Title: **Determination of Benzo[a]pyrene in Tobacco Products by GC-MS**

Quality System Compliance Review and Approval

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A. SCOPE

1. This method is used to quantitatively determine the amount of benzo[a]pyrene (B[a]P) (CAS # 50-32-8) in smokeless tobacco products using gas chromatography-mass spectrometry (GC-MS). The tobacco is weighed and extracted using methanol, followed by solid-phase extraction (SPE) and then concentration. The concentrated sample is analyzed by GC-MS in the selected ion monitoring (SIM) mode. The results are reported as nanograms of B[a]P per gram of sample.
2. This method is applicable to the analysis of smokeless tobacco products (snus pouches, moist snuff, dry snuff, and chewing tobacco), ground tobacco and tobacco filler.

B. DEFINITIONS

1. %RCR – an abbreviation for Percent Relative Concentration Residual. %RCR is calculated to show the degree of deviation of individual concentration points to the established calibration equation.
2. %CV - Coefficient of Variation. %CV is equal to the Percent Relative Standard Deviation (%RSD).
3. CCS - an abbreviation for Calibration Check Standard. The CCS is prepared independently from the calibration standards. It provides quality control and it is used to verify the calibration after the calibration and during sample analysis.
4. RRF - Relative Response Factor. Refers to the response of a target analyte relative to the response of the corresponding internal standard
5. LOQ - Limit of quantitation
6. LOD - Limit of detection
7. QC - Quality Control
8. SST - System Suitability Test
9. SPE - Solid Phase Extraction
10. B[a]P - Benzo[a]pyrene
11. B[a]P-d_{12} - Benzo[a]pyrene-d_{12}, deuterated isotope of B[a]P, used as an internal standard

C. RESPONSIBILITIES

1. The designated trained analyst performing the method is responsible for following all steps of the procedure and documenting and reporting any procedural deviations from the method to lab management.
2. Personnel using this test method are responsible for conducting the analysis in a manner consistent with the safety policies of ALCS.

D. VALIDATION

1. This test method was validated in regard to, calibration, recovery, precision, selectivity, LOD/LOQ, sample stability and system suitability. Complete information is in the 2014 validation report titled "**Determination of Benzo[a]pyrene in Tobacco Products by GC-MS.**" A summary of the validation results is presented below:

   a. **Accuracy:** The recovery of the analytes was demonstrated using the CORESTA Reference Products (CRP 1, CRP 2, CRP 3 and CRP 4), and the 3R4F Kentucky Reference cigarette filler fortified with B[a]P at three concentration levels. The average recoveries were all within ±15% of the target.
   b. **Precision:** The precision of the test method was evaluated by analyzing replicate samples of the CRPs. The repeatability (intra-day precision) of the method on three different days ranged from 0.4 to 9.1%RSD for all tobacco matrices. The inter-day precision (%RSD) on three different days ranged from 1.4 to 10.3% for all tobacco matrices.
   c. **LOD and LOQ:** The LOQ for each analyte was determined to be the lowest concentration calibration standard (0.5 ng/mL) which is sufficiently low for the analysis of B[a]P in various smokeless tobacco products

E. EQUIPMENT AND APPARATUS

1. Equipment and Apparatus Required

Note: If necessary, equivalent items may be used in place of those specified below, with prior authorization from lab management.

   a. Orbital shaker or wrist action shaker or multi-tube vortexer
   b. GC column: DB-17MS (Agilent cat# 122-4732, 30 m x 0.25 mm I.D., 0.25 µm film thickness) or equivalent
   c. Split/Splitless inlet liner (4.0 mm I.D), straight design packed with wool
   d. Extraction vial (40 mL amber)
   e. Amber autosampler 2 mL vials
   f. Eppendorf pipettor/Repeater (10-100 µL and 1 - 10,000 µL)
   g. Eppendorf pipette tips (100 - 5000 µL)
   h. Volumetric flasks (50 and 100 mL).
   i. Graduated cylinder (500 mL).
   j. Gastight® syringes (1-1.25 mL)
   k. Disposable plastic syringe (10-15mL)
   l. Disposable culture tubes, 16x100 mm
   m. RapidTrace® SPE Workstation with controller software and computer, Biotage Corporation or manual SPE manifold.
   n. Zymark TurboVap® LV Evaporator, Zymark Corporation.
   o. Strata-X 60 mg 33 µ polymeric reverse phase cartridge, 3mL, Catalog # 8B-S100-UBL, Phenomenex, Torrance, CA.
   p. Vortex mixer, Touch Mixer Model 231 (Fisher Scientific), or equivalent

q. Glass Pasteur pipets, 5.75 in
r. Whatman 25 mm PVDF syringe filters, 0.45 µm pore size

2. Instrument Parameters Setup

a. Set up program for the operation of the RapidTrace® SPE WorkStation: Refer to the instrument manual for information as to how to operate the RapidTrace®.

