



**Cooperation Centre for Scientific Research
Relative to Tobacco**

**Tobacco and Tobacco Products Analytes
Sub-Group**

**CORESTA Recommended Method
No. 82**

**DETERMINATION OF
BENZO[a]PYRENE IN
TOBACCO PRODUCTS BY GC-MS**

March 2018



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Title:

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Status: Valid

Note: This document will be periodically reviewed by CORESTA

Document history:

| Date of review | Information |
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| March 2016 | Version 1 |
| June 2016 | Version 2 - 4.6: catalogue number corrected |
| July 2017 | Version 3 - Reproducibility results for CRP2.1 and CRP3.1 improved |
| March 2018 | Version 4 - Extension of scope to include ground cigars |
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DETERMINATION OF BENZO[a]PYRENE IN TOBACCO PRODUCTS BY GC-MS

(March 2018)

0. INTRODUCTION

In 2015, the CORESTA Smokeless Tobacco Sub-Group (STS), now named Tobacco and Tobacco Products Analytes Sub-Group (TTPA), conducted a collaborative study for the determination of benzo[a]pyrene (B[a]P) in cigarette filler and smokeless tobacco products (STP) using gas chromatography with mass spectrometric detection (GC-MS). Seventeen laboratories participated in the study. This study was the basis for this CORESTA Recommended Method (CRM) as initially implemented.

In 2017, two additional collaborative studies were conducted. The goal of the first 2017 study was to provide additional detail for the analysis of tobacco products with higher levels of B[a]P than were included in the 2015 study and also to provide repeatability and reproducibility values for the 2016 CORESTA Reference Products. The second 2017 collaborative study (CORESTA TTPA-150-1 Technical Report - 2017 CORESTA Collaborative Study on Ammonia and Benzo[a]pyrene in Tobacco Products) was conducted to expand the scope of the Recommended Method to include ground cigars. This Recommended Method has been shown to be fit for purpose for the analysis of different tobacco varieties and tobacco products including a range of smokeless tobacco products, cigarette filler, and ground cigars. The repeatability and reproducibility values of this method have been assessed according to ISO 5725-2:1994.

1. FIELD OF APPLICATION

This CRM is applicable to the determination of B[a]P in tobacco, cigarette filler, smokeless tobacco (e.g. moist snuff, snus, chewing tobacco, and dry snuff), and ground cigars. The calibration range specified is from 0,5 ng/ml to 300 ng/ml. This range correlates to 0,15 ng/g to 90 ng/g as is, wet weight. Samples with higher levels of B[a]P may be analysed by extracting less tobacco to bring the samples within the calibration range.

2. NORMATIVE REFERENCES

- 2.1 CORESTA Smokeless Tobacco Sub-Group. *Smokeless Tobacco Glossary*
- 2.2 CORESTA Guide N° 11 - *Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products*

3. PRINCIPLE

The B[a]P content of tobacco products is determined by extracting the tobacco with methanol followed by solid-phase extraction (SPE) and subsequent concentration prior to gas chromatography/mass spectrometric (GC-MS) analysis in the selected ion monitoring (SIM) mode. The results are reported as nanograms of analyte per gram of tobacco (ng/g).

4. APPARATUS

Normal laboratory apparatus are required, in particular, the following items:

- 4.1 Analytical balance (0,0001 g accuracy)
- 4.2 Syringe filter, 0,45 µm polytetrafluoroethylene (PTFE) or equivalent
- 4.3 Volumetric flasks of capacities 100 ml, 250 ml and 1000 ml
- 4.4 Mechanical pipettes with disposable plastic tips 10 µl - 1000 µl
- 4.5 GC column: 50 %-Phenyl-methylpolysiloxane column of mid polarity (30 m × 0,25 mm I.D., 0,25 µm film thickness)¹
- 4.6 Polymeric reversed phase SPE cartridge; 3 ml volume, and 60 mg packing per cartridge²
- 4.7 Gas chromatograph-mass spectrometer (GC-MS)
- 4.8 Glass 4,0 mm I.D. liner with glass wool³
- 4.9 16 mm × 100 mm culture tubes
- 4.10 Amber glass extraction vial (approximately 40 ml)
- 4.11 Orbital shaker or wrist action shaker
- 4.12 SPE automated workstation or manual SPE manifold
- 4.13 Solvent evaporation system

5. REAGENTS

Use only reagents of recognized analytical grade.

