PAHs in Tobacco Filler and Cigarette Smoke

Mark Williams

Jana Ticha

British American Tobacco Group R&D UK

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PAHs in Tobacco Filler and Cigarette Smoke

A. Describe the different extraction steps used when analyzing PAHs in tobacco filler, smokeless tobacco, and cigarette smoke particulate and any applicable clean-up techniques used.

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Tobacco and STPs		TPM
Homogenisation[a] (grinding, cryomilling) Hydration (1:1 w/v overnight, unless >50% water)	**Sample pre-treatment**	Cigarette conditioning and smoking (rotary smoking machine, 20 cigs/sample)
Labelled internal standards Multi-step extraction (shaking) Saponification (alkaline ethanol) followed by liquid-liquid extraction[b] Soxhlet extraction	**Extraction**	Labelled internal standards Exhaustive extraction (shaking, ASE)
Base-modified silica adsorption chromatography SPE (silica, NH_2)	**Clean-up**	Base-modified silica adsorption chromatography SPE (mixed mode C_{18}/NH_2) SPE (silica, cyclohexyl)
HPLC/FLD HRGC/LRMS (SIM) HRGC/HRMS	**Analytical determination**	GC/LRMS GC/HRMS

[a]BAT GR&D internal methods

[b]Future work and/or work from collaboration partners

PAHs in Tobacco Filler and Cigarette Smoke

B. Discuss the optimal solvents, extraction solutions, standards, and reference tobacco product(s) needed during the extraction of PAHs from tobacco filler or, as applicable, a Cambridge filter pad.

Tobacco and STPs		TPM
Hexane/acetone (90/10, v/v)[a] Alkaline ethanol[b] Dichloromethane	Solvents	Cyclohexane Methanol Dichloromethane Toluene / Ethanol (9:1 v/v) (Masala *et al,* Anal Bioanal Chem. 401:3305-15)
Add labelled IS & equilibrate (30 min)	Standards	Add labelled IS & equilibrate (30 min) (+ add syringe std before GC/MS)
Mechanical shaking Centrifugation	Extraction solutions	Shaking (orbital) ASE Soxhlet extraction
3R4F tobacco CORESTA STP reference products	Reference materials	Control cigarette 3R4F CORESTA test piece CM7

[a]*BAT GR&D internal methods*

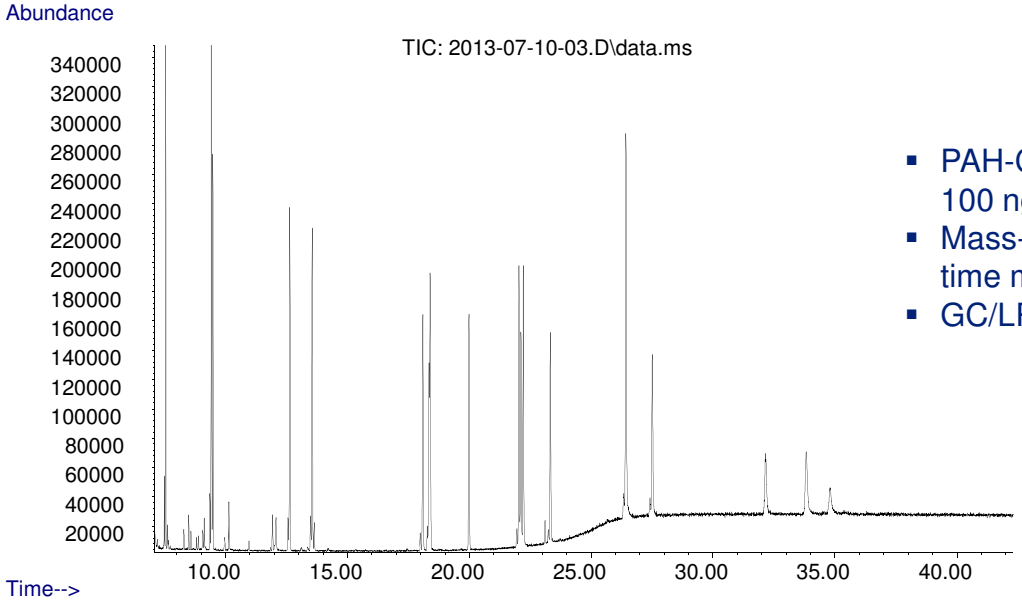
[b]*Future work and/or work from collaboration partners*

PAHs in Tobacco Filler and Cigarette Smoke

C. Discuss the rationale for using isotopically labelled internal standards instead of targeted surrogates or external standards for PAHs. Provide the number of isotopically labelled internal standards needed to calculate the amount of PAHs in a sample. .