Step	Source	Output	Volume (mL)	Flow Rate (mL/min.)
Purge-Cannula	Hexane	Cannula	6	40.0
Purge-Cannula	MeOH	Cannula	6	40.0
Condition	MeOH	Waste 1	3	12.0
Load	Sample	Waste 1	3	2.0
Load	Sample	Waste 1	3	2.0
Rinse	50% MeOH - 50% H_2O	Waste 1	2	2.0
Rinse	IPA	Waste 1	2	2.0
Rinse	Hexanes	Waste 1	0.3	2.0
Collect	Toluene-Isooctane	FRACT1	3	2.0
Purge-Cannula	Hexane	Cannula	6	40.0

b. GC-MSD Settings: Refer to the instrument manual for information as to how to adjust the instrument to these settings. Small modifications to these parameters may be made (with lab management approval) to improve quality of analytical results, provided that there has been no compromise of system suitability.

1) Instrument: Gas Chromatograph: Agilent 6890 Plus or equivalent
   - Column: J&W DB-17MS (or equivalent), 30m x 0.25mm I.D., 0.25 µm film thickness
   - Mode: constant flow
   - Flow rate: 1.0 mL/min
   - Detector: Agilent 5973 Mass Selective Detector, or equivalent
2) Oven Temp:
   - Initial 200 °C; hold for 1.0 min
   - Ramp 25 °C/min to 280 °C
   - Ramp 40 ºC/min to 325 ºC, hold for 6.67 min
   - Run time: 12.0 min
3) Carrier Gas: Helium, with head pressure set at 11.65 psi
4) Injection Mode: Pulsed Splitless (25 psi initial pressure, 0.95 min pulse time, 1.0 min purge activation time)
5) Inlet temp: 300 ºC / Transfer line temp: 315 ºC
6) Injection volume: 1 µL injection
7) Analysis performed in the Selective Ion Mode (SIM)

- MS Quad 200 ºC, MS Source 250 ºC
- Ion Dwell Time: 100 msec
- Peak Threshold: 8.0
- Solvent delay 6.00 min

8) The masses (m/z) of the fragments used to perform quantitation and identification are shown in the table below with the approximate retention time.

MSD Quantitation/Qualifier Ions

Analyte	Retention time (min)	Quantitative Ion	Qualifier Ion
B[a]P-d_{12}	9.04	264	132
B[a]P	9.10	252	126

Attachment J contains typical selected ion chromatograms (Fig. 1 to 7) for the mid-level calibration standard (5 ng/mL) and B[a]P in the CRP matrices and 3R4F filler, with compounds and retention times identified.

3. Instrument Maintenance

a. System Maintenance: Regular maintenance of the GC/MSD system includes changing the GC liner and septum as needed and annual preventative maintenance including cleaning the electron ionization source. If a regular maintenance schedule is followed, then the condition of the analytical column is monitored by a visual inspection of the chromatographic resolution of B[a]P and adjacent peak(s) found in the tobacco reference samples. Significant degradation in resolution indicates the necessity of maintenance of the GC inlet and/or analytical column.

F. CHEMICALS AND REAGENTS

1. Chemicals Required

a. Certified benzo[a]pyrene stock, 1000 µg/mL in dichloromethane (used for Calibration standards), SPEX CertiPrep (cat# S-430), Supelco (catalog#CRM40071), or equivalent.
b. Certified benzo[a]pyrene stock, 200 µg/mL in dichloromethane (used for CCSs), Supelco (cat# CRM48665) or equivalent.
c. Certified benzo[a]pyrene-d12 stock, 1000 µg/mL in dichloromethane (used for ISTD), SPEX CertiPrep (cat# S-431) or equivalent.
d. Isooctane, Optima grade, Catalog # O301-4, Fisher Scientific, or equivalent.
e. Toluene, Optima grade, Catalog # T291-4, Fisher Scientific, or equivalent.
f. Hexanes, Optima grade, Catalog # H303-4, Fisher Scientific, or equivalent.
g. Methanol, Optima grade, Catalog #A454-4, Fisher Scientific, or equivalent.
h. 2-Propanol, A.C.S. grade, Catalog # A520-4, Fisher Scientific, or equivalent.
i. Helium, UHP Grade.
j. Nitrogen, UHP Grade.