- 5.1 Benzo[a]pyrene solution (1 mg/ml in methylene chloride)
- 5.2 Benzo[a]pyrene-d₁₂ solution (1 mg/ml in methylene chloride)
- 5.3 Reagent water, Resistivity ≥ 18,2 MΩ·cm^{5.4}
- 5.4 Isooctane, Reagent grade
- 5.5 Toluene, Reagent grade
- 5.6 Hexane, Reagent grade
- 5.7 Methanol, Reagent grade
- 5.8 2-Propanol, A.C.S. grade

¹ The following column is an example of a suitable product available commercially: DB-17MS, Catalog # 122-4732, Agilent Technologies, part number 25420-083. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

² The following SPE cartridge is an example of a suitable product available commercially: Strata-X 60 mg 33 µm polymeric reversed phase cartridge, 3ml, Catalog # 8B-S100-UBJ, Phenomenex. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

³ The following liners are examples of suitable products available commercially: (1) splitless, single taper, glass wool, Catalog # 5190-2293, Agilent Technologies or (2) split, straight, glass wool, Catalog # 5190-2294, Agilent Technologies. This information is given for the convenience of users of this document and does not constitute an endorsement of these product.

NOTE: Benzo[a]pyrene and benzo[a]pyrene-d₁₂ are Group 1 (IARC) carcinogens. Appropriate safety precautions shall be taken when handling these compounds or any solution containing these compounds.

6. PREPARATION OF SOLUTIONS

- 6.1 **50/50 (v/v) Toluene/Isooctane:** Use two 500 ml graduated cylinders to measure 500 ml of toluene and 500 ml of isooctane and mix together, and store in an amber 1000 ml bottle with a Teflon-lined cap. Store at room temperature.
- 6.2 **50/50 (v/v) Methanol/Water:** Use two 500 ml graduated cylinders to measure 500 ml methanol and 500 ml reagent water. Mix together, and store in a 1000 ml bottle with a Teflon-lined cap. Store at room temperature.

7. STANDARDS

- 7.1 **Primary B[a]P Stock Solution:** Purchase or prepare a 1 mg/ml benzo[a]pyrene stock solution in methylene chloride.
- 7.2 **Secondary B[a]P Stock Solution (2nd B[a]P Stock, 2,5 µg/ml B[a]P):** Transfer 0,250 ml of the primary analyte stock solution to a 100 ml volumetric flask containing approximately 50 ml of toluene. Dilute to volume with toluene and mix.
- 7.3 **Primary Internal Standard Stock Solution:** Purchase or prepare a 1 mg/ml benzo[a]pyrene-d₁₂ stock solution in methylene chloride.
- 7.4 **Secondary Internal Standard Stock Solution (2nd IS Stock, 10 µg/ml B[a]P-d₁₂):** Transfer exactly 1,00 ml of the Primary Internal Standard Stock solution to a 100 ml volumetric flask containing approximately 50 ml of toluene. Dilute to volume with toluene and mix.
- 7.5 **Working Internal Standard Solution (WISS, 0,3 µg/ml B[a]P-d₁₂):** Transfer 0,300 ml of the Secondary Internal Standard Stock Solution to a 10 ml volumetric flask containing approximately 5 ml of toluene. Dilute to volume with toluene and mix. This solution is added to the tobacco samples prior to extraction.

7.6 Working Standards

Transfer the specified volumes of B[a]P Secondary Stock Solution (7.2) and Secondary IS Stock Solution (7.4) according to the table below into 50 ml volumetric flasks, containing approximately 25 ml of 50/50 toluene/isooctane. Bring to a final volume with 50/50 toluene/isooctane and mix.

7.7 Storage

The standard solutions should be stored in the freezer at approximately -20 °C and have been shown to be stable for 6 months at these conditions.

