Cooperation Centre for Scientific Research Relative to Tobacco

Tobacco and Tobacco Products Analytes Sub-Group

CORESTA Recommended Method No. 91

DETERMINATION OF 15 PAHs IN TOBACCO AND TOBACCO PRODUCTS BY GC-MS/MS or GC-MS

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0. INTRODUCTION

In 2017/2018, the CORESTA Tobacco and Tobacco Products Analytes Sub-Group (TTPA) conducted a collaborative study for the determination of 18 polycyclic aromatic hydrocarbons (PAHs) in tobacco and tobacco products. A total of 14 laboratories participated and submitted results. The study included the analysis of five smokeless tobacco products, two cigarette fillers, two cigar fillers and one ground tobacco. The method specified in this study was shown to be appropriate for the determination of 15 of the PAHs that were evaluated. These results are the basis for this CORESTA Recommended Method (CRM).

1. FIELD OF APPLICATION

The method is used to quantitatively determine the concentration of 15 PAHs in tobacco and tobacco products using Gas Chromatography (GC) connected to a single mass spectrometer (MS) or tandem mass spectrometer (MS/MS). The method is applicable to single grades of tobacco, cigarette filler, cigar filler, and smokeless tobacco products (e.g. snus, moist snuff, dry snuff, and chewing tobacco). The calibration range specified in the method is from 0,2 ng/ml to 250 ng/ml using GC-MS/MS and 0,5 ng/ml to 250 ng/ml using GC-MS. This range corresponds to 0,2 ng/g to 250 ng/g using GC-MS/MS and 0,5 ng/g to 250 ng/g using GC-MS when 1 g of tobacco is extracted. Samples with higher levels of PAHs may be analyzed by extracting less tobacco to bring the samples within the calibration range. The target analytes for the method are:

- Benzo[c]phenanthrene (CAS 195-19-7)
- Benzo[a]anthracene (CAS 56-55-3)
- Chrysene (CAS 218-01-9)
- Benzo[b]fluoranthene (CAS 205-99-2)
- Benzo[k]fluoranthene (CAS 207-08-9)
- Benzo[j]fluoranthene (CAS 205-82-3)
- Benz[j]aceanthrylene* (CAS 202-33-5) + benz[e]aceanthrylene* (CAS 199-54-2)
- Benzo[a]pyrene (CAS 50-32-8)
- Dibenzo[a,h]anthracene (CAS 53-70-3)
- Indeno[1,2,3-c,d]pyrene (CAS 193-39-5)
- Benzo[g,h,i]perylene (CAS 191-24-2)
- Dibenzo[a,l]pyrene (CAS 191-30-0)
- Dibenzo[a,e]pyrene (CAS 192-65-4)
- Dibenzo[a,i]pyrene (CAS 189-55-99)
- Dibenzo[a,h]pyrene (CAS 189-64-0)

* These two analytes co-elute and are quantified together as the sum.

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 PAH content of tobacco products is determined by extracting the tobacco with methanol followed by solid-phase extraction (SPE) prior to gas chromatography/mass spectrometric analysis. Internal standards (IS) are added to the sample prior to the extraction. Separation and quantification take place using GC with a capillary column connected to a tandem mass spectrometer (MS/MS) or single quadrupole mass spectrometer (MS) and analyzed in MRM or SIM mode, respectively. The results are reported as nanograms of analyte per gram of tobacco (ng/g).

4. EQUIPMENT AND APPARATUS

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

4.1 Analytical balance (0,0001 g accuracy)

4.2 Mechanical pipettes (positive displacement) with disposable plastic tips 10 µl – 1000 µl

4.3 Volumetric flasks (4 ml, 5 ml, 10 ml, 25 ml, 100 ml, 250 ml)

4.4 Extraction vessel, 50 ml polypropylene centrifuge, or similar

4.5 Orbital shaker or wrist action shaker or tube vortexer

4.6 SPE automated workstation or manual SPE manifold

4.7 16×100 mm culture tubes

4.8 Polymeric reversed phase SPE cartridge; 3 ml volume, and 60 mg packing per cartridge[1]

4.9 GC-MS/MS or GC-MS with data acquisition system and autosampler

4.10 GC column: 50 %-phenyl-methylpolysiloxane column of mid polarity (20 m × 0,18 mm I.D., 0,14 µm film thickness)[2]

4.11 Glass 4.0 mm I.D. liner with glass wool[3]

[1] The following cartridge has been found to provide acceptable performance: Strata-X 60 mg, 33 µm polymeric reversed phase cartridge, 3 ml from Phenomenex. Part No. # 8L-S100-UBJ for manual use or Part No. # 8L-S100-UBJ-A with ASPEC cap for SPE workstation use. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

[2] The following separation column has been found to provide acceptable performance: DB-EUPAH, (20 m x 0.18 mm I.D., 0.14 µm film thickness (Catalog # 121-9627, Agilent Technologies). This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

[3] The following liner has been found to provide acceptable performance: Single taper, Ultra Inert Liner with glass wool, Catalog # 5190-2293, Agilent Technologies. This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

5. REAGENTS

Only use reagents of recognized analytical grade or better.

5.1 Methanol, >99 %, reagent grade

5.2 Isopropanol, HPLC grade

5.3 Hexane, HPLC grade

5.4 Toluene, >99.5 %, ACS reagent

5.5 Isooctane, >99 %, ACS reagent

5.6 Reagent water ≥ 18,2 MΩ-cm

5.7 PAH mixture containing Benzo[a]anthracene, Benzo[j]fluoranthene, Benzo[k]fluoranthene, Benzo[a]pyrene, Benzo[b]fluoranthene, Benzo[g,h,i]perylene, Chrysene, Dibenzo[a,h]anthracene, Dibenzo[a,l]pyrene, Dibenzo[a,e]pyrene, Dibenzo[a,i]pyrene Dibenzo[a,h]pyrene and Indeno[1,2,3-c,d]pyrene (10 ng/µl of each compound in cyclohexane)[4]

5.8 Benzo[c]phenanthrene (10 µg/ml in cyclohexane)

5.9 70 % Benz[j]aceanthrylene + 30 % benz[e]aceanthrylene, solid[5]

5.10 Deuterated PAH mixture containing Benzo[a]anthracene-d12, Chrysene-d12, Benzo[b]fluoranthene-d12, Benzo[k]fluoranthene-d12, Benzo[a]pyrene-d12, Indeno[1,2,3-c,d]pyrene-d12, Dibenzo[a,h]anthracene-d14 and Benzo[g,h,i]perylene-d12 (10 µg/ml of each compound in cyclohexane)[6]

5.11 Dibenzo[ai]pyrene-d14 (200 µg/ml in toluene-d8)[7]

Note: Some PAHs are group 1 (IARC) carcinogens. Appropriate safety precautions shall be taken when handling all PAH compounds or any solution containing these compounds.

