TSNAs in Tobacco Filler and Cigarette Smoke

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TSNAs in Tobacco Filler and Cigarette Smoke

A. Describe the different extraction steps used when analyzing TSNAs in tobacco filler, smokeless tobacco, and cigarette smoke particulate.

Tobacco Filler and STPs		Smoke
Homogenisation (grinding, cryomilling)	**Sample pre-treatment**	**Cigarette conditioning and smoking**
Hydration 1:1 (w/v) for in house method		
• Labelled or unlabelled internal standards • Extraction with methanol, ammonium acetate or dichloromethane • Extraction by shaking or sonication	**Extraction**	• Labelled or unlabelled internal standards • Extraction with methanol, ammonium acetate or dichloromethane • Extraction by shaking or sonication
• Filtration using PTFE/PVDF filter • Reversed Phase SPE • Cation exchange SPE	**Clean-up**	• Filtration using PTFE filter • SPE on alumina column
LC-MS/MS **GC-TEA**	**Analytical determination**	**LC-MS/MS** **GC-TEA**

BAT Group R&D procedures **Procedures used by research partner laboratories**

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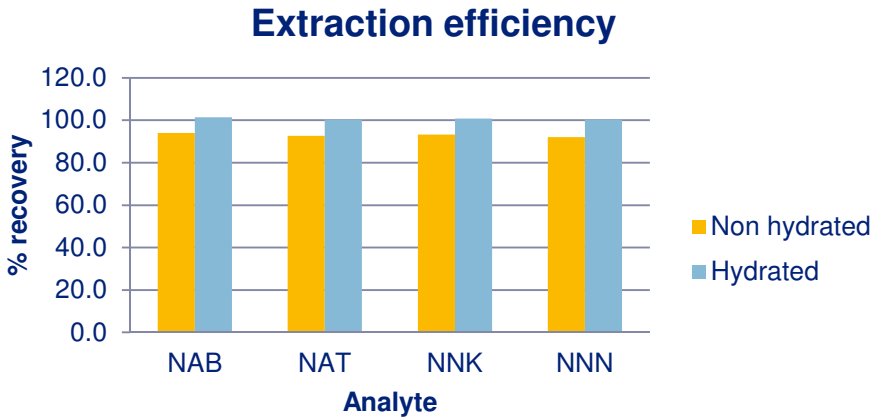
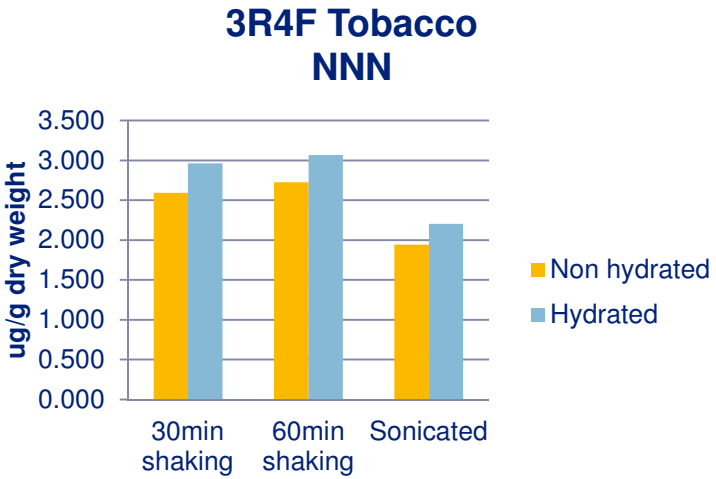
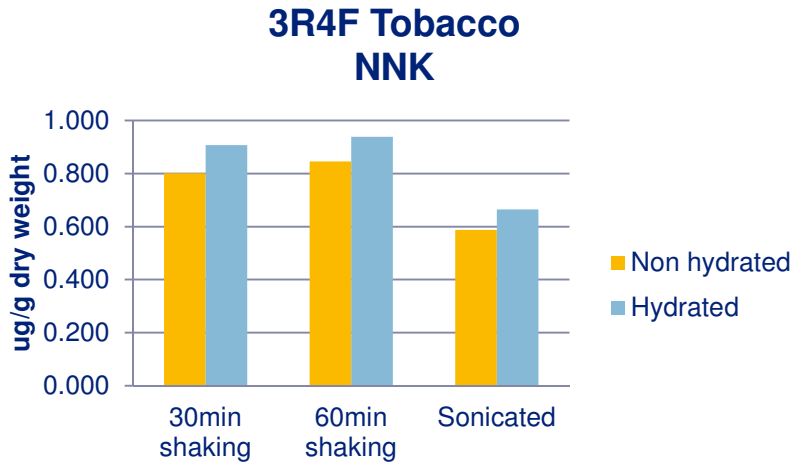
A. Describe the different extraction steps used when analyzing TSNAs in tobacco filler, smokeless tobacco, and cigarette smoke particulate.