Table 1 - Preparation of Working Calibration Standards

| Calibration Standards | Volume of 2° B[a]P Stock (ml) | Volume of 2° IS Stock (ml) | Final Conc. of B[a]P (ng/ml) | Final Conc. of B[a]P-d ₁₂ (ng/ml) |
|-----------------------|-------------------------------|----------------------------|------------------------------|--|
| 1 | 0,010 | 0,250 | 0,50 | 50,00 |
| 2 | 0,020 | 0,250 | 1,00 | 50,00 |
| 3 | 0,100 | 0,250 | 5,00 | 50,00 |
| 4 | 0,200 | 0,250 | 10,0 | 50,00 |
| 5 | 1,00 | 0,250 | 50,0 | 50,00 |
| 6 | 2,50 | 0,250 | 125,0 | 50,00 |
| 7 | 4,00 | 0,250 | 200,0 | 50,00 |
| 8 | 6,00 | 0,250 | 300,0 | 50,00 |

8. SAMPLE PROCEDURE

8.1 Sample Handling

Refer to CORESTA Guide N° 11, *Technical Guideline for Sample Handling of Smokeless Tobacco and Smokeless Tobacco Products* for sample handling guidelines.

8.2 Sample Preparation

8.2.1 Cigarette filler, ground tobacco, or ground cigar/cigar filler: Weigh 1,00 g ± 0,02 g of tobacco into a suitable glass extraction vessel.

8.2.2 Portioned smokeless tobacco products: Analyze unit portions (pouches) by cutting the pouch in half and adding both parts of the tobacco and pouch material directly to the extraction vessel.

Note: Samples that exceed the calibration range of the method shall not be diluted but must be prepared again using a reduced sample mass (0,5 g is recommended). It is not acceptable to dilute already prepared samples since the samples are prepared by solid phase extraction. When using a reduced sample mass it is important to ensure the sample is sufficiently homogeneous and if in doubt, the sample should be ground.

8.2.3 Add 50 µl Working Internal Standard Solution (WISS, section 7.5) to each sample vial followed by 10 ml of methanol.

8.2.4 Shake samples on an orbital shaker or a wrist action shaker for 30 minutes. Use a setting that allows for vigorous shaking. Once sample extraction is complete, allow any solids to settle to the bottom of tubes (approximately 15 min).

8.2.5 Decant the 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 ml to 8 ml of sample extract is decanted. Filter the sample extracts into 16 mm × 100 mm culture tubes.

Perform Solid Phase Extraction (SPE) using the procedure described below. When performing SPE manually using a manifold, the procedure should be performed at ambient pressure (without vacuum). If necessary, a small amount of positive pressure may be applied to cartridges to initiate a flow rate of

approximately 1-2 drops per second. Follow the steps detailed below for all samples.

- Condition the SPE cartridge with 3 ml methanol and discard to waste.
- Load the entire filtered sample extract (8.2.5) and discard to waste.
- Wash with 2 ml methanol:water (1:1) and discard to waste.
- Wash with 2 ml isopropanol and discard to waste.
- Wash with 0,3 ml hexane and discard to waste.
- Elute with 3 ml toluene:isooctane (1:1) and collect eluent in 16 mm × 100 mm culture tube. Vacuum should be briefly applied following the last step to draw the remaining eluent from the SPE cartridge.

8.2.6 Using a solvent evaporation system, evaporate the solvent in each sample to near dryness. This may be performed at approximately 50 °C, under a nitrogen stream to aid evaporation. The samples may be brought to dryness; however, the time at dryness should be kept to an absolute minimum.

8.2.7 Remove the sample tubes immediately and reconstitute in 300 µl of 50/50 toluene:isooctane. Vortex each culture tube briefly, and transfer each sample to a labelled amber autosampler vial containing a low volume insert.

9. SAMPLE ANALYSIS

9.1 GC-MS Operating Conditions

Set up and operate the GC-MS system in accordance with the manufacturer's instructions. The following conditions are suitable for analysis:

9.1.1 Injection Parameters:

Mode: constant flow
Flow rate: 1,0 ml/min
Injection Mode: Pulsed Splitless (25 psi until 0,95 min)
Inlet temp: 300 °C
Injection volume: 1 µl injection

9.1.2 Oven Temperature:

Initial 200 °C; hold for 1,0 min
Ramp 25 °C/min to 280 °C
Ramp 40 °C/min to 325 °C, hold for 6,67 min
Run time: 12,0 min

9.1.3 Carrier Gas: Helium

9.1.4 Transfer Line Temperature: 315 °C

9.1.5 MS Parameters:

MS Quad 200 °C, MS Source 250 °C
Ion Dwell Time: 100 ms
Peak Threshold: 8,0
Solvent delay 6,00 min

NOTE: The laboratory should demonstrate that the chromatographic conditions provide sufficient resolution between benzo[a]pyrene and benzo[e]pyrene.

