Determination of Benzo[a]pyrene at low-levels in Olive Oil

Walter B. Wilson[a], Jacolin A. Murray[a], and Blaza Toman[b]

[a] *Chemical Sciences Division, Material Measurement Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, USA*

[b] *Statistical Engineering Division, Information Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, USA*

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are an important class of environmental pollutants originating from a wide variety of natural and anthropogenic sources. Studies have shown that exposure to PAHs can lead to an increased risk of cancer and as such, PAHs are classified as potential carcinogens and makes their presence in foods a health concern. Edible oils and fats are potentially a significant source of PAHs in the human diet due to their lipophilic nature and prevalence in food. Edible oils are susceptible to contamination by various routes including environmental pollution of the raw vegetable material and/or industrial processing steps prior to refining such as seed drying with smoke and solvent extraction. Benzo[a]pyrene (BaP) is one of the markers for the occurrence of PAHs in foods, for which regulatory limits around the world have been set in edible oils to be from 2.0 µg/kg to 10 µg/kg. The determination of PAHs in lipids is difficult due to low concentration levels, the complexity of extraction and clean-up procedures, the need for good chromatographic resolution, and the need for sensitive detectors. Several methods of extraction and clean-up of PAHs from edible oils have been developed, which typically consist of a solvent extraction procedure followed by solid-phase extraction or column chromatography. PAH determination is often carried out by reversed phase liquid chromatography with fluorescence detection (RPLC/FL) or gas chromatography/mass spectrometry (GC/MS). In this study, a new analytical method combining size-exclusion chromatography (SEC) fractionation and RPLC/FL for the determination of BaP in two olive oil samples. The new SEC-RPLC/FL method was compared to a traditional extraction method combined with GC/MS. Both methods provided accurate and precise results; however, the SEC-RPLC/FL allowed for shorter sample preparation times, analysis times, and no requirements of solid-phase extractions.

Materials and Methods

1. Materials and Reagents

Olive oil samples were purchased from a local supermarkets in Gaithersburg, Maryland. SRM 2260a (calibration solution) and SRM 2270 (Internal Standard Mix) were obtained from the Office of SRMs at NIST. HPLC grade water (H_2O), acetonitrile (ACN), dichloromethane (DCM), and n-hexane were purchased from Fisher Scientific, MilliporeSigma, and/or J.T. Baker.

2. Sample Preparation

The two olive oil samples were spiked with SRM 2260a to have a mass fraction value of ≈ 2 µg/kg for BaP and homogenized by vibro-mixing at room temperature for 24 h. Samples were stored in the dark at room temperature prior to analysis. Stock calibration solutions of SRM 2260a or SRM 2270 were prepared in HPLC grade *n*-hexane and kept in the dark at 4 °C. Working solutions containing both SRM solutions were prepared gravimetrically of stock solutions in *n*-hexane. Approximately 100 µL aliquots of the working internal standard solution was mixed with the olive oil sample through sonication for 30 s. The olive oil samples were allowed to settle for 5 min to remove any bubbles formed during the mixing procedure. The olive oil sample was split into four subsamples for analysis.

3. Sample Clean-up via SEC Fractionation

SEC fractionation was performed using a Varian 9012 Solvent Delivery System coupled to Jasco UV-1570 Intelligent UV-vis detector. Samples were fractionated using a semi-prep PLGel column purchased from NETA Scientific Inc. and manufactured by Agilent Technologies with the following characteristics: 30 cm length, 25 mm diameter, 10 µm average particular diameter, and 100 Å pore size. Approximately 500 µL of olive oil was injected into the SEC system and processed through the semi-prep column with a mobile phase of 100 % dichloromethane (DCM) at a flowrate of 10.0 mL/min. The PAH fractions were collected, combined, and evaporated to near dryness under nitrogen. Additional n-hexane (2 mL) was added to the sample and evaporated further to ≈ 250 µL and transferred to an autosampler vial.

4. Reversed-Phase Liquid Chromatography with Fluorescence Detection

The RPLC/FL measurements were performed on an Dionex Ultimate 3000 LC system equipped with a pump, autosampler, column compartment, diode array detector, and FL detector. Separations were carried out on a polymeric (Zorbax Eclipse PAH) C_{18} column purchased from Agilent with the following characteristics: 25 cm length, 4.6 mm diameter, and 5 µm average particle diameter. Separation and detection was achieved using flowrate of 1.5 mL/min, column temperature of 25 °C, and the mobile gradient listed in Table 1. All sample injections were held constant at 2 µL. FL spectra were recorded using a stop-flow function on the instrument at the apex of each chromatographic peak.

Table 1: RPLC/FL operating conditions for BaP measurements in olive oil.