[4] The following standard mix is an example that could be used: PAH Mix 183 (10 ng/µl in cyclohexane) from LGC (DRE-LA20950183CY). This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

[5] The following standard is an example that could be used: Benz[j]aceanthrylene and Benz[e]aceanthrylene (70:30 Mixture) from TRC Inc.(B197910). This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

[6] The following standard mix is an example that could be used: PAH-Mix 9 deuterated (10 µg/ml in cyclohexane) from LGC (DRE-L20950902CY). This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

[7] The following standards are examples that could be used: Dibenzo[ai]pyrene-d14 (200 µg/ml in toluen-d8) from LGC (CIL-DLM-3740-1.2) or from Cambridge Isotope Laboratories (DLM-3740-1.2). This information is given for the convenience of users of this document and does not constitute an endorsement of this product.

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 a 1 L 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 1 L bottle with a Teflon-lined cap. Store at room temperature.

6.3 **Primary stock solution PAH mixture (1800 ng/ml):** Transfer 900 µl of the PAH mixture into a 5 ml volumetric flask, dilute to volume with toluene:isooctane (50:50, v/v) and mix by gently inverting the flask.

Note: PAH mix ampules should be sonicated before preparation of primary stock solutions as Dibenzo[a,e]pyrene can precipitate.

6.4 **Primary stock solution Benzo[c]phenanthrene (2000 ng/ml):** Transfer 2000 µl of the Benzo[c]phenanthrene stock solution into a 10 ml volumetric flask, dilute to volume with toluene:isooctane (50:50, v/v) and mix by gently inverting the flask.

6.5 **Primary stock solution Benz[j]aceanthrylene/benz[e]aceanthrylene (6000 ng/ml):** Transfer 1,50 mg of the Benz[j]aceanthrylene/benz[e]aceanthrylene solid into a 250 ml volumetric flask, dilute to volume with toluene:isooctane (50:50, v/v) and mix by gently inverting the flask.

Note: The given concentration of the solution is of the sum of benz[j]aceanthrylene and benz[e]aceanthrylene.

6.6 **Primary stock solution Dibenzo[a,i]pyrene-d14 (24000 ng/ml):** Transfer 600 µl of the Dibenzo[a,i]pyrene-d14 stock solution into a 5 ml volumetric flask, dilute to volume with toluene:isooctane (50:50, v/v) and mix by gently inverting the flask.

6.7 **Secondary (2°) PAH mixture stock solution 1 (50 ng/ml):** Transfer 278 µl PAH mixture primary stock solution, 250 µl Benzo[c]phenanthrene primary stock solution and 83 µl Benz[j]aceanthrylene/Benz[e]aceanthrylene primary stock solution into a 10 ml volumetric flask and bring to final volume with 50/50 (v/v) toluene:isooctane.

6.8 **Secondary (2°) PAH mixture stock solution 2 (500 ng/ml):** Transfer 2780 µl PAH mixture primary stock solution, 2500 µl Benzo[c]phenanthrene primary stock solution and 830 µl Benz[j]aceanthrylene/Benz[e]aceanthrylene primary stock solution into a 10 ml volumetric flask and bring to final volume with 50/50 (v/v) toluene:isooctane.

6.9 **Working Internal Standard Solution (WISS, 500 ng/ml):** Transfer 1250 µl of the deuterated PAH mixture stock solution (5.10) and 1550 µl Dibenzo[a,i]pyrene-d14 primary stock solution into a 25 ml volumetric flask containing approximately 10 ml of methanol. Dilute to 25 ml with methanol and mix by gently inverting the flask. This solution is added to the tobacco samples prior to extraction.

Note: The prepared concentration of Dibenzo[a,i]pyrene-d14 is 1500 ng/ml.

6.10 **Working Calibration standards:** Transfer the specified volumes of PAH Secondary stock solutions and the Working Internal Standard Solution (WISS) into the indicated volumetric flasks according to the table below. Bring to final volume with 50/50 (v/v) toluene:isooctane and mix.

6.11 **Storage:** All standard solutions should be stored in the freezer at approximately -20 ºC and should be sonicated at room temperature before use.

Table 1. Preparation of Working Calibration Standards

Calibration standards	Volume WISS (µl)	Volume of (2°) PAH Stock Soln 1 (µl)	Volume of (2°) PAH Stock Soln 2 (µl)	Final volume (µl)	Final Conc. (ng/ml)
1	1000	40	-	10	0.2
2	1000	100	--	10	0.5
3	1000	400	--	10	2
4	1000	--	--	10	10
5	1000	--	100	10	50
6	1000	--	2500	10	125
7	1000	--	5000	10	250

7. SAMPLE PROCEDURE

7.1 Sample Handling

Refer to CORESTA Guide No 11, *Technical Guideline for Sampling Handling of Smokeless Tobacco and Smokeless Tobacco Products,* for sample handling guidelines.

7.2 Sample Preparation

7.2.1 Loose smokeless tobacco, cigarette filler, ground tobacco, or ground cigar/cigar filler: Weigh 1,00 g ± 0,02 g of tobacco into a suitable glass extraction vessel. Record the exact weight to 0,0001 g.

7.2.2 Portioned smokeless tobacco products: Analyze unit portions (pouches) by cutting the pouch in half and adding both the tobacco and pouch material directly to the extraction vessel. Use a sufficient number of pouches where the final weight will come closest to 1 g. Record the exact weight to 0,0001 g.

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.

Note: Care should be taken in handling samples stored in freezer. Ensure sufficient time is given to allow the samples to reach room temperature before preparation.

7.2.3 Add 100 µl of Working Internal Standard Solution (WISS) to each sample vessel, wait 10 min and add 10 ml of methanol.

7.2.4 Shake samples on an orbital shaker (set to approximately 130 rpm) or a wrist action shaker for 30 minutes. Once sample extraction is complete, centrifuge the extraction vessel for 10 min at 2500 rpm.

7.2.5 Decant the sample extract into a 16×100 mm culture tube, typically 7 ml to 8 ml of sample extract is decanted.

7.2.6 Perform Solid Phase Extraction (SPE) using Strata-X 60 mg 33 µm polymeric reverse phase cartridges following 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 6 ml sample extract and discard to waste.
- Wash with 2 ml methanol:water (1:1, v/v) and discard to waste.
- Wash with 2 ml isopropanol and discard to waste.
- Wash with 0,3 ml hexane and discard to waste. Do not dry the cartridge after this step.
- Elute with 1 ml toluene:isooctane (1:1, v/v) and collect eluent in 16×100 mm culture tube. Vacuum should be briefly applied following the last step to draw the remaining eluent from the SPE cartridge.
- Transfer each sample to a labelled amber autosampler vial.

8. SAMPLE ANALYSIS

8.1 Instrument Operating Conditions

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

8.1.1 Injection Parameters

Mode: constant flow

Carrier gas: Helium

Flow rate: 1,8 mL/min

Injection Mode: Splitless (43 psi until 0,8 min)

Inlet temp: 325 °C

Purge flow to split vent 100 mL/min at 0,8 min

Injection volume: 2 µl injection

8.1.2 Oven temperature

Initial 110 °C; hold for 0,8 min

Ramp 70 °C/min to 180 °C; hold for 0 min

Ramp 7 °C/min to 230 °C; hold for 6 min

Ramp 40 °C/min to 280 °C; hold for 5 min

Ramp 5 °C/min to 300 °C; hold for 0 min

Ramp 25 °C/min to 335 °C; hold for 4 min

Run time: 30,6 min

Post run: 335 °C for 6 min or preferably set up and use column backflushing to reduce post run time.