Table 2 - MSD Quantitation/Qualifier Ions with Approximate Retention Times

| Analyte | Retention time (min) | Quantitative Ion | Qualifier Ion |
|-----------------------|----------------------|------------------|---------------|
| B[a]P-d ₁₂ | 9,04 | 264 | 132 |
| B[a]P | 9,10 | 252 | 126 |

NOTE: Qualifier Ions are used for confirming peak identification only. Relative abundances should be established by the individual laboratories. Samples near the method limit of quantitation may not contain usable qualifier ions; therefore, peaks may need to be manually identified.

9.2 Calibration of the GC-MS

Create an internal standard calibration method in the instrument operating software. A calibration curve is generated by calculating a linear regression of the area ratios of B[a]P to B[a]P-d₁₂ as a function of the concentration ratios of B[a]P to B[a]P-d₁₂. 1/X weighting is recommended. Ensure that the calibration curve is linear. If the curve is not linear, the calibration range should be reduced.

9.3 Determination of the Concentration of B[a]P

Inject each sample and calculate the area ratio of B[a]P to B[a]P-d₁₂ for each sample and obtain the concentration ratio by comparing the area ratio with the calibration curve.

The amount of B[a]P in the tobacco samples is quantified by the internal standard method. The concentration of B[a]P in the samples is reported in ng/ml by the chromatography software. Examples of chromatograms are shown in Appendix 1A-1F.

NOTE: Samples that exceed the calibration curve shall not be diluted, but must be prepared again using a reduced sample mass. Refer to section 8.2.2 for instruction.

9.4 Determination of the B[a]P Content of Samples

The concentration of B[a]P expressed in nanograms per gram of tobacco is calculated with the formula below:

$$\text{B[a]P (ng/g)} = \frac{C}{M} \times \frac{M_{IS}}{C_{IS}}$$

Where:

C = is the concentration obtained from the calibration curve (ng/ml)

M = is the mass of tobacco extracted (g)

M_{IS} = is the amount of internal standard added to the samples (15 ng)

C_{IS} = is the concentration of internal standard added to the standards (50 ng/ml)

NOTE: The internal standard quotient shown in the formula above is a correction factor to account for the different amounts of internal standard added to the samples and standards where 15 ng of internal standard is added to the samples and 50 ng/ml of internal standard is added to the calibration standards. This gives a correction factor 15 ng/50 ng/ml or 0,3 ml.

This correction factor does not need to be applied if the correct internal standard concentrations are entered into the instrument software (50 ng/ml for the standards and 15 ng for the samples).

10. REPEATABILITY AND REPRODUCIBILITY

In 2015, an international collaborative study involving 16 laboratories was conducted using cigarette filler and smokeless tobacco products by the CORESTA Smokeless Tobacco Sub-Group (STS).⁴ Results were analysed according to ISO 5725-2:1994 and ISO/TR 22971:2005. After removal of outlying data, the final repeatability (r) and reproducibility (R) results were calculated. The r&R results for the study are presented in Table 3.

At the beginning of 2017 the TTPA conducted an international collaborative study involving 15 laboratories using the four CRPs manufactured in 2016 to evaluate minor changes to the Recommended Method to improve the repeatability and reproducibility for samples with high levels of B[a]P.⁵ Results were analysed according to ISO 5725-2:1994 and ISO/TR 22971:2005. After removal of outlying data, the final repeatability (r) and reproducibility (R) results were calculated and are presented in Table 3.

In June 2017 a study was conducted involving 15 laboratories to expand the scope of the Recommended Method to include ground cigars.⁶ Results were analysed according to ISO 5725-2:1994 and ISO/TR 22971:2005. After removal of outlying data, the final repeatability (r) and reproducibility (R) results were calculated and are presented in Table 3.

⁴ STS Technical Report: 2015 Collaborative Study on Benzo[a]pyrene in Tobacco Products, March 2016.

⁵ TTPA Technical Report: 2017 CORESTA Collaborative Study on Benzo[a]pyrene in Tobacco Products, July 2017.