Comparison of extraction efficiency of hydrated vs non-hydrated tobacco



Comparison of extraction technique and hydration on extraction efficiency





TSNAs in Tobacco Filler and Cigarette Smoke

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

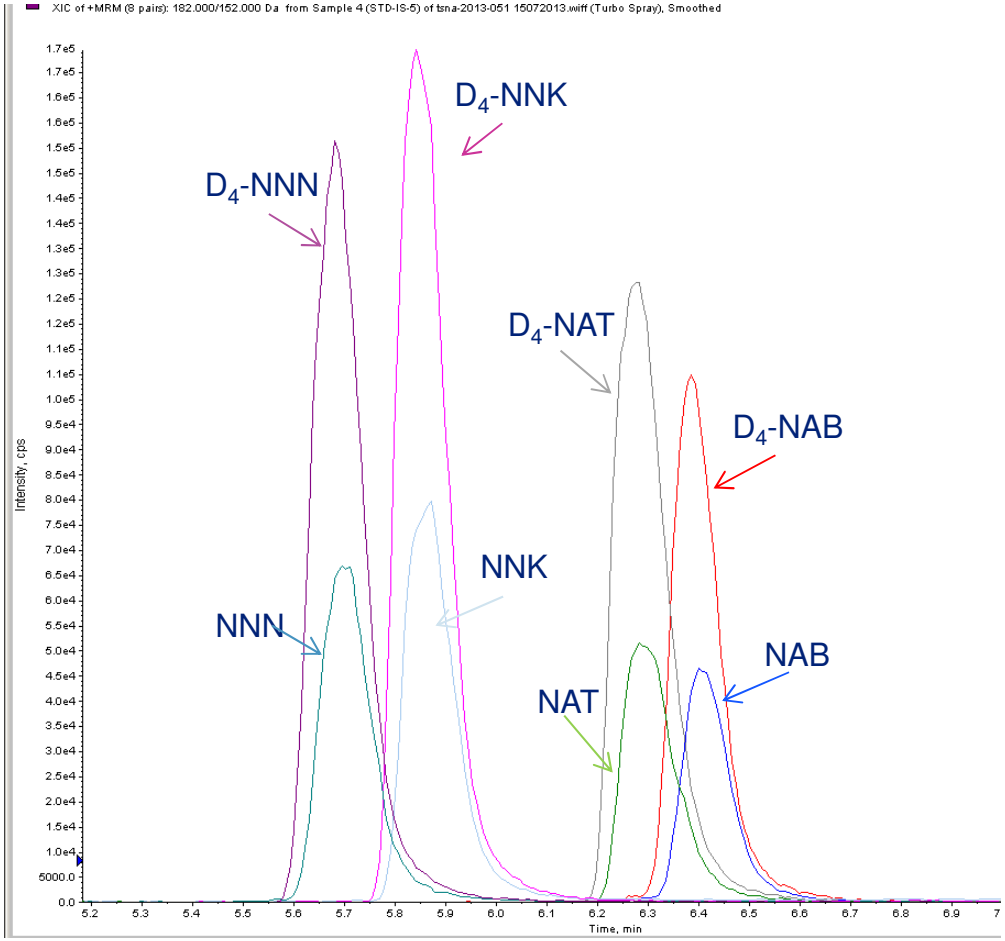
Tobacco and STPs		Smoke
Methanol after hydration (1:1 w/v, overnight) Ammonium acetate (100 mM) Dichloromethane	Solvents	Methanol Ammonium acetate (100 mM) Dichloromethane
Add labelled IS	Standards	Add labelled IS
Mechanical shaking (180 rpm, 60 min) Sonication	Extraction solutions	Mechanical shaking (200 rpm, 30 min) Sonication
3R4F tobacco	Reference materials	Control cigarettes 3R4F