Time	H_2O (%)	ACN (%)	Excitation (nm)	Emission (nm)
0.0	50	50	404	440
3.0	50	50		
23.0	0	100		
24.6			290	411
40.0	0	100		

5. Gas Chromatography – Mass Spectrometry

The GC/MS measurements were performed on an Agilent HP 6890 Series GC system coupled to a quadrupole mass spectrometer with electron ionization (HP 5973 MSD, Agilent). Separations were carried out on a 50 % phenyl GC stationary phase (SLB-PAHms) purchased from MilliporeSigma (Bellefonte, PA) with the following characteristics: 60 m x 0.25 mm i.d. x 0.25 µm film thickness. The chromatographic conditions for analysis included an ultra-high purity helium gas flow rate of 1.1 mL/min, transfer line temperature of 325 °C, ion source temperature of 230 °C, quadrupole temperature of 150 °C, and the oven temperature program described in Table 1. All sample injections were on-column and held constant at 1 µL. A 5 m guard column from Restek was used for all analysis.

Table 2: GC/MS operating conditions for BaP measurements in olive oil.

Time	Temperature (°C)	Ramp (°C/min)
0.0	70	0.0
1.0	70	30.0
3.7	150	0.0
6.7	150	2.0
86.7	310	0.0
146.7	310	0.0

Results and Discussion

1. RPLC/FL Analysis of BaP and six MM 252 PAH isomers in SRM 2260a

Previous studies at NIST developed a RPLC/UV method using a Pinnacle II PAH column (250 mm x 4.6 mm) with a 5 µm particle size for the certification of SRM 1647e. The mobile phase gradient elution program was the following: 3 min hold at 50 % H_2O/ACN; linear gradient to 100 % ACN over 15 min; and hold for 15 min. The new RPLC/FL method established here for BaP and six MM 252 PAH isomers used a different column with the same dimensions and a similar mobile phase gradient (Table 1). The fluorescence wavelengths selected in this study were based on the fluorescence spectra (Figure 1) recorded using the stop-flow function of the RPLC system.

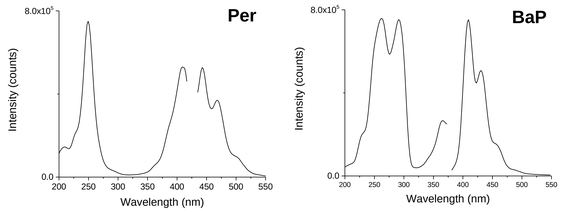

Figure 1: Stop-flow fluorescence spectra.

Quantitative analysis of PAHs in complex samples via RPLC/FL is normally based on the internal standard calibration method to account for variations in detection response and injection irreproducibility. Isotopically labeled internal standards are preferred for quantitation and readily available. Two solution SRMs are available with perdeuterated PAHs for use as internal standards (SRM 2269 and SRM 2270).

SRM 2270 contains six isotopically labeled PAHs with a range of MM: naphthalene-d_8 (136 g/mol), acenaphthene-d_{10} (164 g/mol), pyrene-d_{10} (212 g/mol), BaP-d_{12} (252 g/mol), perylene-d_{12} (Per-d_{12}, 252 g/mol), and benzo[ghi]perylene-d_{12} (288 g/mol). SRM 2270 is prepared in *n*-hexane/toluene mixture (96:4 volume fraction) and is miscible with the calibration solution SRM 2260a.

The target PAH in the olive oil samples is BaP with a MM 252 g/mol, so the internal standard of choice could be either BaP-d_{12} or Per-d_{12}., which have identical fluorescence spectra to their parent PAHs shown in Figure 1. The RPLC/FL chromatogram obtained for SRM 2270 and for a mixture of SRM 2270/SRM 2260a are shown in Figure 2.

Of the 35 PAHs in SRM 2260a, six isomers of BaP are present in the solution: Per, benzo[*e*]pyrene (BeP), benzo[*a*]fluoranthene (BaF), benzo[*b*]fluoranthene (BbF), and benzo[*k*]fluoranthene (BkF). Per and Per-d_{12} have significantly different fluorescence profiles than the co-eluting MM 252 isomers with maximum excitation and emission wavelengths of 406 nm and 440 nm, respectively. Under these fluorescence conditions for the first 24.6 min, the four MM 252 isomers in red are not detectable as shown in Figure 2.

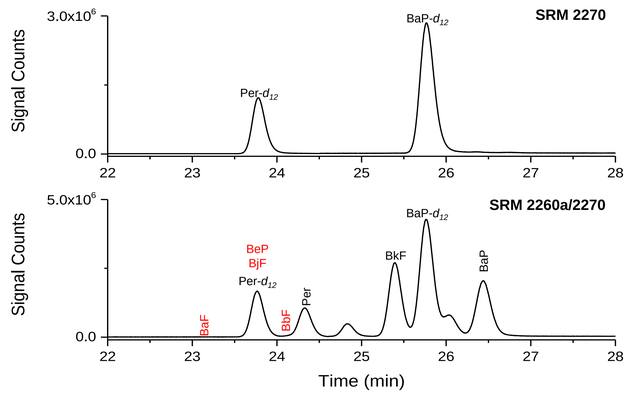

Figure 2: RPLC/FL chromatograms obtained using the separation conditions in Table 1. The red labels refer to the four additional MM 252 PAHs present in SRM 2260a.