⁶ TTPA Technical Report: 2017 CORESTA Collaborative Study on Ammonia and Benzo[a]pyrene in Tobacco Products, March 2018.

Table 3 - Results from 2015³ and 2017^{4,5} Interlaboratory Studies

| Sample Type | N ¹ | Mean B[a]P (ng/g) ² | Repeatability | | Reproducibility | |
|---|----------------|--------------------------------|---------------|-------|-----------------|-------|
| | | | r | r (%) | R | R (%) |
| 1R5F reference cigarette filler ³ | 16 | 9,0 | 0,97 | 11 | 4,42 | 49 |
| 1R6F reference cigarette filler ³ | 14 | 5,0 | 0,77 | 15 | 2,53 | 51 |
| 3R4F reference cigarette filler ³ | 14 | 8,5 | 1,20 | 14 | 2,27 | 27 |
| CORESTA Monitor 8 (CM8) filler ³ | 14 | 59,6 | 5,85 | 10 | 21,7 | 36 |
| CRP1 - Swedish-style snus pouch ³ | 15 | 0,70 | 0,14 | 20 | 0,55 | 79 |
| CRP2 - American-style loose moist snuff ³ | 16 | 56,2 | 3,30 | 6 | 12,2 | 22 |
| CRP3 - American-style loose dry snuff powder ³ | 16 | 40,3 | 4,71 | 12 | 8,69 | 22 |
| CRP4 - American-style loose-leaf chewing tobacco ³ | 16 | 1,11 | 0,36 | 32 | 0,71 | 64 |
| CRP1.1 - Swedish-style snus pouch ⁴ | 15 | 0,83 | 0,35 | 42 | 1,00 | 121 |
| CRP2.1 - American-style loose moist snuff ⁴ | 15 | 159 | 15,5 | 10 | 58,4 | 37 |
| CRP3.1 - American-style loose dry snuff powder ⁴ | 14 | 145 | 12,5 | 9 | 55,0 | 38 |
| CRP4.1 - American-style chopped loose-leaf chewing tobacco ⁴ | 15 | 1,21 | 0,50 | 42 | 0,95 | 79 |
| Flavoured cigar (ground wrapper, binder, and filler) ⁵ | 11 | 10,4 | 2,20 | 21 | 5,89 | 57 |
| Traditional dark-air cured cigar (ground wrapper, binder and filler) ⁵ | 10 | 4,38 | 0,42 | 10 | 1,97 | 45 |
| <ol style="list-style-type: none"> 1. The number of laboratory data sets remaining after removal of outliers. 2. Results are presented on an as is basis, without correction for moisture. 3. CORESTA STS Technical Report - 2015 Collaborative Study on Benzo[a]pyrene in Tobacco Products, March 2016. 4. CORESTA TTPA Technical Report - 2017 CORESTA Collaborative Study Benzo[a]pyrene in Tobacco Products, July 2017. 5. CORESTA TTPA Technical Report - 2017 CORESTA Collaborative Study on Ammonia and Benzo[a]pyrene in Tobacco Products, February 2018 | | | | | | |