- Stable isotope dilution (SID) is an inherently rugged technique of measurement by ratio.
- Requires mass selective detection, which gives added confidence in chemical identity.
- Mass-labelled analogues of 2 types are available – ^{2}D and ^{13}C.
- Mass-labelled analogues confirm the retention time of the target substance.

  - Theory - a single labelled analogue per homologue group is acceptable.
  - Practice - a labelled analogue per target substance accounts more fully for matrix artefacts.



- PAH-CVS5 (800 ng/mL native PAHs, and 100 ng/mL labelled analogues).
- Mass-labelled analogues act as retention time markers for native PAHs.
- GC/LRMS, scan data acquisition mode.

PAHs in Tobacco Filler and Cigarette Smoke

D. Discuss the challenges with isotopically labelled internal standards, including:
(1) The commercial availability of internal standards or their analogues

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- Suites of PAH standards (native and mass labelled) are commercially available *e.g.* from Wellington laboratories, Cambridge Isotope Laboratories (CIL) and Sigma Aldrich (native PAHs only).

Wellington laboratories:

- PAH-CVS-A calibration kit (23 native compounds and their 20 deuterated analogues, concentration range 2 ng/mL – 800 ng/mL over 5 concentration levels, 5 x 1.0mL): $1414
    - This calibration kit includes most of the PAHs included in the HPHC list, but not benz[j]aceanthrylene, benzo[b]furan and benzo[c]phenanthrene
- PAH-ISS-A syringe standards (3 labelled PAHs, 1.2mL, 2000 ng/mL): $158
- PAH-LCS-A recovery standards (17 labelled PAHs, 1.2ml, 2000 ng/mL): $423
- Benz[j]aceanthrylene, benzo[b]furan and benzo[c]phenanthrene – not part of the suites - total cost for pure standards approx. $461
- *Note:* No commercial supplier was found for benz[j]aceanthrylene. Mix of benz[j]aceanthrylene and benz[e]aceanthrylene (70:30) available from Toronto Research Chemicals (50mg, $1400)

Cambridge Isotope Laboratories

- CIL ES-4087 (US EPA 16, 13C-labelled) costs $1350 for 1.2mL @ 5µg/mL in nonane – but does not include all analogues from FDA list
- CIL ES-5164-1.2 (EPA 16, 2H-labelled) costs $425 for 1.2mL @ 200µg/mL

Suppliers of individual standards

- QMX, Sigma Aldrich, Toronto Reseach Chemicals

PAHs in Tobacco Filler and Cigarette Smoke

D. Discuss the challenges with isotopically labeled internal standards, including:
(2) Individual v. mixture of internal standards, cost of internal standards.

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- Pre-mixed PAH standards reduce effort needed for preparation and reduce risk of errors in preparation or differences in application between laboratories.
- Certification provides increased traceability (ISO 17025), but manufacturers do not indicate ISO Guide 34 accreditation.
- Good repeatability (concentration, purity) from batch to batch facilitates method stability over time and between laboratories.
- Standard suites include batch-matched calibration, recovery and surrogate standards from the same source to minimise risk of errors or product quality failure (*e.g.* contamination with unlabelled PAHs).
- Suites of standards provided commercially are compatible with EPA method approaches (*e.g.* EPA 8270, EPA 625, EPA 610).

Cost of standard consumption per sample

- For a batch of 20 samples with addition of 100ng (50 µL) of ISS and 100ng (50 µL) of LCS to each test extract and 5-point calibration at start & end of batch, cost of standards per sample is approximately $29.

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D. Discuss the challenges with isotopically labeled internal standards, including:
(3) Deuterated v. 13C labeled internal standards.

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- ^{13}C-labelled analogues have 2 advantages in comparison to ^{2}D-labelled analogues;
  - unaffected by deuterium-protium exchange under acidic and basic conditions;
  - provide similar mass spectra to native substance (deuterated analogues can undergo different mass losses if a deuterated moiety fragments).
- ^{13}C-labelled analogues are provided by fewer suppliers (CIL), in lower quantities, at higher cost, longer lead time and at different concentration ranges to ^{2}D analogues.