2. Determination of BaP in Olive Oil Samples via SEC-RPLC/FL

Previous work completed at NIST has used SEC fractionation to isolate PAHs and polychlorinated biphenyl congeners from complex fatty matrixes prior to GC/MS analysis. SEC fractionation removes most of the lipid and biogenic material in the mussel tissue samples and fish oil samples. More recently, Drabov and co-workers studied the potential use of SEC for the isolation of PAHs in olive oil samples with no prior sample extraction. These studies suggested that SEC was insufficient to adequately clean-up olive oil samples and a silica gel solid-phase extraction (SPE) step was implemented to remove any remaining interferences prior to GC/MS measurements.

Within this context, the current study evaluated the use of a single SEC fractionation step to clean up the olive oil sample prior to RPLC/FL measurements. The SEC fractionation chromatogram obtained for one olive oil sample spiked with SRM 2260a and SRM 2770 is shown in Figure 3. PAHs were collected between the time interval of 9.5 to 11.5 min. The SEC fractionation procedure was repeated four times, fractions were combined, and evaporated to near dryness for analysis via RPLC/FL. The RPLC/FL chromatograms for a calibrant, blank olive oil sample, and spiked olive oil sample are shown in Figure 4. The stop-flow fluorescence spectra of BaP and Per in Figure 1 were used to confirm peak identity in the calibrants and spiked olive oil samples.

A summary of the determined BaP mass fraction values for the SEC-RPLC/FL method is reported in Table 3. The determined mass fraction values for BaP in the olive oil samples is in good agreement with the gravimetric value and GC/MS data not shown here for a conventional extraction procedure. However, the mass fraction values were significantly higher when Per-d_{12} was used as the internal standard. The significantly different mass fraction values suggest that changes in the labeled internal standards occur during sample processing. If Per-d_{12} is not fully recovered during cleanup (*i.e.*, relative to the recovery of BaP), this would positively bias the unknown compared with full recovery, which is assumed for BaP-d_{12}. To illustrate, the Per mass fraction values were determined in the same spiked oil samples to be almost identical to the gravimetric value (1.85 ± 0.04 µg/kg and 1.87 ± 0.03 µg/kg, respectively).

SEC-GC/MS was utilized to investigate these results because the seven MM 252 isomers are baseline resolved using a 50 % phenyl stationary phase. The MS sensitivity degraded immediately after the first SEC fraction injection as shown in Figure 5. The lost in sensitivity can be attributed to the oil sample not being cleaned up enough prior to analysis indicating the need for an additional SPE step for GC/MS analysis.

Table 3: Mass Fraction values for spiked olive oil samples.

	Gravimetric (µg/kg)	SEC-RPLC/FL (µg/kg)
Sample 1	1.99	2.07 ± 0.26
Sample 2	2.54	2.30 ± 0.17

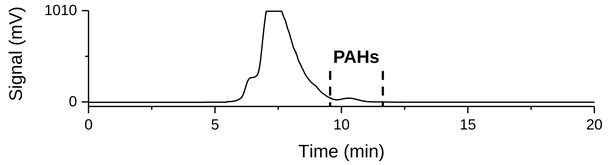

Figure 3: SEC fractionation chromatogram of spiked olive oil sample.

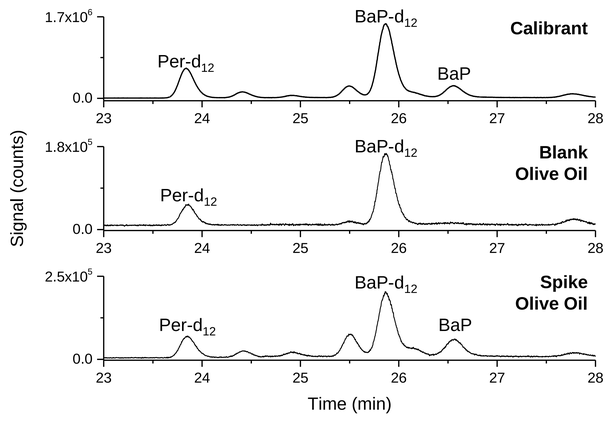

Figure 4: RPLC/FL chromatograms obtained for the calibrant, blank olive oil, and spiked olive oil samples after SEC fractionation.

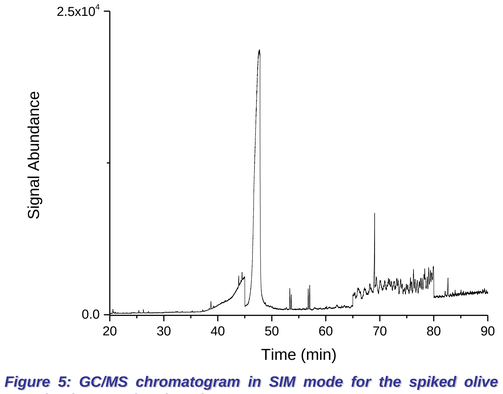

Figure 5: GC/MS chromatogram in SIM mode for the spiked olive oil sample after SEC fractionation.

Conclusion

The results presented here demonstrate the ability to use SEC fractionation prior to RPLC/FL analysis. The SEC-RPLC/FL method allowed for the quantitation of BaP in olive oil samples at very low concentration levels.