8.1.3 MS Parameters:

Transfer line: 350 ˚C

MS Quad: 180 ˚C. MS source: 340 ˚C

Solvent delay: 6,00 min

Collision gas: See manufacturer's instructions

In Table 2 the target analyte names and abbreviations are listed together with retention time data and MS acquisition parameters. The table also specifies which internal standard to use for each target analyte.

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.

Table 2. Quantitation/Qualifier Ions with Approximate Retentions Times

Name	**Abbreviated**	**Retention time** [min]	**Time segment** # [start in min]	**Internal Standard to use**	**MS/MS Quantifier** Precursor/product/Collision Energy[1,2]	**MS/MS Qualifier** Precursor/product/ Collision Energy[1]	**MS Qualifier Ion**	**Dwell Time** [ms]
Benzo[c]phenanthrene	**B[c]PA**	12,31	2 [10.5]	B[a]A-d12	228/228/10	228/202/40	202	40
Benzo[a]anthracene-d12	B[a]A-d12	13,25	2 [10.5]	-	240/240/15	240/212/40	212	40
Benzo[a]anthracene	**B[a]A**	13,40	2 [10.5]	B[a]A-d12	228/228/10	228/202/40	114	40
Chrysene-d12	CHR-d12	13,73	2 [10.5]	-	240/240/15	240/212/40	212	40
Chrysene	**CHR**	13,93	2 [10.5]	CHR-d12	228/228/10	228/202/40	114	40
Benzo[b]fluoranthene-d12	B[b]F-d12	17,51	3 [17]	-	264/264/20	264/260/40	132	40
Benzo[b]fluoranthene	**B[b]F**	17,58	3 [17]	B[b]F-d12	252/252/15	252/250/40	126	40
Benzo[k]fluoranthene-d12	B[k]F-d12	17,59	3 [17]	-	264/264/20	264/260/40	132	40
Benzo[k]fluoranthene	**B[k]F**	17,65	3 [17]	B[k]F-d12	252/252/15	252/250/40	126	40
Benzo[j]fluoranthene	**B[j]F**	17,73	3 [17]	B[k]F-d12	252/252/15	252/250/40	126	40
Benz[j]aceanthrylene + benz[e]aceanthrylene	**B[j]A+ B[e]A**	18,11	3 [17]	B[k]F-d12	252/252/15	252/250/40	-	40
Benzo[a]pyrene-d12	B[a]P-d12	18,74	3 [17]	-	264/264/20	264/260/40	132	40
Benzo[a]pyrene	**B[a]P**	18,83	3 [17]	B[a]P-d12	252/252/15	252/250/40	126	40
Indeno[1,2,3-c,d]pyrene-d12	I[cd]P-d12	23,17	4 [21]	-	288/288/20	-	-	40
Indeno[1,2,3-c,d]pyrene	**I[cd]P**	23,21	4 [21]	I[cd]P-d12	276/276/20	276/274/40	138	40
Dibenzo[a,h]anthracene-d14	*DB[ah]A-d14*	23,27	4 [21]	-	292/292/20	-	-	40
Dibenzo[a,h]anthracene	**DB[ah]A**	23,41	4 [21]	DB[ah]A-d14	278/278/20	-	-	40
Benzo[g,h,i]perylene-d12	B[ghi]Pl-d12	24,44	4 [21]	-	288/288/20	-	-	40
Benzo[g,h,i]perylene	**B[ghi]Pl**	24,55	4 [21]	B[ghi]Pl-d12	276/276/20	276/274/40	138	40
Dibenzo[a,l]pyrene	**DB[al]P**	27,48	5 [26]	DB[ai]P-d14	302/302/15	-	-	100
Dibenzo[a,e]pyrene	**DB[ae]P**	28,19	5 [26]	DB[ai]P-d14	302/302/15	-	-	100
Dibenzo[a,i]pyrene-d14	DB[ai]P-d14	28,53	5 [26]		316/316/40	-	-	100
Dibenzo[a,i]pyrene	**DB[ai]P**	28,63	5 [26]	DB[ai]P-d14	302/302/15	-	-	100
Dibenzo[a,h]pyrene	**DB[ah]P**	28,88	5 [26]	DB[ai]P-d14	302/302/15	-	-	100

[1] *Collision Energy should be optimized for each instrument manufacturer/model*
[2] *For GC-MS operation use the precursor mass as quantifier.*

8.2 **Calibration**

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 the analyte to the internal standard used as a function of the concentration ratios of the analyte to the internal standard used. 1/X weighting is recommended.

If the calibration range is poorly fitted using a linear curve (due to non-linearity) the calibration range should be reduced by excluding the higher calibration concentration(s).

Examples of chromatograms are shown in Appendix 1A-G. In Appendix 2 guidance is given for peak identification useful at evaluation.

8.3 **Determination of the concentrations of the analytes**

Inject each sample and calculate the area ratio of the analyte to the internal standard used for each sample and obtain the concentration ratio by comparing the area ratio with the calibration curve.

The amount of the individual analyte in the tobacco samples is quantified by the internal standard method. The concentration of the individual analyte in the sample extracts is reported in ng/ml by the chromatography software.

8.4 **Determination of the analyte content of samples**

The concentration of each analyte expressed in nanograms per gram of tobacco is calculated with the formula below:

$$\text{Analyte concentration in sample (ng/g)} = \frac{C}{M} \times \frac{M_{IS}}{C_{IS}}$$

Where:

C = the concentration obtained from the calibration curve (ng/ml).
M = the mass of tobacco extracted (g).
M_{IS} = the amount of internal standard added to the samples (50 ng for all internal standards except for DB[ai]P-d14 which is 150 ng).
C_{IS} = the concentration of internal standard added to the standards (50 ng/ml for all internal standards except for DB[ai]P-d14 which is 150 ng/ml).

Note: The internal standard quotient shown in the formula above is a correction factor to account for the amounts of internal standard added to the samples and standards. By strictly following this CRM the correction factor will have a value equal to 1.

B[j]A and B[e]A are calibrated as the sum of the two compounds and shall therefore be reported as the sum of B[j]A and B[e]A.

9. REPEATABILITY AND REPRODUCIBILITY

An international collaborative study involving 14 laboratories that used the specified test method was conducted by the CORESTA TTPA Sub-Group in 2017/2018[8]. Two of the laboratories submitted results using both MS and MS/MS detection technique. The study included the analysis of five smokeless tobacco products, two cigarette fillers, a cigar filler, a ground cigar, and one ground tobacco. The products used in the collaborative study are listed in Table 3.

Table 3: Products Used in the Collaborative Study

Product	Description
2016 CRP1.1	Swedish style snus pouch
2016 CRP2.1	American-style loose moist snuff
2016 CRP3.1	American-style loose dry snuff
2016 CRP4.1	American-style loose-leaf
2009 CRP3	American-style loose dry snuff powder
Cigar Filler#1 05/17	Ground, flavored cigar filler
CigarM16 05/17	Ground, traditional dark-air cured cigar
1R6F Ground Filler	American blended cigarette filler
1R5F Ground Filler	American blended cigarette filler (high burley)
RTDAC	Dark air cured ground tobacco

The data were statistically evaluated in basic conformance with the recommendations of ISO 5725-5, using robust estimators of the within lab and between lab variability. The mean values, repeatability (r), reproducibility (R), %r, and %R for ten of the analytes are presented in Table 4. The r&R values were calculated combining the MS and MS/MS results. The results were combined both because comparison of the data showed little, if any, difference between MS and MS/MS results and in order to have an adequate number of laboratories for the r&R calculations.