- PAHs are chemically stable (away from direct light) with potentially long shelf life for standards; control of concentration (*e.g.* tracking mass change due to solvent loss) becomes important.
- Accurate dispensing of small volumes (*e.g.* 50 µL) requires careful use of appropriate positive displacement pipettors and gravimetric audit trail.

PAHs in Tobacco Filler and Cigarette Smoke

D. Discuss the challenges with isotopically labeled internal standards, including:
(4) Concerns of proton exchange with deuterated labeled internal standards.

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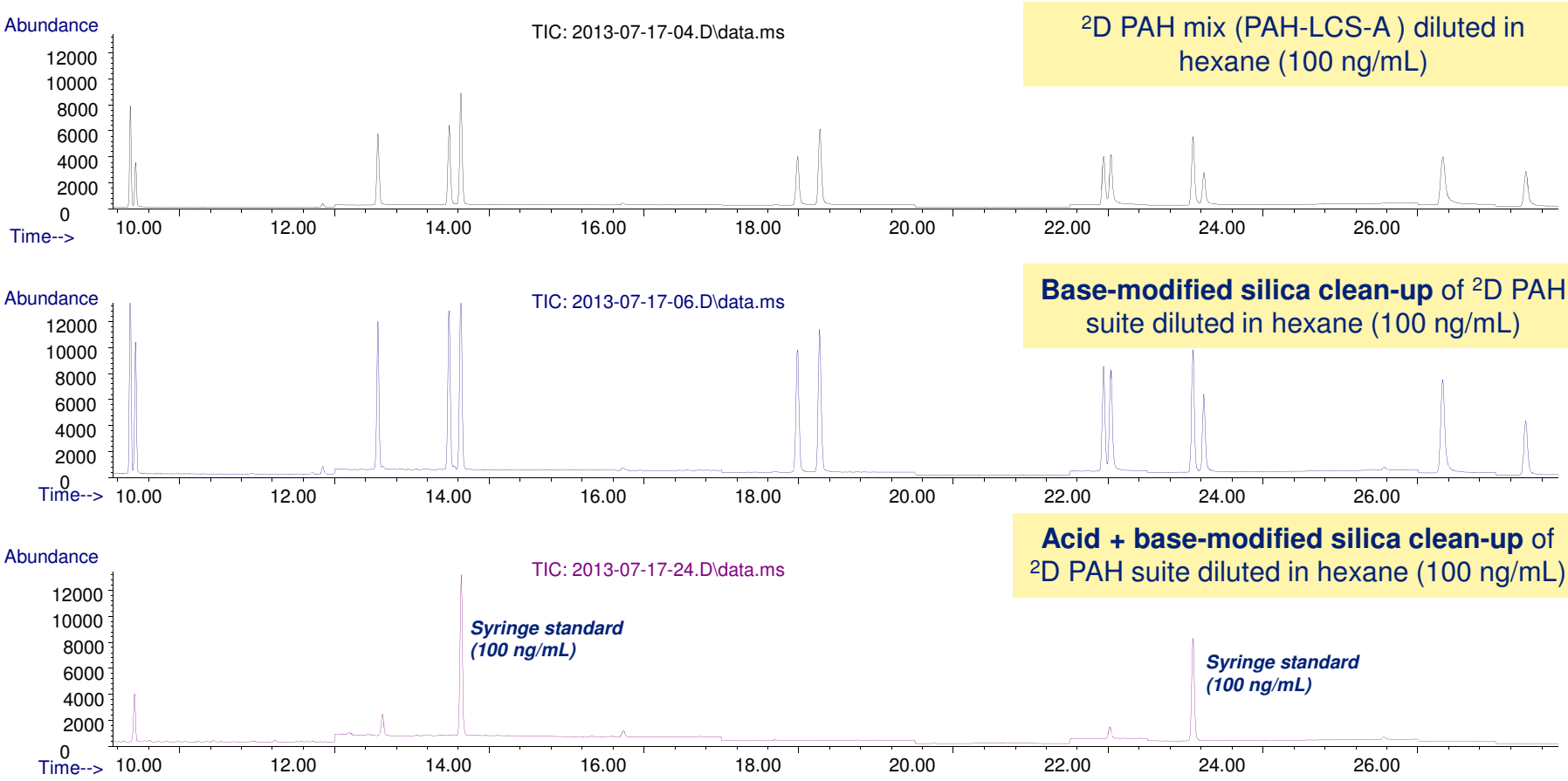

^{2}D PAH mix (PAH-LCS-A) diluted in hexane (100 ng/mL)

Base-modified silica clean-up of ^{2}D PAH suite diluted in hexane (100 ng/mL)

Acid + base-modified silica clean-up of ^{2}D PAH suite diluted in hexane (100 ng/mL)

Deuterated analogues are unstable in strongly acidic media

PAHs in Tobacco Filler and Cigarette Smoke

E. Discuss the typical concentration ranges for each of the PAHs listed in this document and any potential method adjustments to accommodate for different cigarette strengths and physical parameters.