k. Millipore Milli-Q water purification system with 18 MΩ product, or equivalent

2. Reagent and Stock Solutions Preparation

a. 50:50 (v/v) Toluene/Isooctane: Using a graduated cylinder, combine equal volumes (typically 500 mL each) of toluene and isooctane into a suitably-sized glass storage bottle and mix well. Store at room temperature. This solution expires six months from date of preparation.
b. 50:50 (v/v) Methanol / Water: Using a graduated cylinder, combine equal volumes (typically 500 mL each) of methanol and Milli-Q water (18 MΩ) into a suitably-sized glass storage bottle and mix well. Store at room temperature. This solution expires six months from date of preparation.
c. Primary (1°) Analyte Stock Solution for calibration standards (CALs): A certified solution of benzo[a]pyrene (1 mg/mL) must be used to prepare calibration standards.
d. Primary (1°) Analyte Check Stock Solution for calibration check standards (CCS): A certified solution of benzo[a]pyrene (200 µg/mL) obtained from a different lot or supplier, other than that used for the CALs, must be used to prepare calibration check standards.
e. Secondary (2°) Analyte Stock Solution (CALs) (2.5 µg/mL B[a]P): Transfer 0.250 mL of the 1000 µg/mL primary analyte stock solution (CALs) to a 100-mL volumetric flask containing approximately 50 mL of toluene. Dilute to volume with toluene and mix well. Store in freezer (-20 ± 5°C) when not in use. This solution expires six months from date of preparation (or upon expiration of primary standard used, if sooner) when stored in freezer.
f. Secondary (2°) Analyte Check Stock Solution (CCS) (5 µg/mL B[a]P): Transfer 0.500 mL of the 200 µg/mL primary analyte stock solution (CCS) to a 20-mL volumetric flask containing approximately 10 mL of toluene. Dilute to volume with toluene and mix well. Store in freezer (-20 ± 5°C) when not in use. This solution expires six months from date of preparation (or upon expiration of primary standard used, if sooner) when stored in freezer.
g. Primary (1°) ISTD Stock Solution: A certified solution of benzo[a]pyrene-d12 (1000 µg/mL) must be used to prepare the working internal standard solution.
h. Secondary (2°) ISTD Stock Solution (10 µg/mL B[a]P-d12): Transfer 1.0 mL of the 1000 µg/mL primary ISTD stock solution to a 100-mL volumetric flask containing approximately 50 mL of toluene. Dilute to volume with toluene and mix well. Store in freezer (-20 ± 5°C) when not in use. This solution expires six months from date of preparation (or upon expiration of primary standard used, if sooner) when stored in freezer.
i. Working Internal Standard Solution (WISS, 1.0 µg/mL B[a]P-d_{12}): Transfer 1.0 mL of the secondary ISTD Stock Solution to a 10-mL volumetric flask containing approximately 5 mL of toluene. Dilute to volume with toluene and mix well. This solution expires six months from date of preparation (or upon expiration of primary standard used, if sooner) when stored in freezer.

3. Standard Preparation

a. Preparation of B[a]P Calibration Standard Solutions

Prepare calibration standards as outlined in the table below. All solutions are prepared in Class A volumetric flasks, diluted to volume with 50:50 toluene: isooctane, and mix well prior to use. Protect the solutions from light in amber bottles & vials. Store all standards in the freezer (-20 ± 5°C). Solutions expire within six months of preparation (or upon expiration of secondary stock standards used, if sooner).

CAL Level	Volume of 2° CALs Stock Solution (mL)	Volume of 2° ISTD Stock Solution (mL)	Final Volume (mL)	Final Conc. B[a]P (ng/mL)	Final Conc. B[a]P-d_{12} (ng/mL)
1	0.010	0.25	50	0.5	50
2	0.020	0.25	50	1.0	50
3	0.100	0.25	50	5.0	50
4	0.200	0.25	50	10.0	50
5	1.000	0.25	50	50.0	50
6	2.500	0.25	50	125.0	50

b. Preparation of B[a]P Calibration Check Standard Solutions

Prepare calibration check standards as outlined in the table below. All solutions are prepared in Class A volumetric flasks, diluted to volume with 50:50 toluene: isooctane, and mixed well prior to use. Store all standards in freezer (-20±5°C), in amber bottles & vials to protect compounds from light. Solutions expire within six months of preparation (or upon expiration of secondary stock standards used, if sooner).

CCS Level	Volume of 2° CCS Stock Solution (mL)	Volume of 2° ISTD Stock Solution (mL)	Final Volume (mL)	Final Conc. B[a]P (ng/mL)	Final Conc. B[a]P-d_{12} (ng/mL)
CCS-Low	0.050	0.25	50	5.0	50
CCS-High	0.500	0.25	50	50.0	50

G. SAMPLE REQUIREMENTS

1. The sample weight used per determination should be approximately 1.0 g. For pouched products with individual unit weights significantly less than one gram, it may be necessary to combine several pouches to give a sample weight of approximately one gram. The sample weight used per determination should be approximately 1.0 g. For pouches with individual unit weights of one gram or more, a single pouch would be used per determination. Both the pouches and the tobacco are extracted together.

2. A method control sample (e.g., 3R4F) will be used during the routine application of the method. These should be prepared and analyzed with each batch of samples, with a minimum of two replicates prepared per analytical batch.
3. Care should be taken in handling the smokeless tobacco products stored in the freezer. Sufficient time should be given to allow the samples to reach room temperature and equilibrate before preparation and analysis.