Table 4: Repeatability and Reproducibility Limits

Analyte	Product	Mean (ng/g)#	No. Labs * Total (MS+MSMS)	r (ng/g)	r (% of mean)	R (ng/g)	R (% of mean)
B[c]PA	2016 CRP1.1	0,54	14 (6+8)	0,159	29,3 %	0,731	135 %
	2016 CRP2.1	108,3	15 (7+8)	11,22	10,4 %	44,60	41 %
	2016 CRP3.1	122,8	14 (6+8)	12,48	10,2 %	75,25	61 %
	2016 CRP4.1	1,04	13 (6+7)	0,294	28,3 %	0,844	81 %
	2009 CRP3	36,43	15 (7+8)	3,82	10,5 %	22,37	61 %
	Cigar Filler#1 05/17	4,40	13 (5+8)	0,599	13,6 %	2,51	57 %

[8] CORESTA Tobacco and Tobacco Products Analytes Sub-Group Technical Report – PAHs in Tobacco and Tobacco Products, 2017/2018 Collaborative Study – March 2019

Analyte	Product	Mean (ng/g)[#]	No. Labs * Total (MS+MSMS)	r (ng/g)	r (% of mean)	R (ng/g)	R (% of mean)
	CigarM16 05/17	5,75	13 (5+8)	1,122	19,5 %	3,61	63 %
	1R6F Ground Filler	2,68	13 (6+7)	0,489	18,3 %	1,869	70 %
	1R5F Ground Filler	1,47	14 (6+8)	0,274	18,7 %	1,288	88 %
	RTDAC	1,178	13 (6+7)	0,242	20,5 %	1,267	108 %
B[a]A	2016 CRP1.1	1,26	15 (7+8)	0,330	26,3 %	0,800	64 %
	2016 CRP2.1	567,0	15 (7+8)	49,91	8,8 %	197,6	35 %
	2016 CRP3.1	621,9	14 (6+8)	35,26	5,7 %	240,5	39 %
	2016 CRP4.1	3,28	14 (6+8)	1,378	42,0 %	1,849	56 %
	2009 CRP3	185,9	15 (7+8)	13,04	7,0 %	68,01	37 %
	Cigar Filler#1 05/17	18,99	13 (5+8)	1,042	5,5 %	4,11	22 %
	CigarM16 05/17	17,17	13 (5+8)	1,547	9,0 %	4,64	27 %
	1R6F Ground Filler	12,89	14 (6+8)	1,477	11,5 %	3,941	31 %
	1R5F Ground Filler	6,92	14 (6+8)	0,801	11,6 %	2,146	31 %
	RTDAC	3,256	14 (6+8)	0,539	16,5 %	1,353	42 %
CHR	2016 CRP1.1	2,89	14 (6+8)	0,648	22,4 %	1,850	64 %
	2016 CRP2.1	751,9	15 (7+8)	56,76	7,5 %	326,0	43 %
	2016 CRP3.1	794,2	14 (6+8)	57,44	7,2 %	291,2	37 %
	2016 CRP4.1	6,26	13 (5+8)	1,509	24,1 %	3,049	49 %
	2009 CRP3	258,9	15 (7+8)	16,45	6,4 %	122,4	47 %
	Cigar Filler#1 05/17	26,24	13 (5+8)	2,143	8,2 %	12,40	47 %
	CigarM16 05/17	31,76	13 (5+8)	2,481	7,8 %	16,16	51 %
	1R6F Ground Filler	22,14	14 (6+8)	2,351	10,6 %	10,231	46 %
	1R5F Ground Filler	10,73	14 (6+8)	1,062	9,9 %	4,264	40 %
	RTDAC	7,156	14 (6+8)	1,086	15,2 %	3,372	47 %
B[b]F	2016 CRP1.1	1,08	14 (7+7)	0,180	16,7 %	0,721	67 %
	2016 CRP2.1	161,6	14 (7+7)	7,12	4,4 %	40,41	25 %
	2016 CRP3.1	150,9	13 (6+7)	9,12	6,0 %	45,65	30 %
	2016 CRP4.1	1,87	13 (6+7)	0,607	32,5 %	0,721	39 %
	2009 CRP3	47,66	14 (7+7)	2,96	6,2 %	17,29	36 %
	Cigar Filler#1 05/17	10,66	12 (5+7)	0,696	6,5 %	3,38	32 %
	CigarM16 05/17	8,60	12 (5+7)	0,979	11,4 %	3,23	38 %
	1R6F Ground Filler	10,41	13 (6+7)	1,530	14,7 %	3,716	36 %
	1R5F Ground Filler	4,78	13 (6+7)	0,584	12,2 %	1,634	34 %

Analyte	Product	Mean (ng/g)[#]	No. Labs * Total (MS+MSMS)	r (ng/g)	r (% of mean)	R (ng/g)	R (% of mean)
	RTDAC	2,325	13 (6+7)	0,524	22,5 %	1,105	48 %
B[k]F	2016 CRP1.1	0,50	14 (7+7)	0,086	17,2 %	0,272	55 %
	2016 CRP2.1	71,81	14 (7+7)	4,38	6,1 %	11,44	16 %
	2016 CRP3.1	67,04	13 (6+7)	4,45	6,6 %	14,61	22 %
	2016 CRP4.1	0,82	13 (6+7)	0,303	37,1 %	0,386	47 %
	2009 CRP3	21,01	14 (7+7)	1,93	9,2 %	6,12	29 %
	Cigar Filler#1 05/17	6,21	12 (5+7)	0,470	7,6 %	1,80	29 %
	CigarM16 05/17	4,22	12 (5+7)	0,471	11,2 %	1,59	38 %
	1R6F Ground Filler	5,41	13 (6+7)	0,645	11,9 %	1,517	28 %
	1R5F Ground Filler	2,61	13 (6+7)	0,386	14,8 %	0,876	34 %
	RTDAC	0,994	13 (6+7)	0,225	22,6 %	0,566	57 %
B[j]F	2016 CRP1.1	0,77	14 (7+7)	0,207	26,8 %	0,614	80 %
	2016 CRP2.1	88,67	14 (7+7)	7,06	8,0 %	19,01	21 %
	2016 CRP3.1	82,10	13 (6+7)	6,96	8,5 %	22,36	27 %
	2016 CRP4.1	1,07	13 (6+7)	0,344	32,1 %	0,505	47 %
	2009 CRP3	26,01	14 (7+7)	1,72	6,6 %	7,78	30 %
	Cigar Filler#1 05/17	7,38	12 (5+7)	0,752	10,2 %	2,86	39 %
	CigarM16 05/17	6,21	12 (5+7)	0,667	10,7 %	2,16	35 %
	1R6F Ground Filler	6,13	13 (6+7)	0,720	11,7 %	2,116	34 %
	1R5F Ground Filler	3,01	13 (6+7)	0,474	15,8 %	1,117	37 %
	RTDAC	0,98	13 (6+7)	0,218	22,3 %	0,812	83 %
B[a]P	2016 CRP1.1	0,63	15 (7+8)	0,162	25,8 %	0,790	125 %
	2016 CRP2.1	152,5	16 (8+8)	8,55	5,6 %	52,25	34 %
	2016 CRP3.1	140,5	15 (7+8)	10,40	7,4 %	54,22	39 %
	2016 CRP4.1	1,16	15 (7+8)	0,451	39,0 %	1,010	87 %
	2009 CRP3	40,52	16 (8+8)	2,46	6,1 %	12,60	31 %
	Cigar Filler#1 05/17	10,11	14 (6+8)	0,743	7,4 %	4,61	46 %
	CigarM16 05/17	4,05	14 (6+8)	0,724	17,9 %	1,63	40 %
	1R6F Ground Filler	9,63	15 (7+8)	1,252	13,0 %	3,951	41 %
	1R5F Ground Filler	4,47	15 (7+8)	1,035	23,1 %	1,906	43 %
	RTDAC	1,157	15 (7+8)	0,263	22,7 %	1,107	96 %
I[cd]P	2016 CRP1.1	0,55	14 (7+7)	0,151	27,5 %	0,419	76 %
	2016 CRP2.1	45,42	14 (7+7)	3,38	7,5 %	18,35	40 %