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STP[a]

Analyte	Average PAH concentration for each product type (ng/g DWB)							
	Loose Snus	Portion Snus	Chewing Tobacco	Dry Snuff	Hard Pellet	Soft Pellet	Moist Snuff	Plug
Naphthalene	96.6	112	54	84.9	69.7	76.5	110	53.4
1-methylnaphthalene	32.2	43	20.1	75.2	19.2	74.2	62	18.3
2-methylnaphthalene	17.6	27	10.4	60	10.5	65.6	46.9	9.51
Acenaphthylene	4.4	6.68	5.3	62.6	4.79	81.6	52.9	2.68
Acenaphthene	4.86	8.65	3.4	49.5	5.31	79.6	48.3	4.39
Fluorene	21.8	29.3	15.6	429	21.3	695	437	8.89
Phenanthrene	73	76.3	207	2925	40	4541	3339	71.1
Anthracene	10.1	11.6	38.6	625	7.58	1210	731	13.3
Fluoranthene	57.7	47.7	183	1512	9.84	2550	1854	67.9
Pyrene	42.8	36.6	163	1520	10.6	2519	1856	63.9
Benzo[a]anthracene	7.57	6.85	32.9	456	2.6	832	545	14.8
Chrysene	12.6	11.8	37.1	468	3.57	789	543	17
Benzo[b]fluoranthene	3.36	3.03	7.3	74.2	0.938	97.6	80	3.35
Benzo[k]fluoranthene	2.06	1.95	3.7	32.3	0.644	38.4	34	1.87
Benzo[j]fluoranthene	2.48	2.39	5.9	55.9	0.688	65	57.9	2.89
Benzo[e]pyrene	2.5	2.26	6	65.8	0.821	77.4	67.7	2.8
Benzo[a]pyrene	2.93	2.53	6	80.4	0.971	117	87.4	3.25
Perylene	0.715	0.7	1.1	11.2	NQ	13.7	11.1	0.307
Dibenz[a,h]anthracene	NQ	0.55	0.5	5.7	BDL	5.67	6.67	NQ
Indeno[1,2,3-cd]pyrene	2	1.47	3.6	31	0.452	26.1	35.8	1.59
Benzo[ghi]perylene	2.03	1.47	3.4	27.9	0.479	17.4	33.7	1.47

NQ = Not Quantified; BDL = Below Detection Limit

[a] from McAdam et al, 2013 (submitted manuscript)

Tobacco filler[b]

B[a]P	Concentration (ng/g)
3R4F blend	8.8
Virginia lamina	45
Burley lamina	8

[b] BAT internal data

TPM: 3R4F, GC/HRMS[c]

Name	Concentration (ng/cig)	LOD (ng/cig)	Recovery (%)
Naphthalene	27.42	0.20	81
Acenaphthylene	36.51	0.23	86
Acenaphthene	49.87	0.16	ND
Fluorene	8.89	0.34	ND
Phenanthrene	31.60	0.10	107
Anthracene	26.84	0.09	ND
Fluoranthene	18.80	0.09	187
Pyrene	16.64	0.09	ND
Benzo[b]napth-thiophene	0.25	0.05	ND
Benzo(c)phenanthrene	1.01	0.02	ND
Benzo(a)anthracene	8.00	0.01	108
Chrysene	10.68	0.01	125
Cyclopenta (c,d)pyrene	0.46	0.01	ND
Benzo(b)fluoranthene	3.77	0.01	111
Benzo(k)fluoranthene	1.45	0.01	115
Benzo(j)fluoranthene	1.83	0.01	ND
Benzo(a)pyrene	3.93	0.01	93
Cholantherene	0.17	0.01	ND
Indeno(1,2,3-cd)pyrene	1.56	0.01	84
Benzo(ghi)perylene	1.36	0.01	87
Dibenzo(ah)anthracene	0.05	0.01	78
Anthranthrene	0.75	0.01	ND
Benzo(a,i)pyrene	0.06	0.01	ND