H. PROCEDURE

1. Sample Handling

   a. For loose tobacco, weigh 1.0 ± 0.1 g of tobacco in a 40-mL amber vial. For pouched products, select a whole number of pouches (minimum of one) so that the total weight is as close to 1 g as possible; cut open pouch(es) and add the tobacco and pouch material to a 40-mL amber vial. Record sample weight to the nearest 0.0001 g. Additionally, for pouched products, record the number of pouches used as well.

      Note: In order to avoid exceeding the range of the calibration curve when analyzing materials with high BaP concentrations, it is allowable to extract a smaller mass of tobacco.

   b. Add 50 µL Working Internal Standard Solution (WISS) to the vial containing the tobacco samples.
   c. Add 10 mL Methanol to the same vial using dispensette. Cap the vial.
   d. Shake sample on an orbital shaker (at 350 rpm) or a wrist action shaker or a vortexer for 30 minutes.
   e. While samples are being extracted, label two sets of 16x100 mm culture tubes.
   f. Once sample extraction is complete, allow solids to settle to bottom of tubes (approximately 15 min.).
   g. Decant the clear sample extract into a 15 mL disposal syringe fitted with a 0.45 µm syringe filter taking care not to add the tobacco to the syringe. Typically 7 to 8 mL of sample extract can be decanted. Filter the sample extract into a labeled 16x100 mm culture tubes.
   h. Perform Solid Phase Extraction (SPE) using Strata-X 60 mg 33 µ polymeric reverse phase cartridges following the manual procedure described below or the RapidTrace procedures listed in E.2.a.

      Note: When performing SPE manually, SPE cleanup should be performed at ambient pressure (without vacuum). If necessary, a small amount of vacuum can be applied to cartridges to initiate or maintain flow rates of approximately 1-2 drops / second. Vacuum should also be briefly applied following step (6) to draw remaining eluent from SPE cartridges.

      1) Condition with 3 mL methanol
      2) Load 6 mL of the filtered sample extract
      3) Wash with 2 mL methanol: water (50:50)
      4) Wash with 2 mL isopropanol

5) Wash with 0.3 mL hexane
6) Elute with 3 mL toluene: isooctane (50:50) into second set of labeled 16x100 mm culture tubes.

i. Using a Zymark TurboVap® or similar sample evaporation system, the samples (3 mL toluene: isooctane) are evaporated almost to dryness at 50°C, under a nitrogen stream. The samples may be brought to dryness; however, the time at dryness should be kept to an absolute minimum.
j. Remove the sample tubes immediately and reconstitute in 300 µL of 50:50 toluene: isooctane. Vortex culture tubes briefly, and transfer to labeled amber crimp-seal autosampler vials with inserts.
k. Inject 1 µL on GC-MS
l. Prepared samples are stable for 2 days at room or refrigerator temperatures.

2. Calibration

a. Prior to performing a calibration, any necessary GC-MSD system maintenance must be performed. Document any maintenance in the instrument logbook.
b. Each time the instrument is calibrated for a run sequence, an MS tune evaluation report should be generated. The report should be reviewed to ensure that there has been no significant shift in mass accuracy, mass peak width, or abundance. Additionally, the tune evaluation report should be reviewed for any increase in air and/or water contamination in the MS background, which might indicate a leak. Significant observed differences in tune evaluation reports indicate a shift in system performance which needs to be addressed before starting an analytical run.
c. A new calibration curve should be run with each batch of samples. The calibration curve can be used up to 48 hours from initial calibration provided all additional samples added to the batch are bracketed with calibration check standards meeting acceptance criteria as outlined in Section H.5, below. Do not use any calibration standards beyond the specified expiration date. Keep all containers tightly sealed and stored in a freezer and protected from light when not in use. Make sure standards are at room temperature prior to their use.
d. Generation of Calibration Curves

1) Consult the appropriate operations manuals for information on using the system software for generating calibration data (i.e., selecting regression models, entering standard concentrations, inputting analytical data, quantitation, etc.).
2) Linear regression ($Y = mX + b$) with 1/x weighting is to be specified in the calibration method for an internal standard quantitation. Y is the peak area ratio, X is the concentration ratio, m is the slope and b is the intercept.
3) The calibration standards are used to generate the calibration curve. The standards should be run in the order of increasing concentrations, i.e., CAL 1 to 6. The concentrations of the calibration standards are entered into the data system software as ng B[a]P per 50 ng ISTD.

Note: Correct concentrations must be entered into the calibration table. Therefore care must be taken to verify the analyte concentrations each time a new commercially prepared stock solution is received or in-house stock solution prepared.

3. Analysis

   a. Before analyzing the samples, build a run sequence and enter calibration standards, sample names and sample multiplier into the appropriate fields of the run sequence table. All standards have a multiplier of 1. The multiplier for the samples is 1 if the same amount of ISTD (50 ng) is used in the sample.

   b. Analyze the calibration standards from low to high concentration and tobacco samples including blank (when applicable), calibration check standards (CCS-High and CCS-Low), and reference samples.

