Application Note 1093

Determination of Benzo(a)pyrene in Sausage and Preserved Ham

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Key Words
PAHs, On-Line SPE, HPLC, Fluorescence Detection, Food Safety Analysis

Goal
To develop an efficient high-performance liquid chromatography (HPLC) method for sensitive and rapid determination of benzo(a)pyrene in meat products such as sausage and preserved ham using on-line solid-phase extraction (SPE) for sample preparation instead of the commonly used off-line SPE method

Introduction
Benzo(a)pyrene (structure shown in Figure 1) is a polycyclic aromatic hydrocarbon (PAH) compound formed from the incomplete combustion of organic matter. Due to its potential carcinogenic and mutagenic properties, most countries have regulations limiting its concentration in drinking water, food additives, cosmetics, workplaces, and factory emissions. Some samples such as sausage and preserved ham have too complex a matrix that interferes with the separation of benzo(a)pyrene, thus preventing use of a routine HPLC method and making benzo(a)pyrene quantification difficult. Therefore, sample preparation steps—including extraction and extract cleanup—are very important for the determination of benzo(a)pyrene by HPLC.

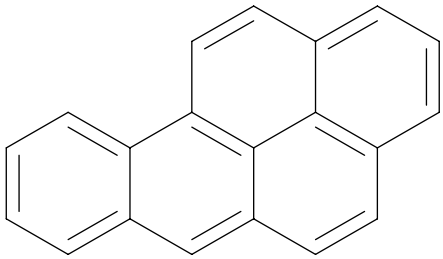

Figure 1. Structure of benzo(a)pyrene.

Liquid-liquid extraction and off-line SPE are among the commonly used sample preparation techniques for meat products and have been applied to many regulatory methods (Table 1).[1-3]

Table 1. Regulations for benzo(a)pyrene analysis required by the China Institute of Standardization (CNIS) in the People's Republic of China.

Regulation	Sample	Pretreatment	Determination Method	MDL* (µg/kg)
NY/T 1666-2008[1]	Meat (Barbecue, Smoked)	Cyclohexane Extraction and Liquid-Liquid Extraction	HPLC with Fluorescence Detection	0.5
SC/T 3041-2008[2]	Aquatic Products	Saponification, *N*-Hexane Extraction, and Off-Line SPE Purification	HPLC with Fluorescence Detection	—
GB/T 5009.27-2003[3]	Food (Meat)	Hexane Extraction and Off-Line SPE Purification	Fluorospectrophotometry	1

*Method detection limit

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Thermo Scientific Application Notes (ANs) 196, 213, and 1085 provide on-line SPE HPLC methods to quantify low concentrations of PAHs in oil and water samples.[4-6] The work shown here provides a sensitive and rapid HPLC method with on-line SPE and fluorescence detection for the determination of benzo(a)pyrene in sausage and preserved ham. This method reduces costs associated with the SPE cartridge, labor, time, and reagents; and results are more consistent because the cleanup step is performed by on-line SPE rather than manual off-line SPE.

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 Dual Rapid Separation LC (RSLC) system, including:
  - DGP-3600RS Dual Gradient Rapid Separation Pump (P/N 5040.0066)
  - SRD-3600 Integrated Solvent and Degasser Rack (P/N 5035.9230)
  - WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted (P/N 5840.0020), equipped with a 100 µL sample loop and a 25 µL syringe
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.000), equipped with one 2–6p valve
  - FLD-3400RS Rapid Separation Fluorescence Detector (P/N 5078.0025)
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) software, version 7.1 or above
- Thermo Scientific™ Sorvall™ ST16 Centrifuge (P/N 75004240)
- RV 10 Basic Rotary Evaporator with Dry Ice Condenser (IKA P/N 8031001)
- T 18 Digital ULTRA-TURRAX® Disperser (IKA P/N 3720000)

Consumables

Thermo Scientific™ Target2™ Polypropylene Syringe Filters (0.45 µm, 30 mm, P/N F2502-9)