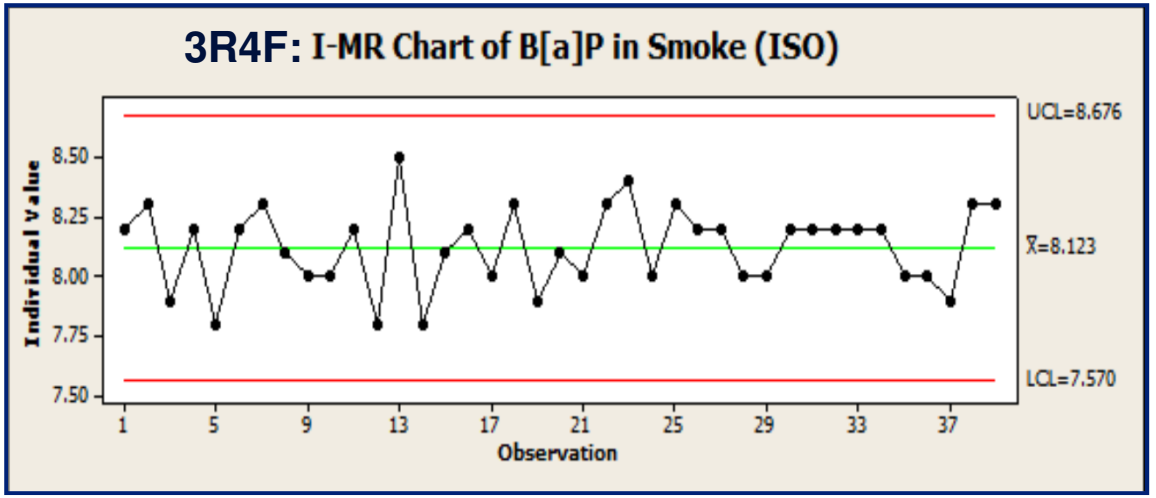
ND = Not Determined

[c] Data provided by Karl Pettit, Marchwood Scientific Services, UK

PAHs in Tobacco Filler and Cigarette Smoke

F. Discuss the major sources of method variability, e.g., include sources from the smoking machine or regimen, sample preparation, separation, and detection of different tobacco product types and strengths.

- For tobacco filler and STPs, sample homogeneity and extraction efficiency require attention to avoid impact on quality of results.
- Matrix artefacts can bias results for PAHs that do not have an exact match IS.
- TPM delivery differs between products but is stable for individual products.
- For smoke samples care is required in clean-up to dry the SPE column before elution.
- Evaporative and adsorptive losses should be minimised during sample workup to avoid effects on LoQ, but are corrected for by SID.
- Naphthalene is not completely retained in TPM and may be further lost during sample handling.



PAHs in Tobacco Filler and Cigarette Smoke

G. Discuss the different methods necessary to separate and detect for PAHs. Provide the number of methods and steps typically used for each from extraction to detection.

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Tobacco, STP

- Saponification
- Hydration (water 1:1, w/v, overnight)
- Extraction → Mixed polarity solvents (hexane / acetone); Shaking (repeated 3x) DCM, ASE, Soxhlet extraction
- Concentration → Turbovap 0.5mL, redissolve hexane
- Clean-up → Base-modified silica adsorption chromatography SPE (silica, CH, -NH_2)
- Concentration → Turbovap 0.5mL, redissolve acetonitrile, iso-octane
- Analysis → HPLC/FLD GC/LRMS (SIM) GC/HRMS (SIM)

TPM

- Extraction → Cyclohexane shaking 1 hour, methanol, DCM Mixed polarity solvents (toluene/ethanol; 9:1, v/v) ASE, Soxhlet extraction
- Clean-up 1 → Base-modified silica adsorption chromatography SPE (mixed mode C_{18}/NH_2)
- (Clean-up 2)* → SPE (silica, CH, -NH_2)
- Concentration → Turbovap 0.5mL, redissolve cyclohexane
- Analysis → GC/LRMS (SIM) GC/LRMS GC/HRMS

**possible requirement for GC/LRMS analysis*

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H. Discuss specific method challenges and limitations when analyzing PAHs, including: (1) Isomer separation and identification