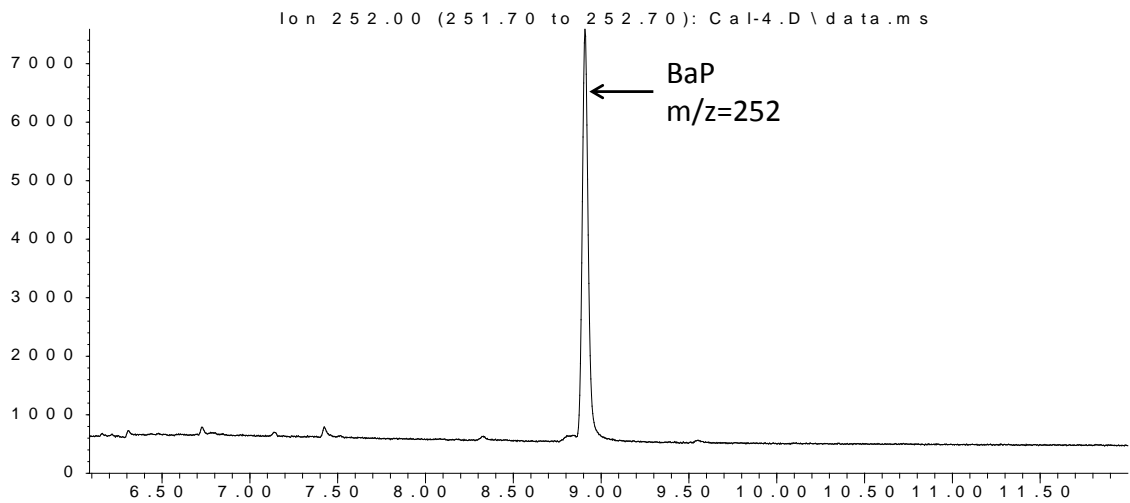
11. TEST REPORT

The expression of the laboratory data depends on the purpose for which the data are required, and the level of laboratory precision. Any statistical analyses should be calculated and expressed before any rounding has taken place. Moisture content may be determined on separate tobacco aliquots if it is necessary to present the final results on a dry-weight basis. This procedure is outlined in CORESTA Recommended Method N° 76: Determination of moisture content (oven volatiles) of tobacco and tobacco products.

APPENDIX 1-A

Example Chromatogram of B[a]P in calibration standard-4 (10 ng/ml)