      1) A *typical* run sequence is as follows:

         1. Solvent wash of 50:50 toluene: isooctane w/o ISTD
         2. Calibration standards (lowest to highest concentration)
         3. Solvent wash 50:50 toluene: isooctane w/o ISTD
         4. Reagent blank (50:50 toluene: isooctane w/ ISTD
         5. CCS-Low
         6. CCS-High
         7. Matrix reference sample (e.g., 3R4F)
         8. Block of test samples*
         9. CCS Low
         10. CCS High
         11. Block of test samples*
         12. Matrix reference sample (e.g., 3R4F)
         13. CCS Low
         14. CCS High
         15. Solvent wash of 50:50 toluene: isooctane w/o ISTD

         Note: A block of test samples would consist of one-half of the analytical samples prepared in a batch or 20 samples (vials), whichever is less. Larger numbers of samples prepared at one time may require several 20-vial blocks to complete. Each block of test samples must be bracketed by calibration check standards.

      2) Process all data using the instrument data system software. Consult the system software operations manual and section H.4., "Calculations and Reporting" for more information on how the calculations are performed. Ensure that all target peaks are identified and properly integrated and reports are appropriately labeled to provide traceability to the sample, system and calibration curves. The resulting B[a]P concentrations will be in units of ng B[a]P per 50 ng ISTD.

4. Calculations and Reporting

   a. Concentrations of B[a]P in sample extracts are calculated based on analyte-to-internal standard peak-area-ratio that is compared against the same peak-area-ratios used to establish the calibration curve.
   b. The amount of B[a]P calculated by the instrument (in nanograms) is then converted to ng/g and reported on a per weight basis:

$$\text{B[a]P (ng/g)} = \frac{\text{Amount of B[a]P Detected (ng)}}{\text{Sample Weight (g)}}$$

   In order to assure appropriate data quality when reporting results, special attention must be directed toward the reporting of results that are near the LOQ. The method reliability is degraded when the resulting data are less than the LOQ. When amounts determined are less than the LOQ, "less than LOQ" will be reported; however, if the results are less than the LOD, "ND" (not detected) will be reported.

5. Quality Control and Acceptance Criteria

   a. The suitability of the system must be checked with every analysis batch by evaluating R^2, the signal to noise ratio of lowest calibration standard, analyzing calibration check standards, reagent blank with internal standard and reference tobacco product (e.g. 3R4F).
   b. Slope and intercept: Since this method is an internal standard method and the GC-MSD response may drift from day to day, a stable and clean analytical instrument should provide consistent slopes and intercepts without large deviation or offset from day to day.
   c. Calibration curves: The coefficient of determination (R^2) for B[a]P calibration curves must be 0.995 or higher. %RCR values for Calibration Standard 1 should not exceed 20% of nominal concentration and 15% of nominal concentration for Calibration Standards 2 - 6. The signal to noise for CAL-1 should be greater than 10 to 1.
   d. Calibration check standards: The validity of the calibration curves must be checked routinely during an analysis batch by injecting a CCS Low and a CCS High. These calibration check standards must be prepared from a separate working stock solution as the working stock solution used to prepare the standards used for calibration. The %RCR must be within 15% of the theoretical value. %RCR results not falling within these limits suggest that the curves are no longer valid, and the cause of the calibration change should be investigated. The results for any samples analyzed after the last passing CCS standard must be reanalyzed after the suitability of the system and calibration curves have been verified.
   e. Tobacco Reference Sample: A reference product (e.g., 3R4F), is used to assess method performance and to help ensure the method is in control. Typically, the tobacco reference sample should be analyzed following the first set of CCSs, as well as once preceding the last set of CCSs.
   f. The B[a]P values from the tobacco reference samples should be compared with the historical data (control charts) for acceptance of the test results in a run batch. If the

B[a]P yields of the Tobacco Reference samples are not within the control limits, data from the given run batch must be examined. If necessary, take corrective action and then reanalyze the existing samples or prepare the samples again for analysis.

I. REFERENCES

1. Method Validation Report of SOP 095-5021 "Determination of Benzo[a]pyrene in Tobacco Products by GC-MS" November 24, 2014.
2. Agilent GC/MSD Operation and Maintenance manuals.
3. Agilent ChemStation and MassHunter software manuals.
4. Biotage RapidTrace® SPE Workstation operation manual.