BAT Group R&D procedures Procedures used by research partner laboratories

TSNAs in Tobacco Filler and Cigarette Smoke

C. Discuss the rationale for using isotopically labeled internal standards, instead of targeted surrogates or external standards for TSNAs. Provide the number of isotopically labeled internal standards needed to calculate the amount of TSNAs 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 for ^{1}H or ^{13}C for ^{12}C.
- Mass labelled analogues confirm the retention time of 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
- Matrix suppression and loss of sensitivity throughout the run means the use of external calibration is inappropriate



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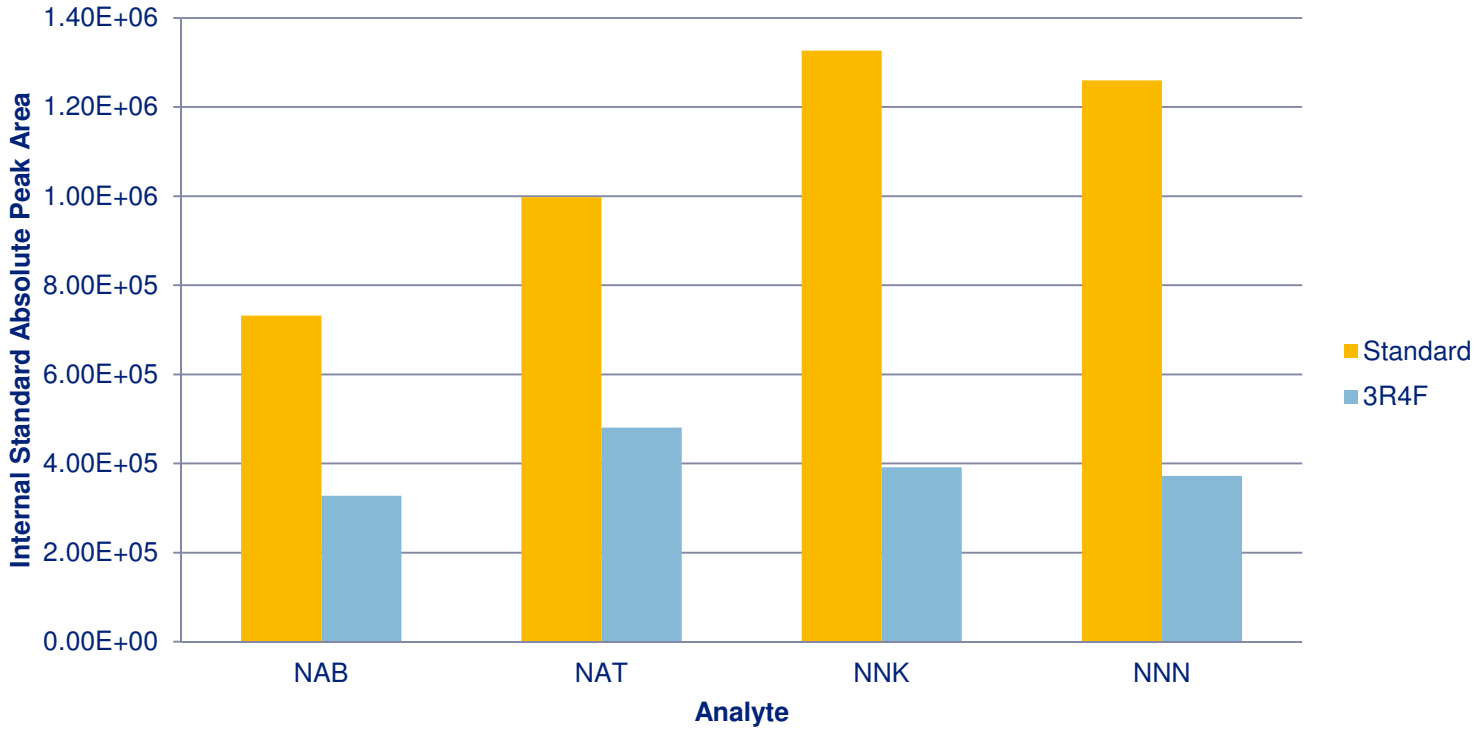
- The effect of matrix suppression.
  Comparison of instrument response for same concentration of analytes in (a) solvent (yellow) and (b) cigarette smoke extract (blue)