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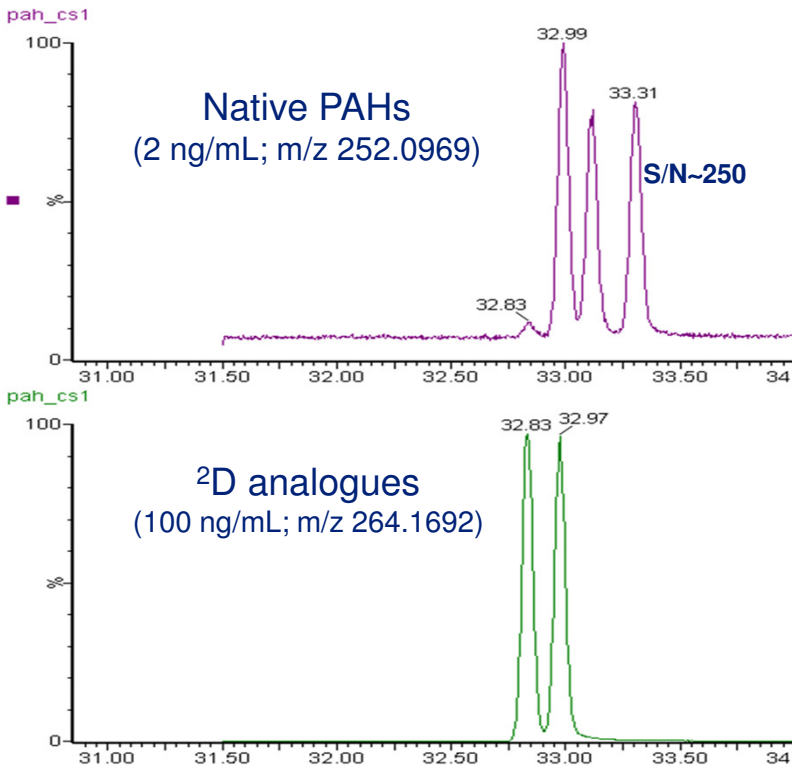
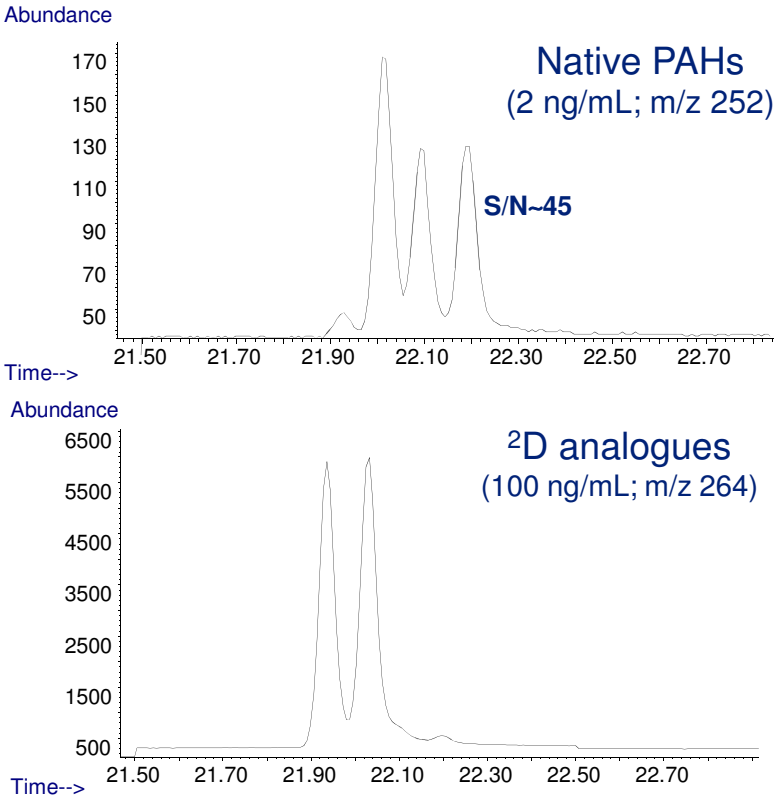
GC/HRMS (magnetic sector)	GC/LRMS (single quad)
DB-EUPAH (60m × 0.25mm × 0.25µm)	VF-17ms (30m × 0.25mm × 0.25µm)

Example: separation of benzofluoranthenes

Standard PAH-A-CS1





GC/HRMS data provided by Karl Pettit, Marchwood Scientific Services, UK

PAHs in Tobacco Filler and Cigarette Smoke

H. Discuss specific method challenges and limitations when analyzing PAHs, including:
(2) Effects of tobacco blend

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- The main effects on tobacco blend / STPs include:
  - Moisture content ⇔ samples require hydration to maximise extraction
  - Mixed polarity solvents
    - *E.g.* hexane / acetone (polarity index 5.1 for acetone and 0 for hexane)