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J. APPENDICES

Attachment 1: Chromatograms

Figure 1: Chromatogram of B[a]P in calibration standard-4 (10 ng/mL)

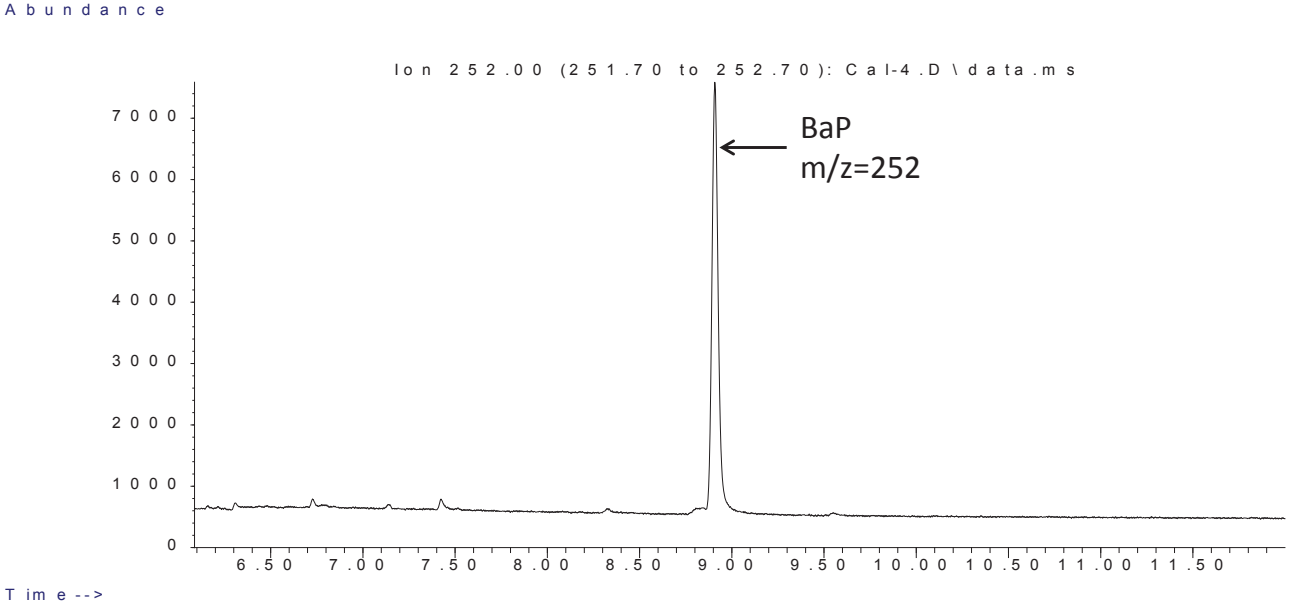