Analyte	Product	Mean (ng/g)#	No. Labs * Total (MS+MSMS)	r (ng/g)	r (% of mean)	R (ng/g)	R (% of mean)
	2016 CRP3.1	44,13	13 (6+7)	3,25	7,4 %	19,27	44 %
	2016 CRP4.1	0,86	13 (6+7)	0,226	26,2 %	0,673	78 %
	2009 CRP3	13,29	14 (7+7)	1,28	9,6 %	5,92	45 %
	Cigar Filler#1 05/17	4,91	12 (5+7)	0,528	10,8 %	2,66	54 %
	CigarM16 05/17	3,73	12 (5+7)	0,685	18,4 %	2,09	56 %
	1R6F Ground Filler	5,37	13 (6+7)	0,969	18,0 %	3,001	56 %
	1R5F Ground Filler	2,48	13 (6+7)	0,346	13,9 %	1,434	58 %
	RTDAC	0,992	13 (6+7)	0,224	22,6 %	0,923	93 %
DB[ah]A	2016 CRP1.1	0,40	7 (5+2)	0,065	16,3 %	1,127	282 %
	2016 CRP2.1	11,36	14 (7+7)	1,32	11,6 %	3,38	30 %
	2016 CRP3.1	11,33	13 (6+7)	0,91	8,0 %	2,63	23 %
	2016 CRP4.1	0,23	6 (4+2)	-	-	-	-
	2009 CRP3	3,58	14 (7+7)	0,48	13,3 %	1,75	49 %
	Cigar Filler#1 05/17	0,73	12 (5+7)	0,096	13,2 %	0,386	53 %
	CigarM16 05/17	0,72	11 (4+7)	0,162	22,4 %	0,666	92 %
	1R6F Ground Filler	0,80	12 (5+7)	0,132	16,6 %	0,567	71 %
	1R5F Ground Filler	0,52	13 (6+7)	0,108	20,8 %	0,534	103 %
	RTDAC	0,283	9 (5+4)	0,149	52,7 %	0,280	99 %
B[ghi]Pl	2016 CRP1.1	0,58	13 (6+7)	0,169	29,4 %	0,552	96 %
	2016 CRP2.1	41,73	14 (7+7)	2,72	6,5 %	11,00	26 %
	2016 CRP3.1	41,63	13 (6+7)	2,74	6,6 %	14,09	34 %
	2016 CRP4.1	0,92	13 (6+7)	0,268	29,1 %	0,364	39 %
	2009 CRP3	12,81	14 (7+7)	1,39	10,8 %	4,74	37 %
	Cigar Filler#1 05/17	5,17	12 (5+7)	0,545	10,6 %	2,64	51 %
	CigarM16 05/17	3,41	12 (5+7)	0,394	11,6 %	1,64	48 %
	1R6F Ground Filler	6,64	13 (6+7)	0,867	13,1 %	3,420	52 %
	1R5F Ground Filler	3,07	13 (6+7)	0,589	19,2 %	1,531	50 %
	RTDAC	1,026	13 (6+7)	0,354	34,5 %	0,663	65 %

* This is the total number of laboratory data sets reported as values and shown in parenthesis is the number broken down per detection technique used.

The mean was estimated using Algorithm A, a robust estimation technique that diminishes the impact of extreme values.

The symbol "-" indicates that too few values were reported to allow statistical analysis. Statistical analysis was only done if 7 or more data points were available.

10. VALIDATION OF FIVE ANALYTES

The following five analytes were either reported by only a few laboratories in some products and/or found at levels below or near the limits of quantification:

- Benz[j]aceanthrylene + benz[e]aceanthrylene
- Dibenzo[a,l]pyrene
- Dibenzo[a,e]pyrene
- Dibenzo[a,i]pyrene
- Dibenzo[a,h]pyrene

The reproducibility limits for these analytes could not be calculated due to insufficient number of data sets or the reproducibility limits were found to be poor, as the levels found were mostly below the limits of quantification. For this reason, a limited validation involving repeatability and accuracy was performed by one participating laboratory. The repeatability and accuracy study is described in CORESTA Tobacco and Tobacco Products Analytes Sub-Group Technical Report – "PAHs in Tobacco and Tobacco Products, 2017/2018 Collaborative Study – February 2019".

10.1 Repeatability and Accuracy by Fortified Matrix Spikes

As described in the Technical Report, an experiment using laboratory fortified matrix spikes was conducted to determine if the analytical method accurately measures the concentration of the analyte in the presence of sample matrix components. The sample types investigated were CRP1.1 and CRP4.1.

The average accuracy in the fortified samples were in the range 81 % - 114 %, except for B[j]A+B[e]A at the high level spiked where the recovery in average was 124 %, see Table 5. The relative standard deviations for the analytes in the fortified samples were in the range 1 - 15 RSD%.

Table 5. Summary of accuracy and repeatability data

Product	Spiked level (ng)		B[j]a+ B[e]A	D[a,l]P	D[a,e]P	D[a,i]P	D[a,h]P
CRP1.1	2,5	**Mean recovery (%)**	**114**	**87**	**81**	**99**	**87**
		Repeatability (RSD%)	9,3	12,6	10,5	6,9	3,7
	50	**Mean recovery (%)**	**124**	**98**	**90**	**92**	**92**
		Repeatability (RSD%)	0,9	2,8	1,3	2,8	3,9
CRP4.1	2,5	**Mean recovery (%)**	**102**	**91**	**97**	**104**	**107**
		Repeatability (RSD%)	9,7	6,1	7,0	15	10,2
	50	**Mean recovery (%)**	**110**	**95**	**100**	**106**	**117**
		Repeatability (RSD%)	9,3	9,6	7,6	6,8	4,4