Reagents and Standards

- Deionized (DI) water, 18.2 M -cm resistivity, generated by the Thermo Scientific™ Barnstead™ GenPure™ Pro UV-TOC (total organic carbon) Water Purification System (P/N 50131948)
- Methanol, 99.8%, HPLC Grade (Fisher Scientific P/N AC610090040)
- Acetonitrile, HPLC Grade (Fisher Scientific P/N AC610010040)
- Ethyl Acetate, HPLC Grade (Fisher Scientific P/N E195-4)
- Sodium Sulfate Anhydrous, Powder (Fisher Scientific P/N S429-500)
- Benzo(a)pyrene, 99.6% (Dr. Ehrenstorfer GmBH P/N 20635000)

Conditions

For On-Line SPE

Column:	Thermo Scientific™ Acclaim™ 120, C8, 5 µm Analytical, 4.6 × 50 mm (P/N 059138)
Mobile Phase:	A. Acetonitrile B. Water C. Ethyl acetate
Gradient:	Table 2
Flow Rate:	1.0 mL/min
Injection Volume:	10 µL on the on-line SPE column

Table 2. Gradient program for loading pump.

Time (min)	% B (Water)	% C (Ethyl Acetate)	Valve Position
0	20	0	1_2
0.3	20	0	6_1
1.8	20	0	1_2
2.0	20	0	1_2
2.5	0	75	1_2
11.5	0	75	1_2
12	20	0	1_2
15	20	0	1_2

For Separation

Column:	Acclaim Phenyl-1, 3 µm Analytical, 4.6 × 150 mm (P/N 071969)*
Mobile Phase:	Acetonitrile:Water (8:2, v/v)
Flow Rate:	1.0 mL/min
Temperature:	35 °C
Detection:	Fluorescence
Excitation:	290 nm
Emission:	410 nm
Response Time:	2.0 s
Data Collection Rate:	5.0 Hz
Sensitivity:	1

*The Thermo Scientific™ Hypersil™ Green PAH Column, 3 µm, 3.0 × 150 mm (P/N 31103-153030) can be used as a substitute.

Preparation of Standard Solutions

Stock Standard Solution 1

Dissolve ~12.5 mg of benzo(a)pyrene in 50 mL of methanol in a 250 mL volumetric flask and dilute to the mark with methanol. The final concentration of benzo(a)pyrene will be 50 mg/L.

Stock Standard Solution 2

Add 1 mL of Stock Standard Solution 1 to a 50 mL volumetric flask and dilute to the mark with methanol. The final concentration of benzo(a)pyrene will be 1 mg/L.

Table 3. Preparation of calibration standards.

Stock Standard Solution of Benzo(a)pyrene	Volume of Stock Standard Solution (µL)	Volume of Methanol (mL)	Final Volume of Calibration Standard (mL)	Final Concn of Calibration Standard (µg/L)
Stock Standard Solution 2 (1 mg/L)	500	9.5	10.0	50
	100	9.9		10
	50	9.95		5
Stock Standard Solution 3 (20 µg/L)	500	9.5		1
	250	9.75		0.5
	50	9.95		0.1

Stock Standard Solution 3

Add 1 mL of Stock Standard Solution 2 to a 50 mL volumetric flask and dilute to the mark with methanol. The final concentration of benzo(a)pyrene will be 20 µg/L.

Working Standard Solutions for Calibration

For calibration, prepare six working standard solutions with different concentrations by diluting the proper amounts of Stock Standards 2 and 3 with methanol. The volumes of each solution needed to make the calibration standards are shown in Table 3.

Sample Preparation

Two kinds of meat product samples, sausage and preserved ham, were purchased from a supermarket in Shanghai, China.

Add 20 mL of ethyl acetate to 2 g of anhydrous sodium sulfate and 2 g of mashed meat product sample in a 50 mL centrifuge tube. Mix for 1 min to extract, centrifuge the extract for 2 min at 8000 rpm, remove the supernatant, then add 20 mL of ethyl acetate to the residue and extract a second time in the same manner. Combine the two supernatants (total volume ~40 mL) and use rotary evaporation at 55 °C to condense the volume to 2 mL. Transfer the condensed solution to a 10 mL volumetric flask and dilute to the mark with ethyl acetate. Prior to injection, filter the solution through a 0.45 µm filter.