- Determination of B[a]P
  - Matrix effects ⇒ chromatographic conditions require adjustment for some matrices to avoid co-elution of matrix artefacts with B[a]P

B[a]P	Average PAH concentration for each tobacco product type (ng/g DWB)[a]								
	Loose Snus	Portion Snus	Chewing Tobacco	Dry Snuff	Hard Pellet	Soft Pellet	Moist Snuff	Plug	3R4F blend[b]
	2.93	2.53	6.0	80.4	0.971	117	87.4	3.25	8.8

[a] *from* McAdam *et al*, 2013 (submitted manuscript)
[b] *internal quality control sample (result not presented in the manuscript)*

PAHs in Tobacco Filler and Cigarette Smoke

H. Discuss specific method challenges and limitations when analyzing PAHs, including:
(3) Low v. high molecular weight PAHs (volatility and sensitivity)

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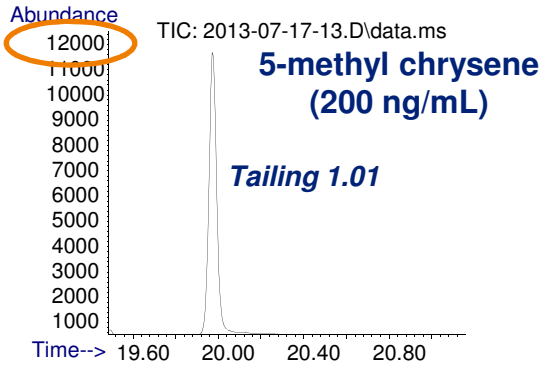
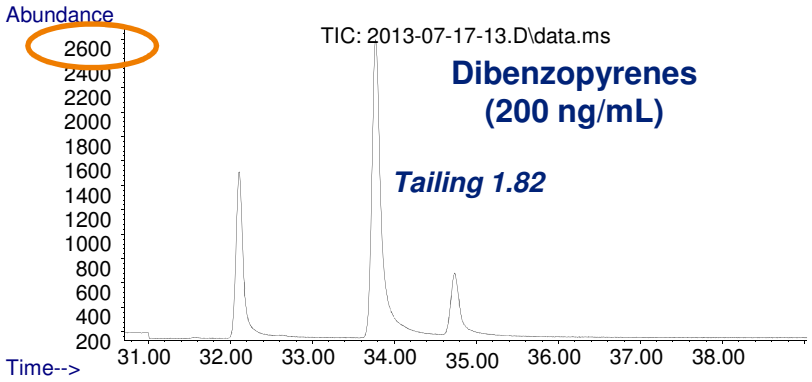
- Naphthalene partition in smoke
  - Vapour phase (ISO): ~ 13 ng/cig 3R4F
  - Particulate phase (ISO): ~ 27 ng/cig 3R4F

- High GC/MS background for 2 and 3 ring substances
- GC conditions are extreme: injector 300℃ or higher; upper isothermal T = 320℃ or higher; transfer line T = 300℃ or higher – otherwise peak broadening for high BP PAHs





- Solvents – aromatic solvents (*e.g.* toluene) theoretically give greater extraction, but increase time and complexity (*e.g.* solvent exchange) and propensity for losses

PAHs in Tobacco Filler and Cigarette Smoke

I. Discuss the differences in separation, detection, and limits of detection/quantitation when comparing gas chromatography/mass spectrometry (GC-MS), liquid chromatography/ultraviolet detection (LC-UV), and liquid chromatography/mass spectrometry (LC-MS) for PAH analysis.