Figure 2: Chromatogram of B[a]P-d12 (50 ng/mL) in calibration standard-4

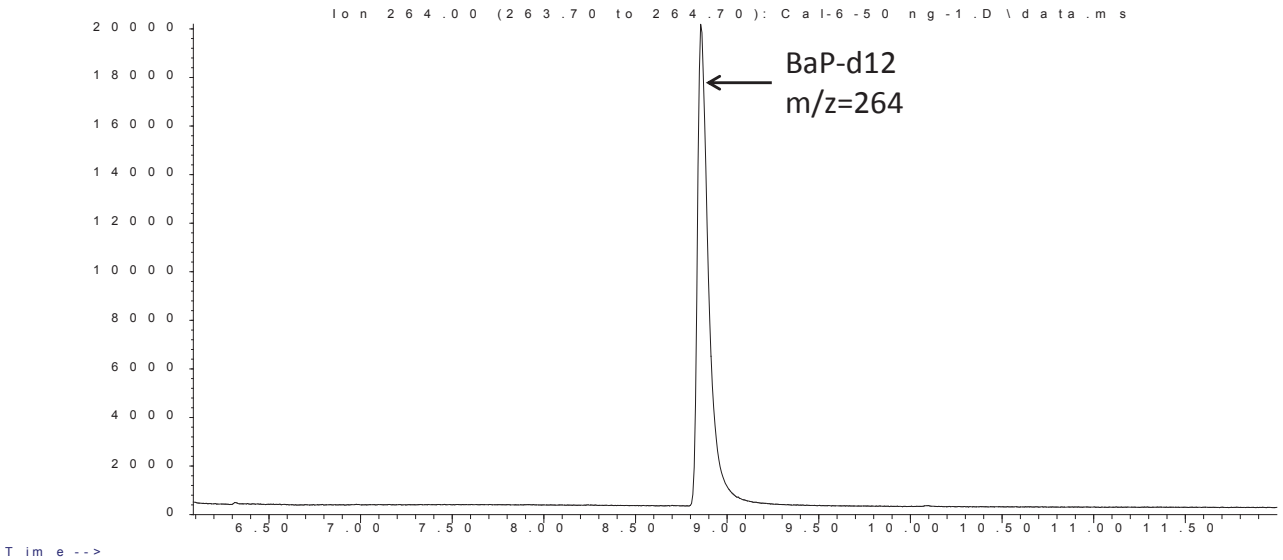

Figure 3: Chromatogram of CRP1 sample extract

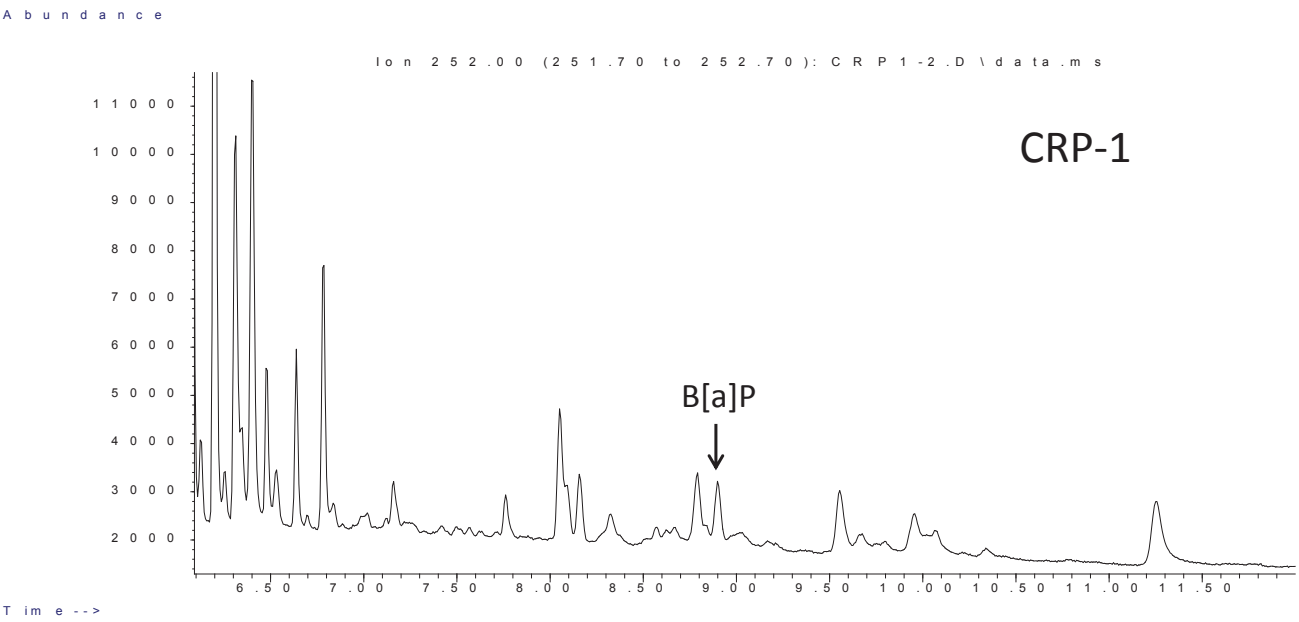

Figure 4: Chromatogram of CRP2 sample extract

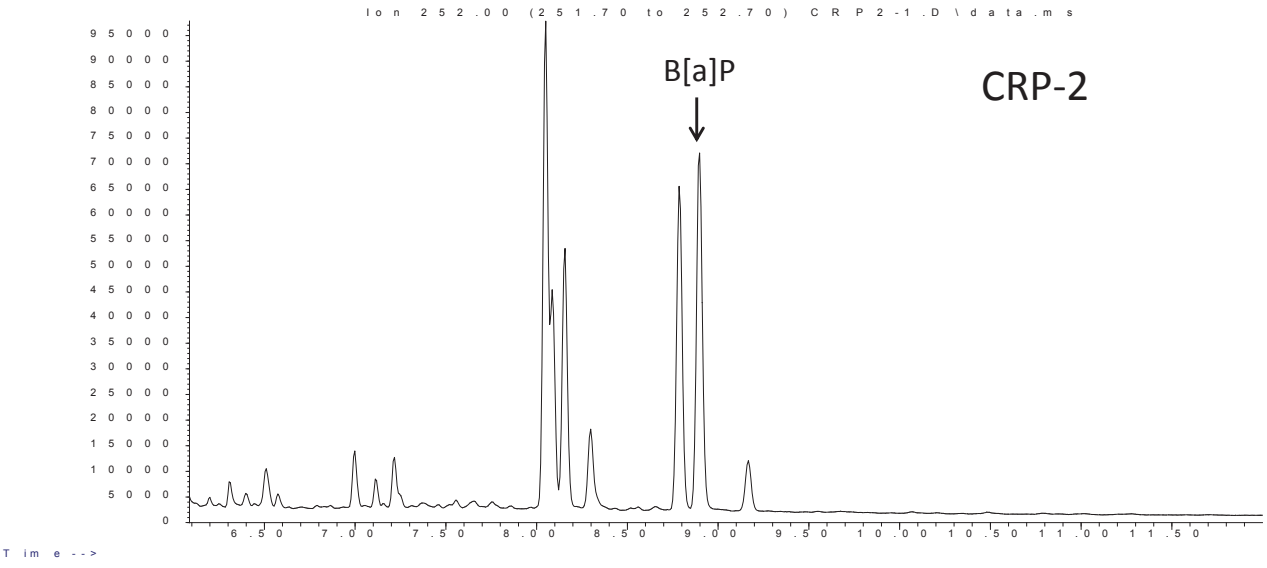