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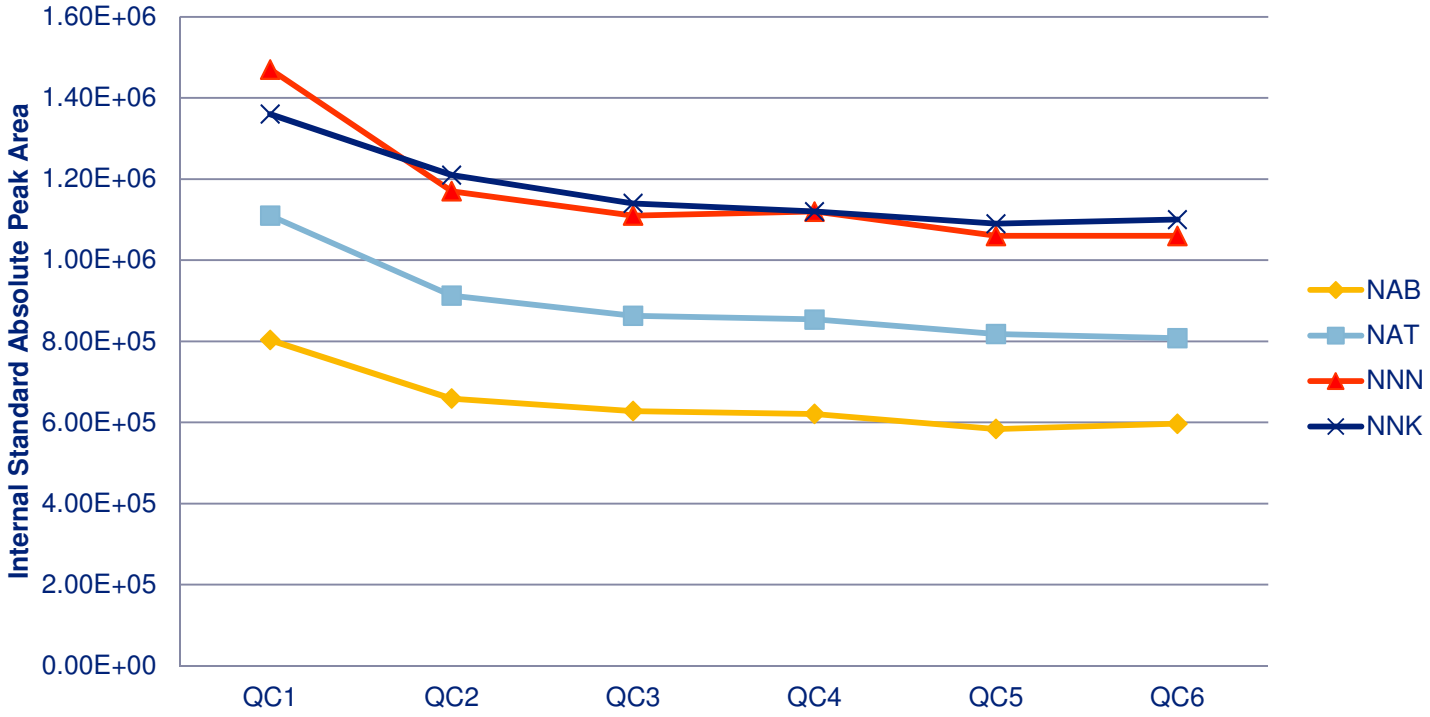
- **Comparison of LC-MS/MS response for drift check standard injected after every 10th sample**