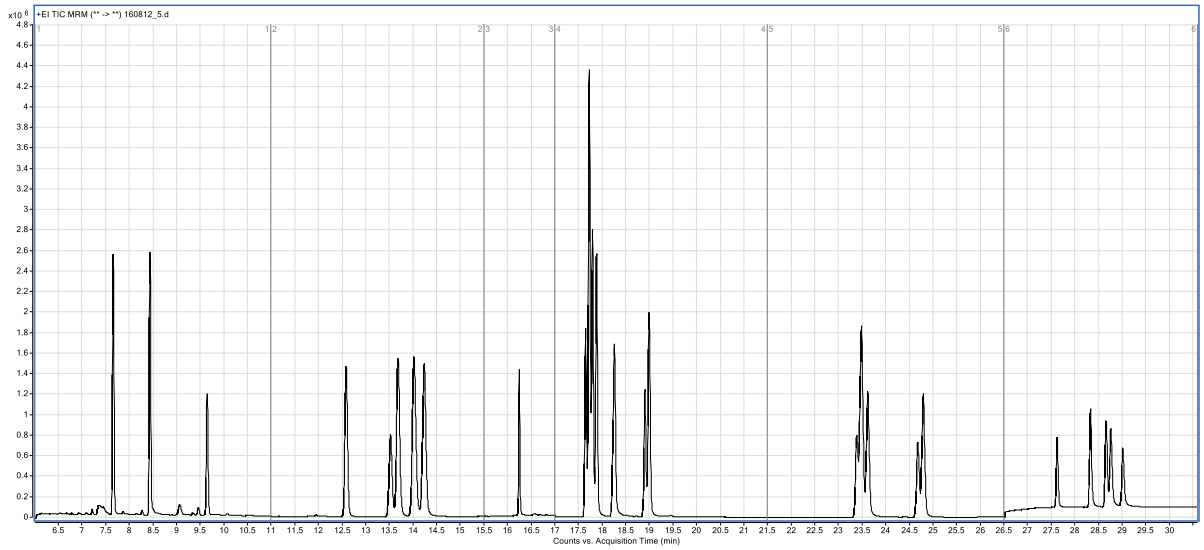
The average recovery data per product fell within the range of 80 % - 120 %, with one exception of 124 %. Based on these results, the method is able to quantify these five analytes but with an unknown level of confidence since no statistical data evaluation from the collaborative study data could be performed.

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 further 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.*

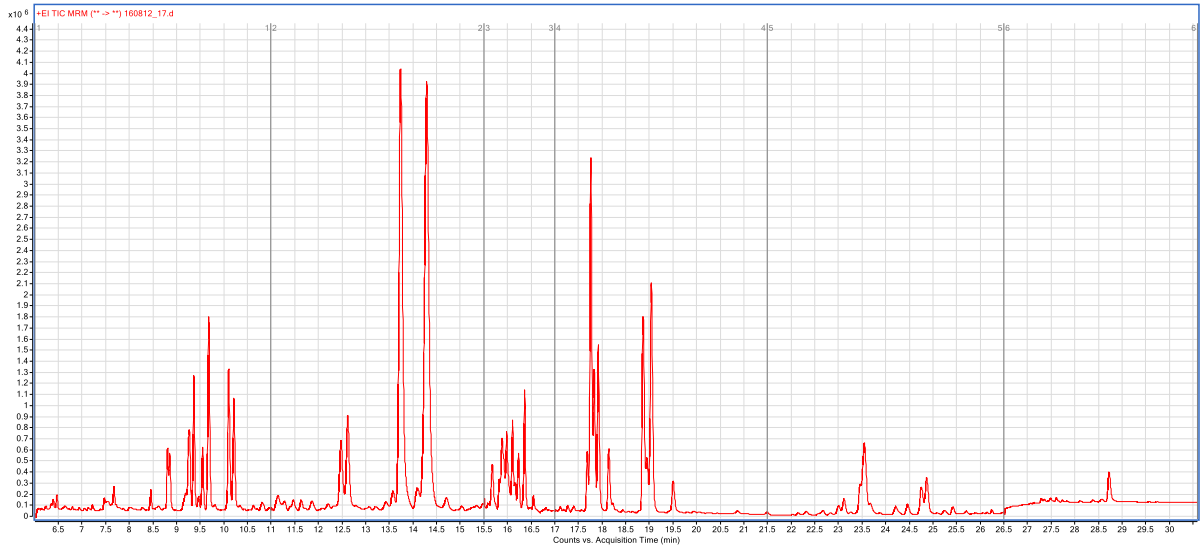
APPENDIX 1.A

TIC chromatogram for calibration standard 5 (50 ng/mL)



