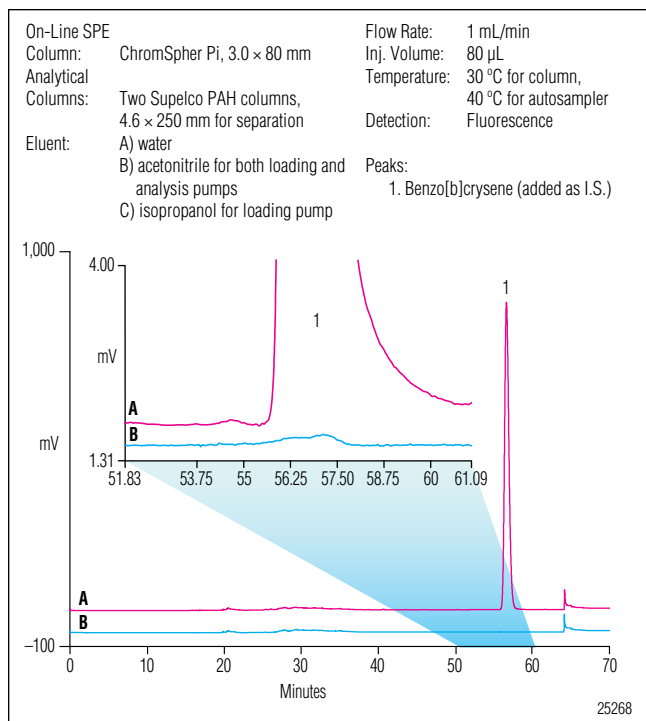


Figure 5. Carryover test on the WPS-3000 autosampler. A) Purified olive oil spiked with 500 µg/kg of benzo[b]crysene (I.S.). B) Purified olive oil prepared as blank, analyzed immediately after A).

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, from which we observe 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 might 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 software.

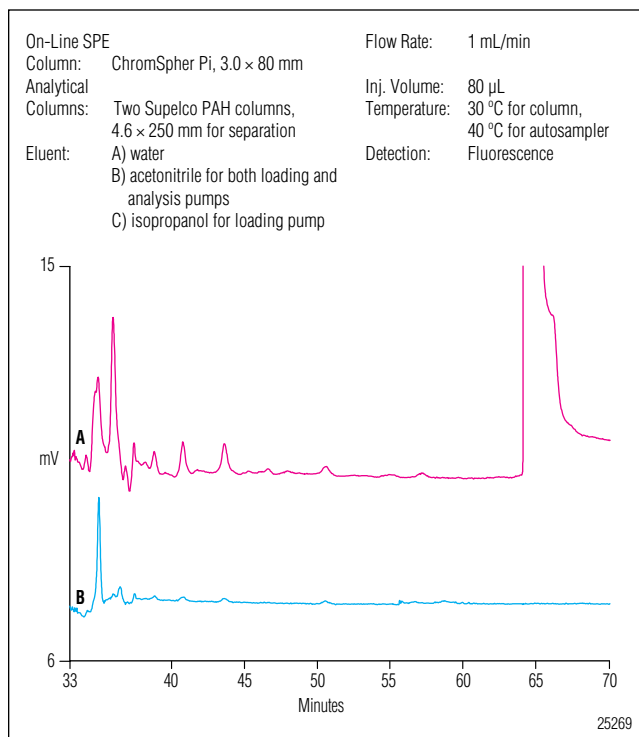


Figure 6. Overlay of chromatograms of A) untreated olive oil, and B) purified olive oil used as a blank.

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 ranges 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.

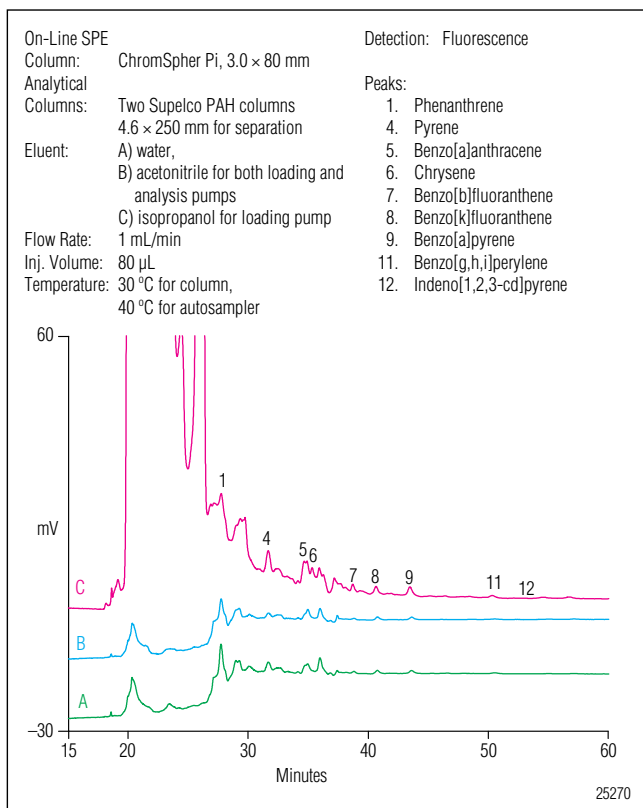


Figure 7. Overlay of chromatograms of A) olive oil 1, B) olive oil 2, and C) sesame oil samples.

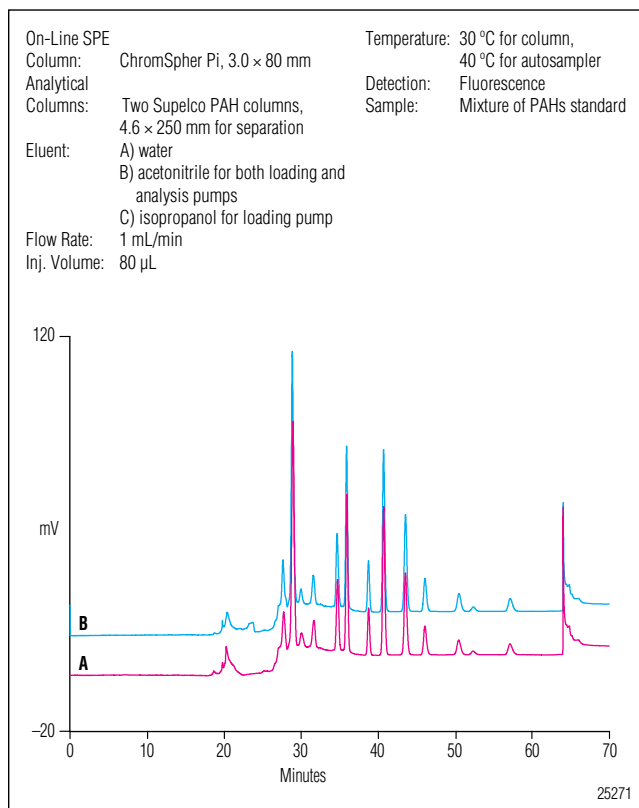


Figure 8. Separation of PAHs in olive oil using different SPE columns. A) SPE column with 600 prior injections; B) new SPE column.

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.

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.

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.

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Dionex Corporation

1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

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U.S. (847) 295-7500
Canada (905) 844-9650

South America

Brazil (55) 11 3731 5140

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