- **Isotopically labelled internal standards adjust automatically for drift in instrument sensitivity and/or matrix suppression**

TSNAs 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.

- D_4 deuterated TSNA standards are available from the following suppliers:
  - Kinesis (supplies from AccuStandard)
  - QMX (supplies from Dr. Ehrenstrofer-Schafers)
  - Toronto Research Chemicals
- Appropriate cost $7000 – $12000 for 30mL of solution (~200 000 tests)
- Lead time approximately 3-4 weeks
- None of the mentioned suppliers have ISO Guide 34 accreditation for producing deuterated TSNAs
- The purity of the standards changes from batch to batch
- Problem with incomplete deuteration

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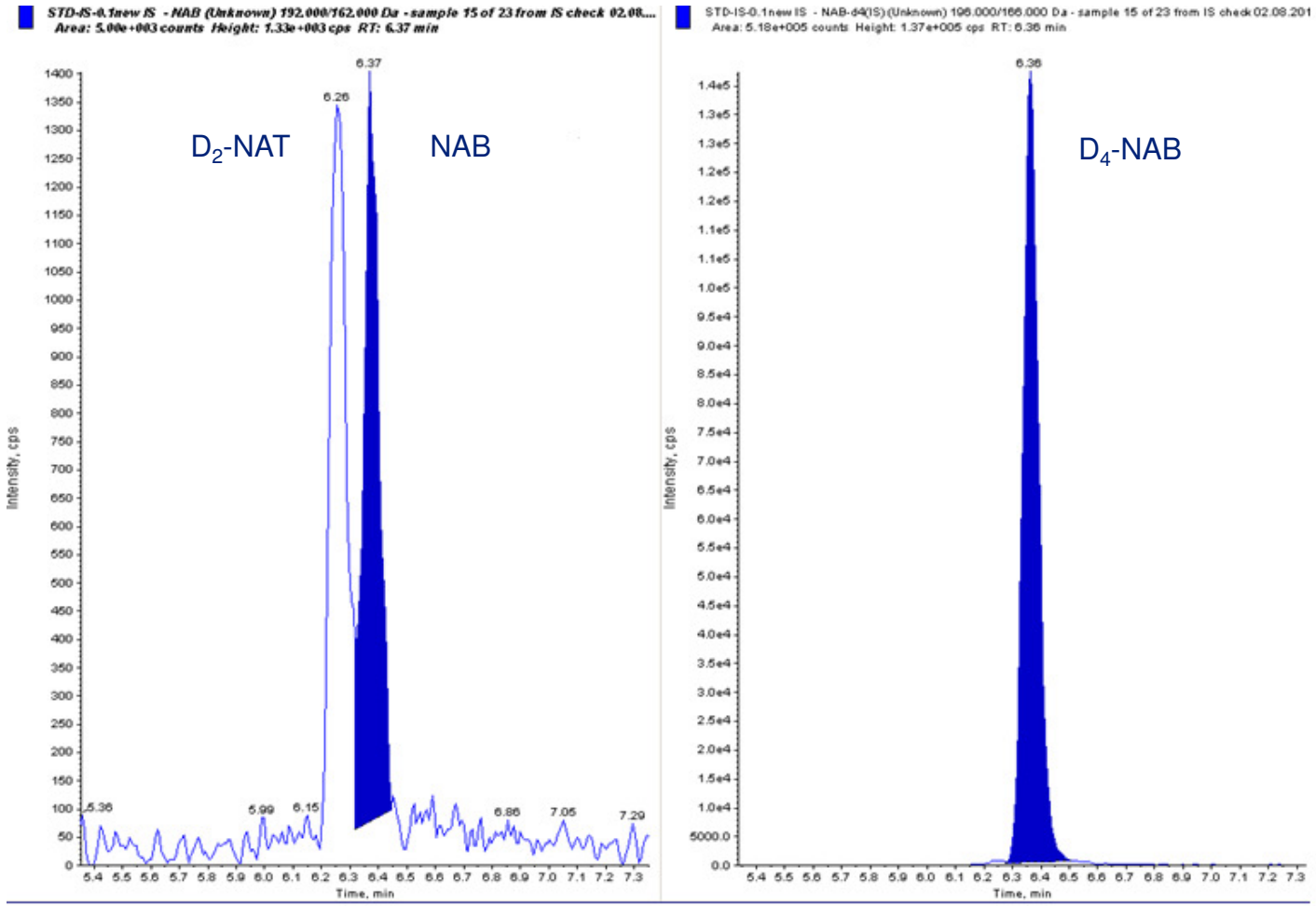