Figure 5: Chromatogram of CRP3 sample extract

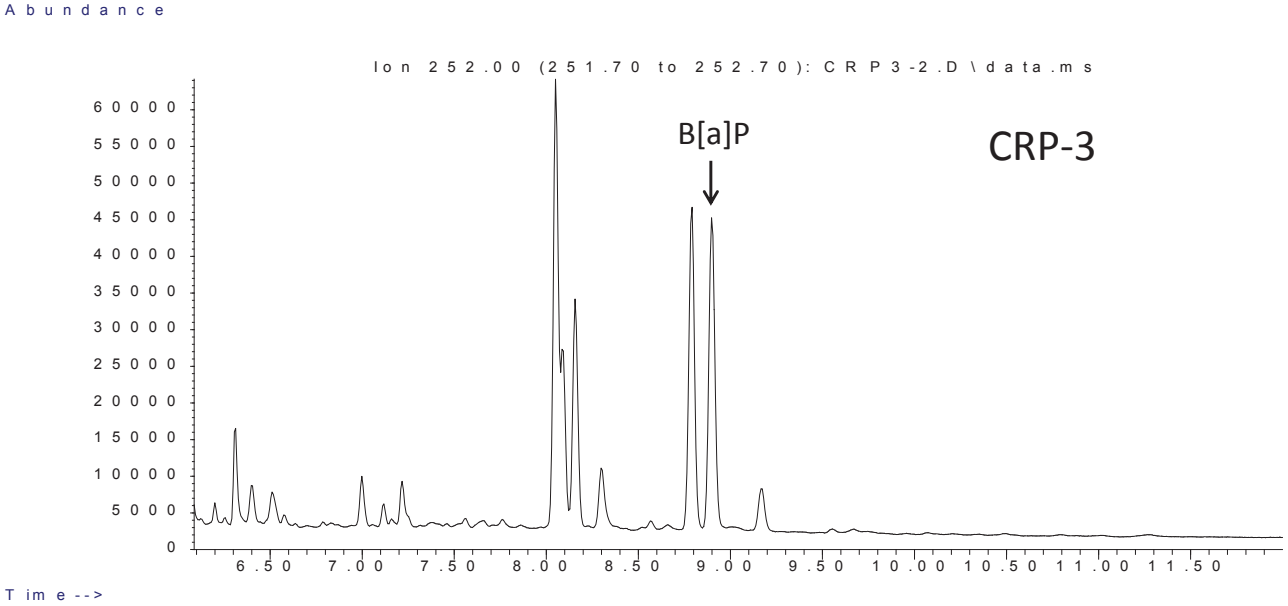

Figure 6: Chromatogram of CRP4 sample extract

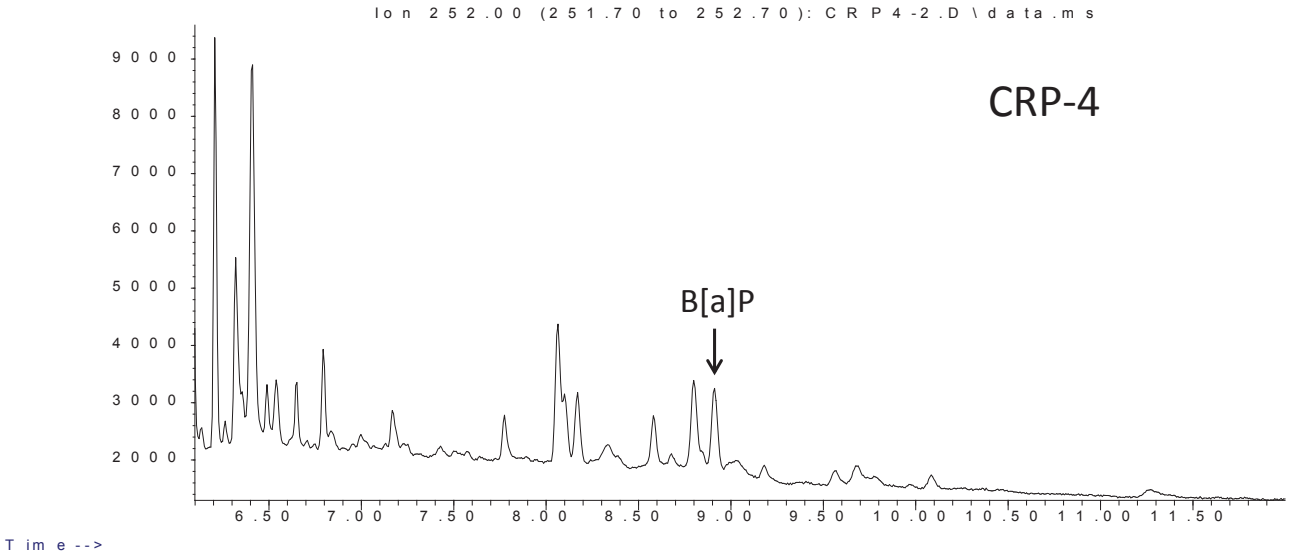

Figure 7: Chromatogram of 3R4F sample extract