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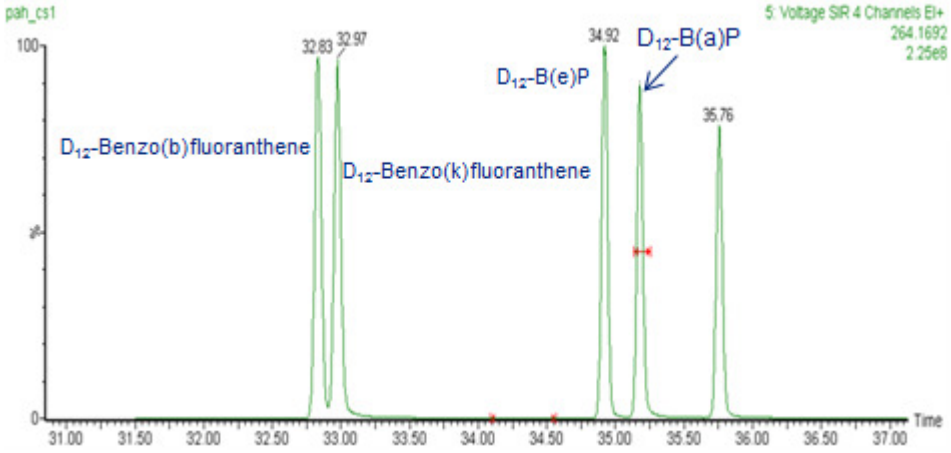
- **HPLC/Fluorescence – constrains use of mass-labelled internal standards**
- **GC/LRMS - limited signal/noise, artefactual inteferences**
- **GC/HRMS – improved signal/noise and much cleaner baseline**
- **HPLC/MS problematic – APPI/MS (Sciex API5000) difficult to optimise, esp. ionisation conditions**

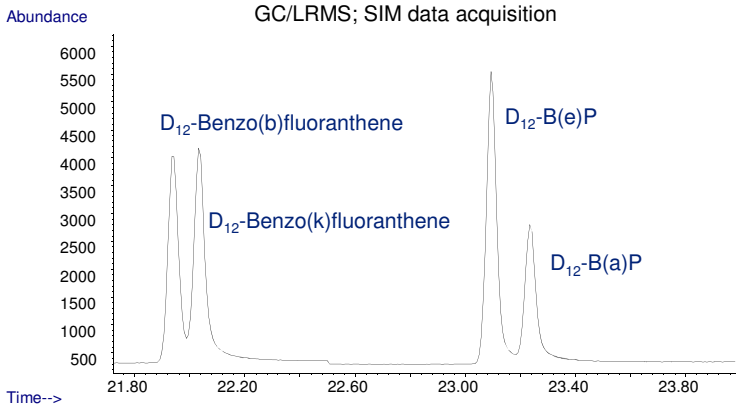
Example: Sensitivity of detection (100 ng/mL PAH internal standard mix)

GC/HRMS (magnetic sector)



GC/HRMS data provided by Karl Pettit, Marchwood Scientific Services, UK

GC/LRMS (single quad)



S/N: D_{12}-B[a]P (m/z 264)	
GC/HRMS	GC/LRMS
1187	145

PAHs in Tobacco Filler and Cigarette Smoke

I. Discuss the differences in separation, detection, and limits of detection/quantitation when comparing gas chromatography/mass spectrometry (GC-MS), liquid chromatography/ultraviolet detection (LC-UV), and liquid chromatography/mass spectrometry (LC-MS) for PAH analysis. (Continued)

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	HPLC/FLD	GC/LRMS	GC/HRMS	HPLC/MS
Number of internal standards	1	20	20	20 (in theory)
Chromatographic selectivity	High (RP[a] or PGC[b])	High (VF-17)	V High (DB-EUPAH, BPX5)	Not established
LoQ for B[a]P in cigarette smoke	Not used	1.25 ng/cig	0.2 ng/cig	Not established
LoQ for B[a]P in STP/filler	0.38 ng/g	0.221 ng/g wwb	0.47 ng/g wwb	Not established

[a]RP = Reverse Phase
[b]PGC = Porous Graphitic Carbon

PAHs in Tobacco Filler and Cigarette Smoke

Summary of main observations

- Stable Isotope Dilution provides enhanced precision of measurement.
- Deuterated analogues are unstable in strongly acidic clean-up media, but are stable in base-modified media.
- The extraction of PAHs from tobacco filler and STPs is maximised by (a) grinding / cryomilling, (b) hydration and (c) the use of mixed polarity solvents.
- Selection of extraction solvent is critical. For example, methanol removes all TPM from the Cambridge Filter Pad, recently published studies suggest that aromatic solvents and intensive extraction (ASE/PLE) can increase the recovery of PAHs from diesel particulates.
- GC/HRMS (Micromass Autospec Ultima, 10 000 resolution) provides enhanced signal/noise and sensitivity over GC/LRMS.
- Capacity of ~ 200 tests per week on a single instrument for B[a]P in cigarette smoke.
- Capacity of ~ 100 tests per week on a single instrument for B[a]P in tobacco filler.