Results and Discussion

Optimization of Sample Preparation

Four solvents with different polarities—acetonitrile, isopropanol, ethyl acetate, and *n*-hexane—were evaluated as extraction solvents for extracting benzo(a)pyrene from a spiked sausage sample. The method recoveries of benzo(a)pyrene obtained using acetonitrile and isopropanol were less than 50% and, therefore, were not further pursued. When using *n*-hexane—a solvent with low polarity—more fat can be extracted from the sample, which can reduce the efficiency of on-line SPE as well as the precision of the method. Using ethyl acetate as the extraction solvent, the average recovery of benzo(a)pyrene was good (98%), obtained by spiking five preserved ham samples with 5 µg/kg benzo(a)pyrene, using one injection for each spiked sample. The peak area RSD was 2.40 and there was a time savings of approximately 5 min, compared to using *n*-hexane in the SPE column regeneration. Therefore, ethyl acetate was chosen as the extraction solvent.

Note: To dehydrate the sample during sample preparation, anhydrous sodium sulfate was added, which can reduce the extraction of high-polarity compounds.

Evaluation of On-Line SPE

Figure 2 shows a typical flow schematic of the on-line SPE column directly coupled to the HPLC column using one 6-port (2–6p) valve. The filtered sample is directly injected onto the system and delivered to the SPE column for enrichment (1_2 position) using one pump of the dual-pump module (labeled For On-Line SPE); the analytical column is simultaneously equilibrated using the other pump of the dual-pump module (labeled For Separation). After analytes are bound to the SPE column and impurities washed out, the SPE column is switched into the analytical flow path (6_1 position) to elute bound analytes separated on the analytical column and detected by the fluorescence detector. This method can be easily performed using an UltiMate 3000 Dual RSLC system.

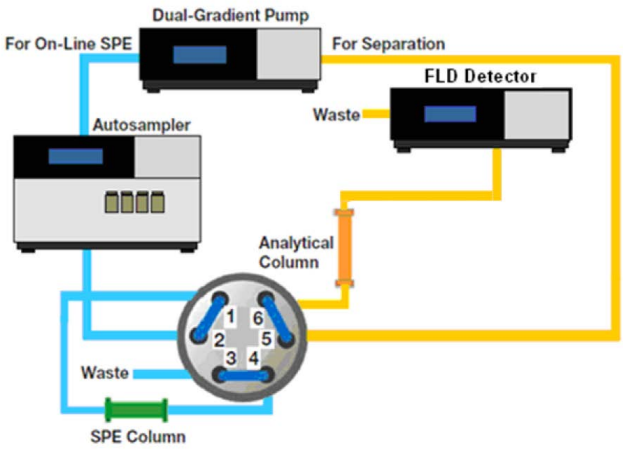

Figure 2. Flow schematic of on-line SPE.

Selection of On-Line SPE and Analytical Columns

As discussed in previous work (ANs 196, 213, and 1085), the Acclaim PolarAdvantage II column is a good choice for on-line SPE preconcentration of large volumes of 100% aqueous samples.[4-6] However, the Acclaim 120 C8 column was chosen as the on-line SPE column for this study because the meat samples have high fat content and fat can be easily eluted from this column during its regeneration.

Baseline separation of 20 PAHs (including the 16 PAHs specified in U.S. Environmental Protection Agency [EPA] Methods 550, 550.1, and 610) in a water sample can be accomplished using the Hypersil Green PAH column that features a specially tailored alkyl-bonded silica with high carbon loading.[7-9] However, the Acclaim Phenyl-1 column, based on covalent modification of silica particles with silane ligand-bearing proprietary alkyl aromatic functionality, can yield good chromatography for a single PAH (e.g., benzo(a)pyrene). As shown in Figure 3, using a combination of the Acclaim 120 C8 column for on-line SPE and the Acclaim Phenyl-1 column for separation, on-line SPE followed by HPLC of benzo(a)pyrene can be accomplished in 15 min. Therefore, the Acclaim Phenyl-1 column was used for the work shown here.