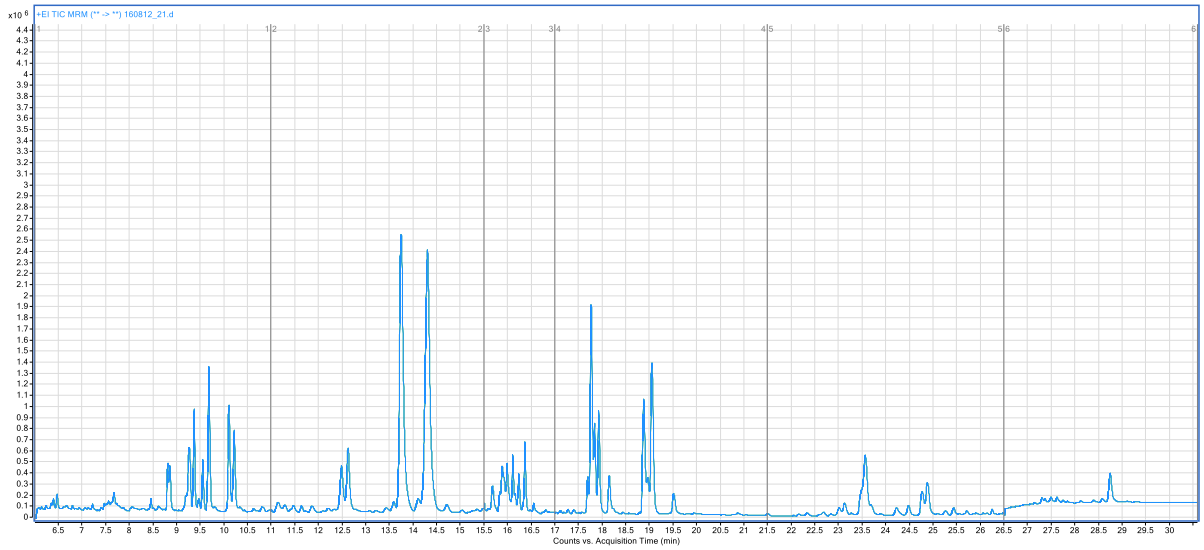
APPENDIX 1.B

TIC chromatogram of CRP2.1



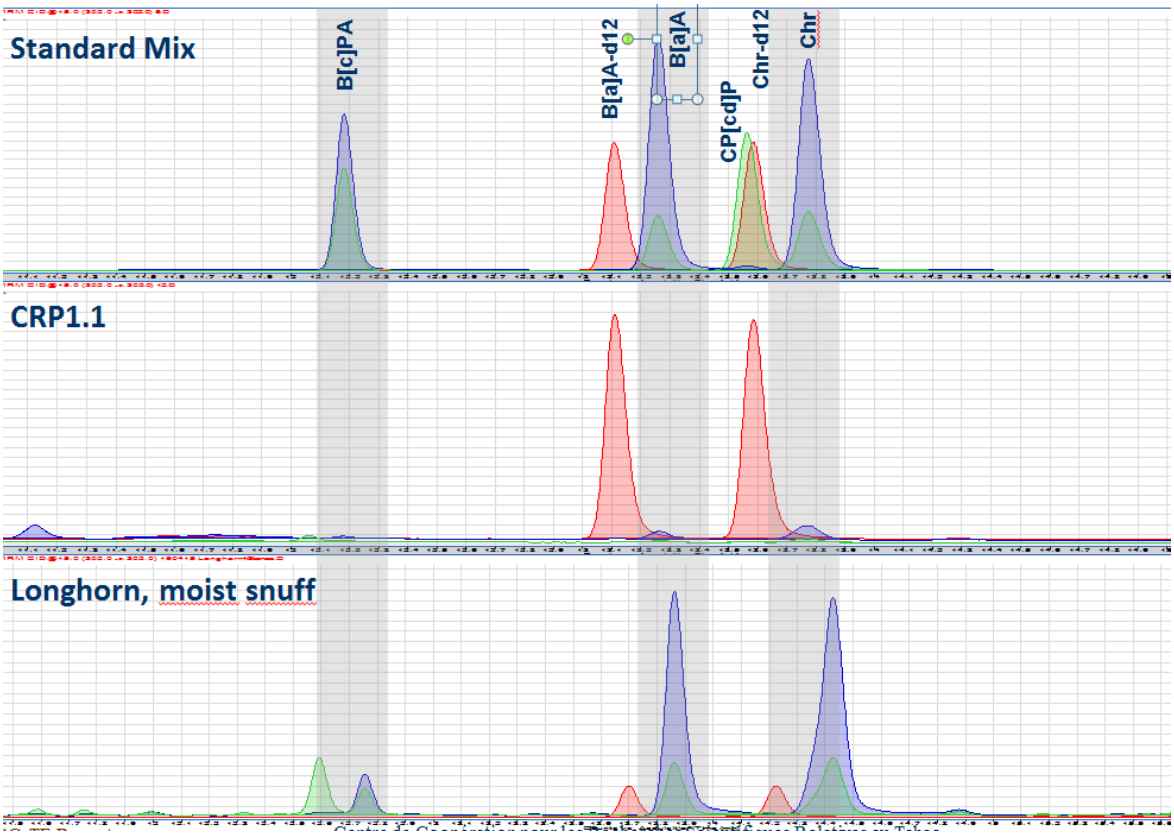
APPENDIX 1.C

TIC chromatogram of CRP3.1.



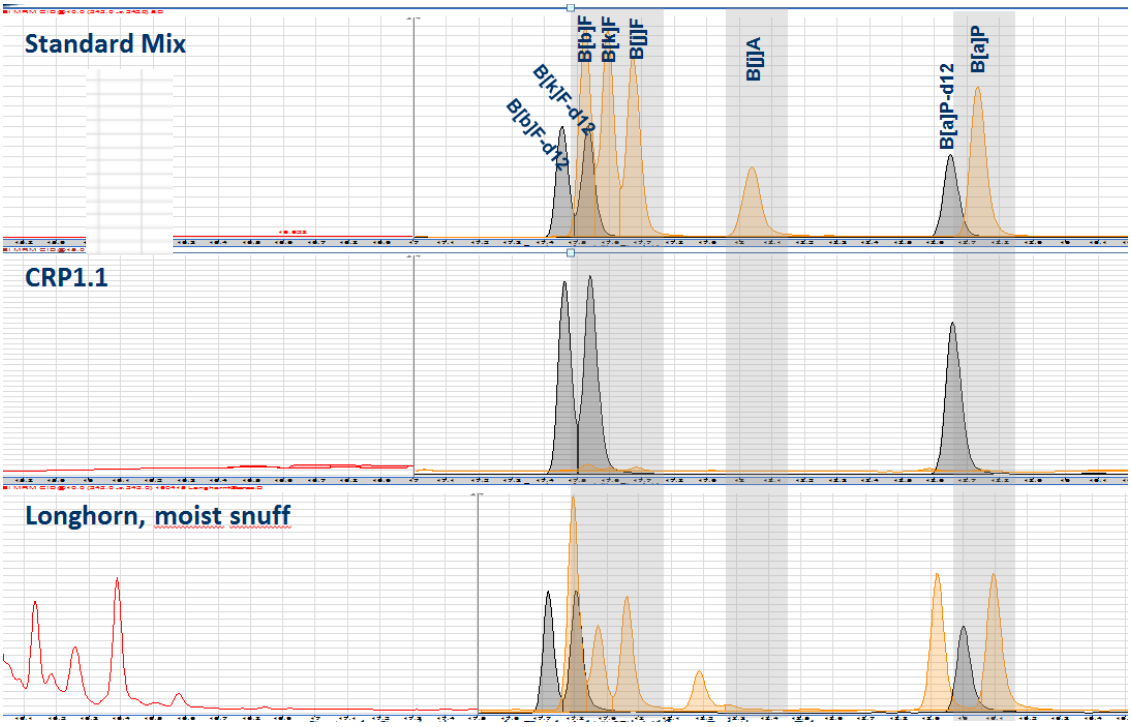
APPENDIX 1.D

MRM chromatograms of time segment 2 for standard mix, CRP1.1 and Longhorn moist snuff.



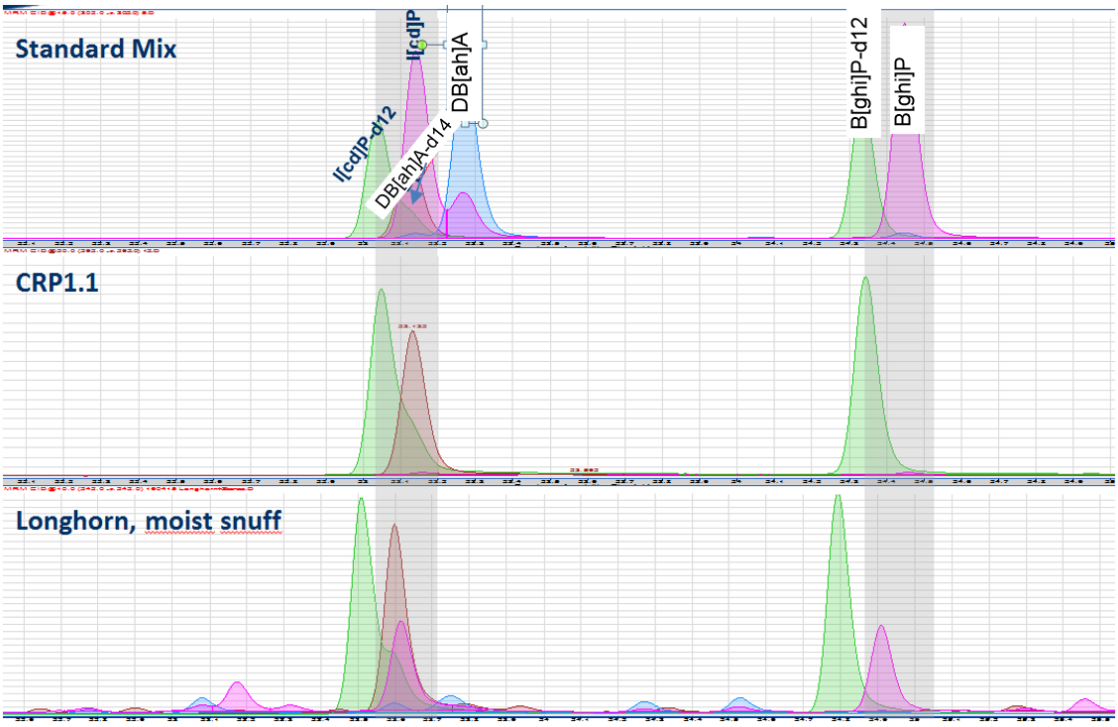
APPENDIX 1.E

MRM chromatograms of time segment 3 for standard mix, CRP1.1 and Longhorn moist snuff.