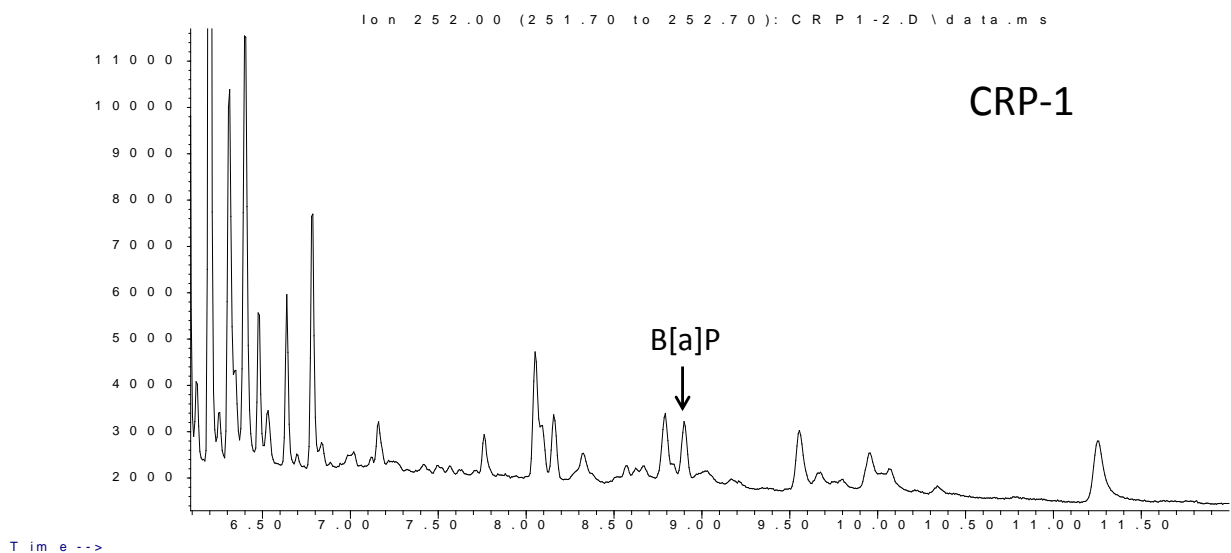
Abundance



APPENDIX 1-B

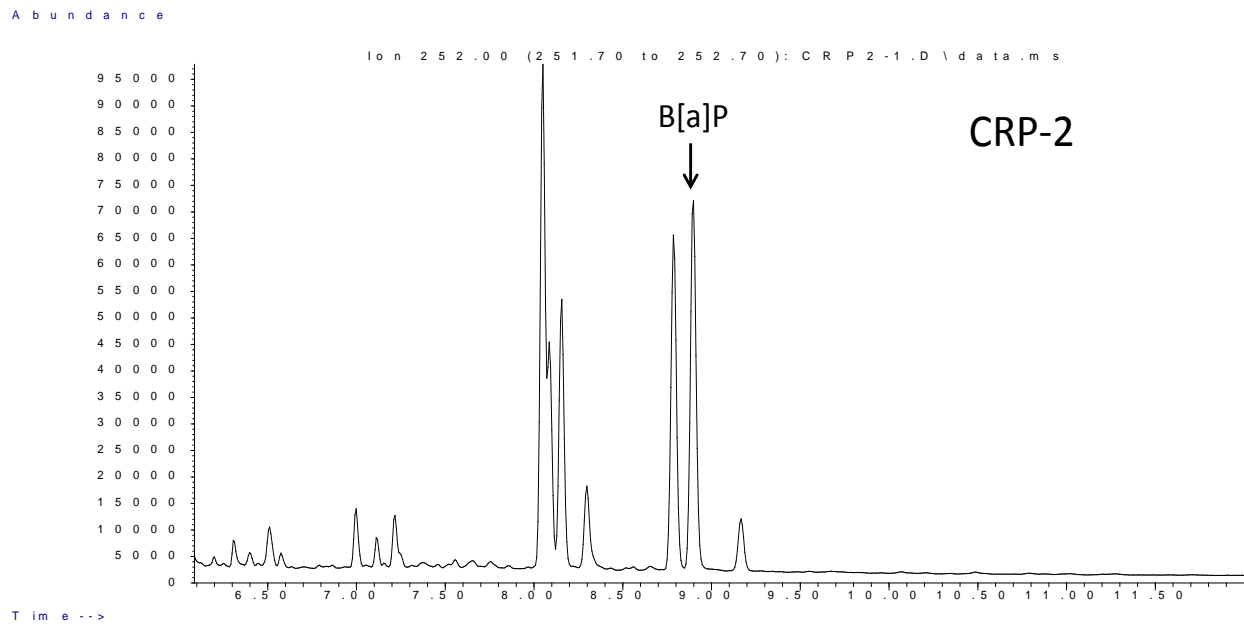
Example Chromatogram of CRP1 sample extract