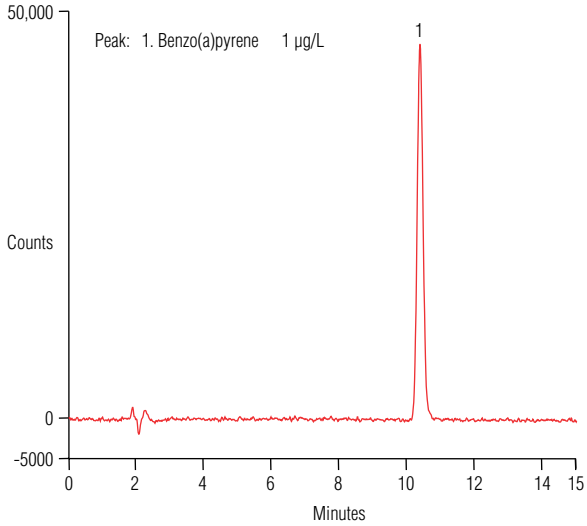

Figure 3. On-line SPE-HPLC determination of benzo(a)pyrene using a combination of the Acclaim 120 C8 and Phenyl-1 columns.

Reproducibility, Linearity, and Detection Limits

Method reproducibility was estimated by making five consecutive injections of a calibration standard with a concentration of 1 µg/L benzo(a)pyrene. The retention time reproducibility RSD is 0.04 and peak area reproducibility RSD is 0.96, demonstrating good short-term precision for this on-line SPE HPLC method.

Sample preparation reproducibility was estimated by spiking five preserved ham samples with 5 µg/kg benzo(a)pyrene, using one injection for each spiked sample. The retention time reproducibility RSD was 0.07 and peak area reproducibility RSD was 2.40, demonstrating good sample preparation reproducibility.

Calibration linearity for fluorescence detection of benzo(a)pyrene was investigated by making five consecutive 10 µL injections of a standard prepared at six different concentrations (i.e., 30 total injections). Linearity was observed from 0.1 to 50 µg/L when plotting concentration versus peak area. The linear regression equation was $A = 8254c + 208.3$, where A represents peak area and c represents concentration of analyte. The coefficient of determination was 1.000. This calibration curve was used to quantify benzo(a)pyrene in sausage and preserved ham samples.

Five replicate injections of a benzo(a)pyrene standard with a concentration of 0.1 µg/L were used for estimating the MDL using a signal-to-noise ratio = 3. The MDL of benzo(a)pyrene was 0.02 µg/L.

Analysis of Meat Product Samples

Table 4 summarizes the results of analyzing sausage and preserved ham samples. Benzo(a)pyrene was found in four of the samples. However, no benzo(a)pyrene was found in any of the meat samples when using the fluorescence spectrophotometric detection method described in GB/T 5009.27-2003.[3] Figure 4 shows a chromatogram of preserved ham samples. To judge method accuracy, five injections were made of Preserved Ham Sample 3 spiked with 5 µg/Kg of benzo(a)pyrene standard. The recovery range was from 95 to 105%.

Table 4. Sample analysis results.

Sample	Detected (µg/kg)	
	Method in this Work	GB/T 5009.27-2003
Sausage 1	0.49	ND*
Sausage 2	1.36	ND
Preserved Ham 1	0.98	ND
Preserved Ham 2	0.53	ND
Preserved Ham 3	ND	ND

*ND = Not Detected

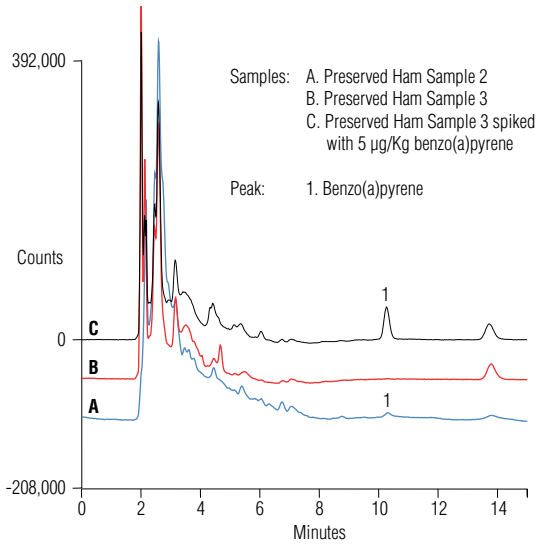

Figure 4. Preserved ham samples.

Conclusion

This work describes an on-line SPE HPLC method with fluorescence detection for a rapid and sensitive determination of benzo(a)pyrene in meat products. The determination was performed using an UltiMate 3000 Dual RSLC system controlled by Chromeleon CDS software.

References

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