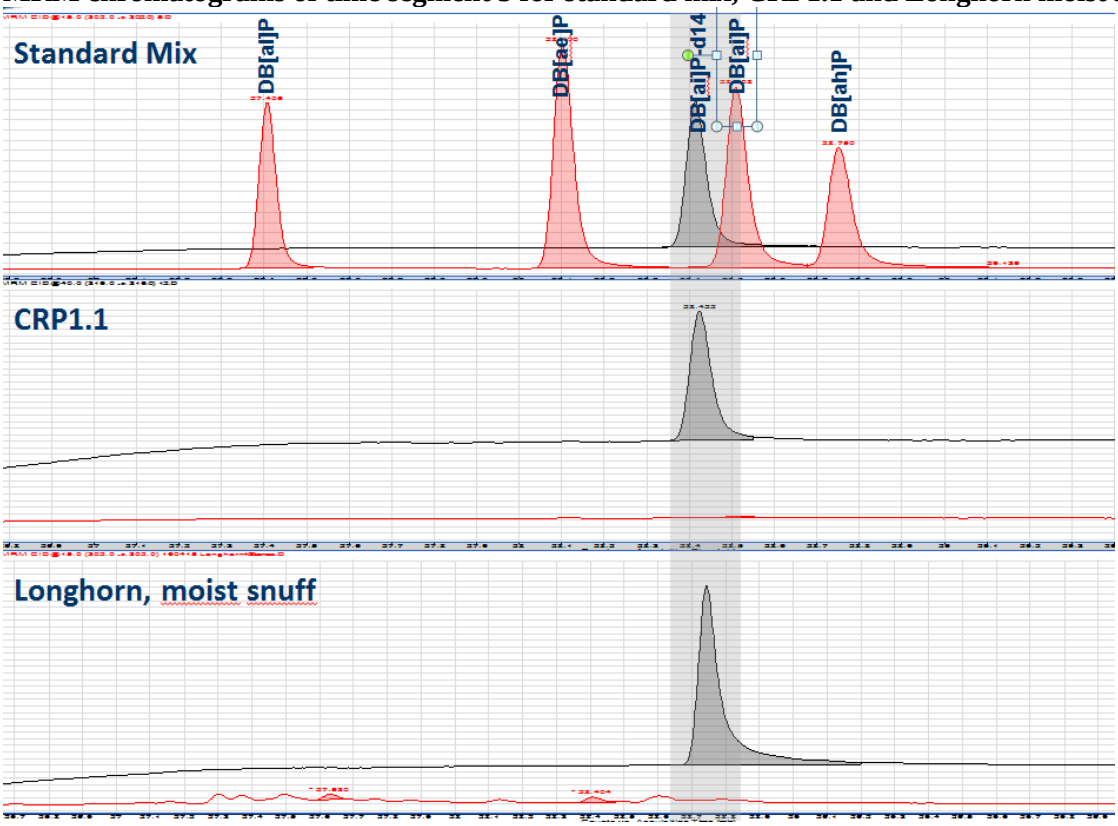
APPENDIX 1.F

MRM chromatograms of time segment 4 for standard mix, CRP1.1 and Longhorn moist snuff.



APPENDIX 1.G

MRM chromatograms of time segment 5 for standard mix, CRP1.1 and Longhorn moist snuff.



APPENDIX 2.

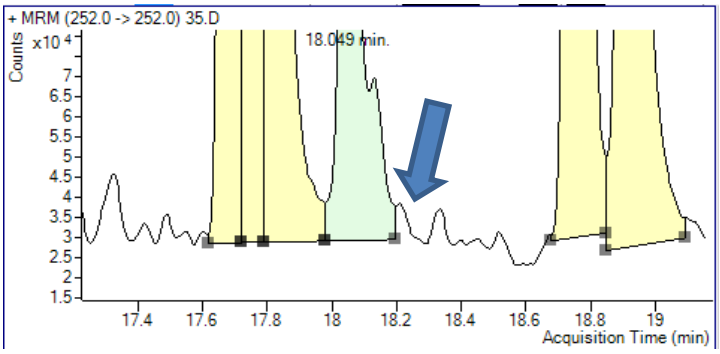
Take precautions when evaluating the chromatograms to obtain the correct identification, especially in these cases:

- B[a]A elutes before CHR and both have the same ion mass 228 (see Appendix 1D, same case for the internal standards).
- The order of elution is the following for these three stereoisomers: B[b]F, B[k]F and B[j]F, ordered by increasing retention time (see Appendix 1E).
- The order of elution is the following for these four stereoisomers: DB[a,l]P, DB[a,e]P, DB[a,i]P, DB[a,h]P, ordered by increasing retention time (see Appendix 1G).

The standard for B[j]A (from TRC) has a purity of only 70 % and the remaining 30 % belongs to the stereoisomer B[e]A which co-elutes with B[j]A, hence, the entire peak is integrated and used for quantification.

Analytes not present or at very low concentrations in a sample can be incorrectly identified as matrix interferences may be present with the same ion mass. In these cases, it is crucial to compare the retention time of the sample analyte to the calibration standards. Furthermore, the retention time window used to identify peaks in the chromatographic software should be minimized. These analytes are prone to be incorrectly identified due to close eluting matrix interferences:

- B[j]A/B[e]A
- DB[a,h]P
- DB[a,l]P



Chromatogram of a CRP2.1 sample extract showing the elution interval for B[j]A and B[e]A (co-elute). Wrong peak has been identified by the software (green filled peak). The correct peak for B[j]A and B[e]A is indicated with a blue arrow.

Special remarks and instrument maintenance:

- Retention times could shift during long analysis batches. It is crucial to verify and correct for these possible shifts before quantifying.
- There could be a substantial signal drop for the last four eluting compounds (dibenzopyrenes) within a long batch (>40 samples). The internal standard corrects for the signal drop but limit of quantification could be affected. It is recommended to run calibration standards at the beginning and the end of each batch.

- It is recommended to run a blank after the highest calibration standard to evaluate degree of carry over.
- Suggested maintenance to restore peak signal intensity: liner change, cut 15 cm off the beginning of the column and heat the system to 340 °C for about 30 min to 2 h. Alternative to cutting the column: use a deactivated fused silica pre-column, e.g. a 1 m × 0,32 mm from Agilent (part no. 160-2325-10), and connect using a press-fit union.