Abundance



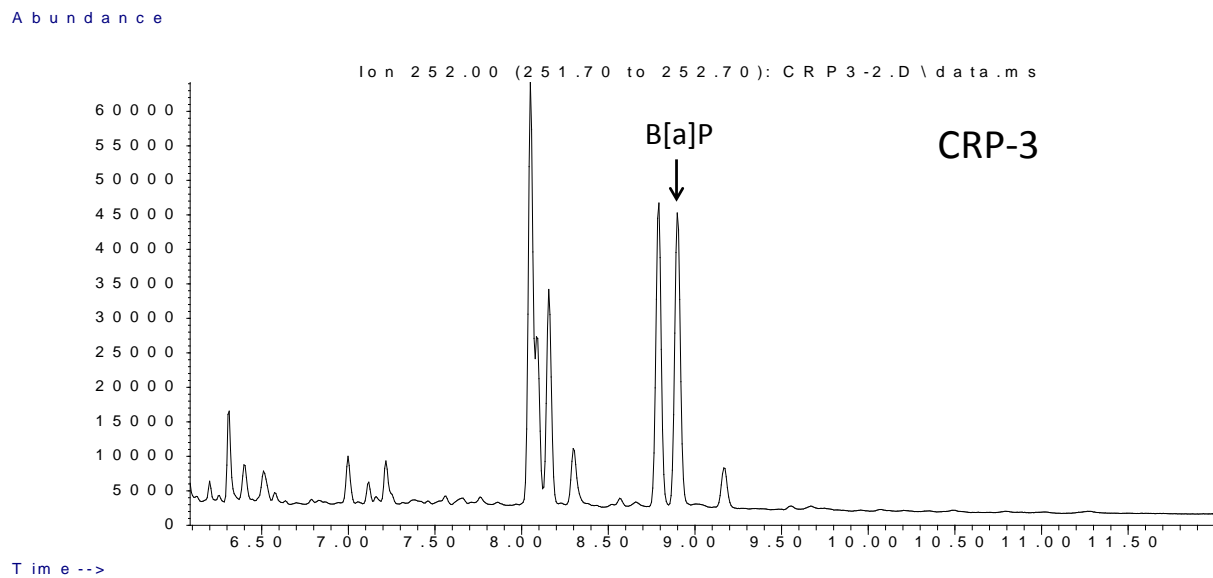
APPENDIX 1-C

Example Chromatogram of CRP2 sample extract



APPENDIX 1-D

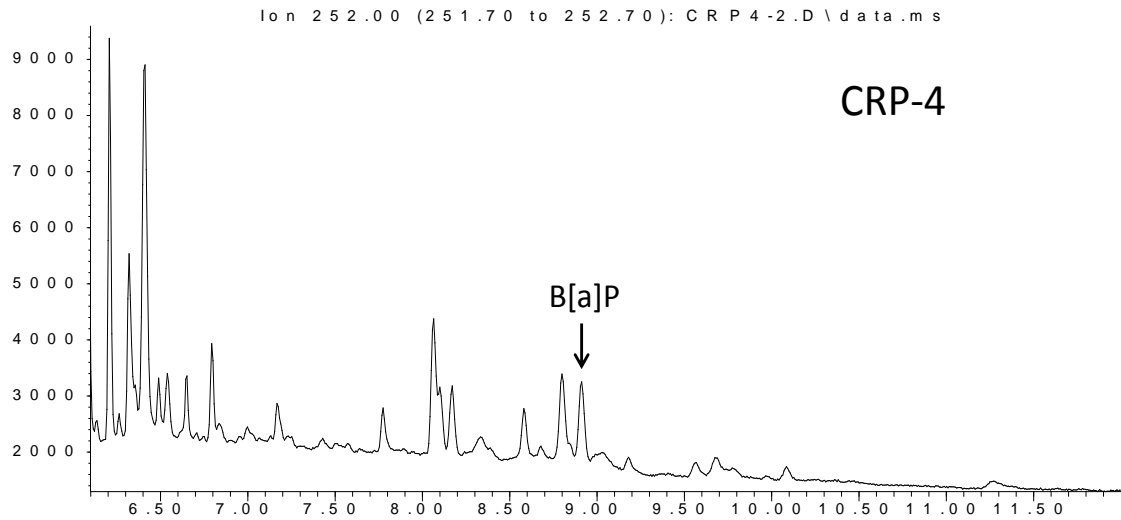
Example Chromatogram of CRP3 sample extract.



APPENDIX 1-E

Example Chromatogram of CRP4 sample extract.

Abundance

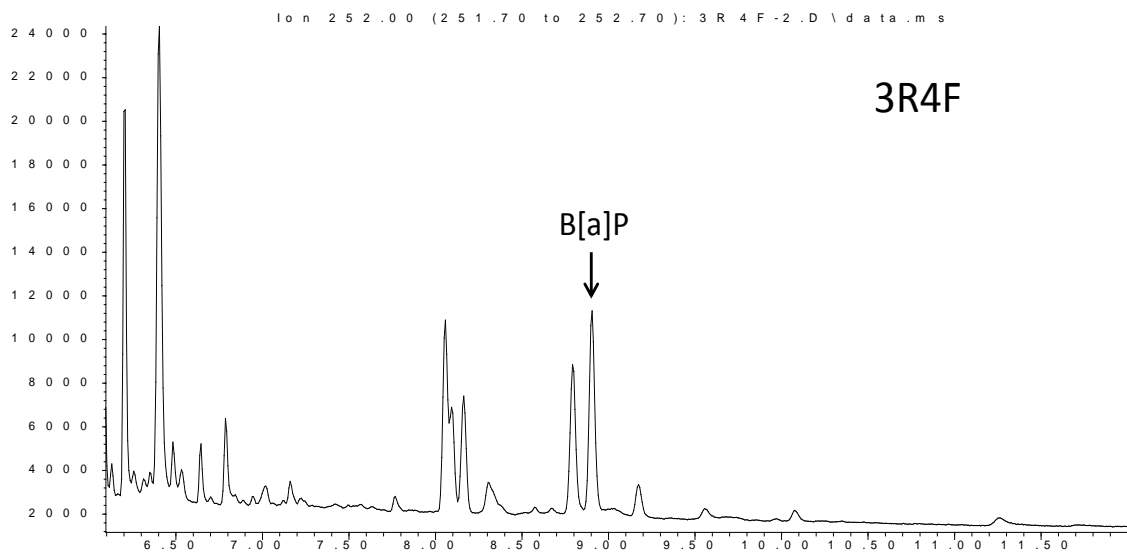


Time -->

APPENDIX 1-F

Example Chromatogram of 3R4F sample extract.

Abundance



Time -->