D_2-NAT can interfere with NAB detection at low concentrations. D_2-NAT is a possible contaminant of D_4-NAT

TSNAs 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

- Pre-mixed TSNA standards reduce effort needed for preparation and reduce risk of errors in preparation or differences in application between laboratories

Cost of D4 mixed internal standard: $7000 – $12000 for 30 mL

Cost of native mixed standard stock: $4000 for 30 mL

- ISO guide 34 unlabelled standards are available
- Uncertainty of certified value:
  - < 5% NAT, NNN, NNK
  - <10% NAB

(3) Deuterated v. 13C labeled internal standards.

We are not aware of a commercial provider of ^{13}C-labelled TSNA standards

TSNAs 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.

- There is no concern about deuterium-protium exchange as the method does not use strongly acidic or basic conditions
- MCX-SPE cation exchange cleanup does not affect recovery of deuterated TSNAs

TSNAs in Tobacco Filler and Cigarette Smoke

E. Discuss the typical concentration ranges for total TSNAs, NNN, and NNK and any potential method adjustments to accommodate for different cigarette strengths and physical parameters.

Range of data reported over 12 month period for in house method

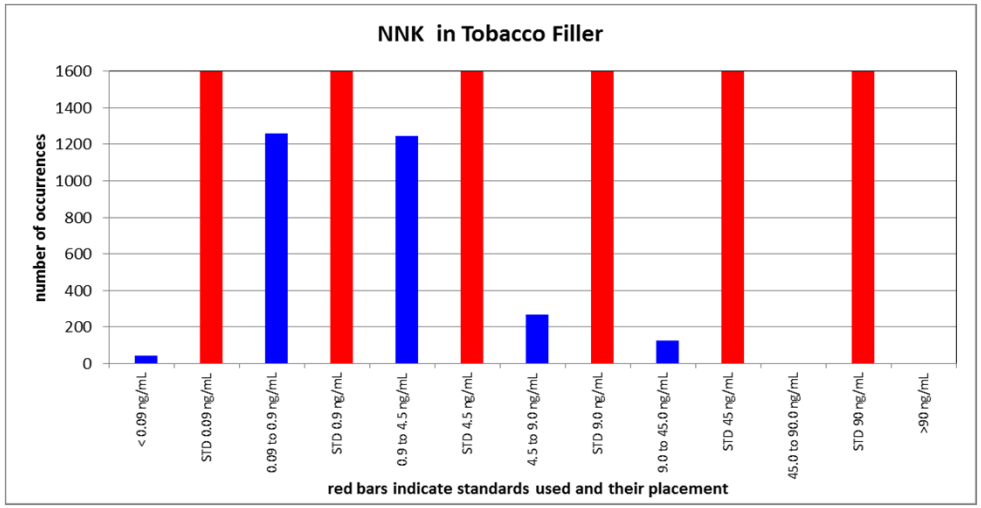
Standards = 0.09 – 90 ng/mL

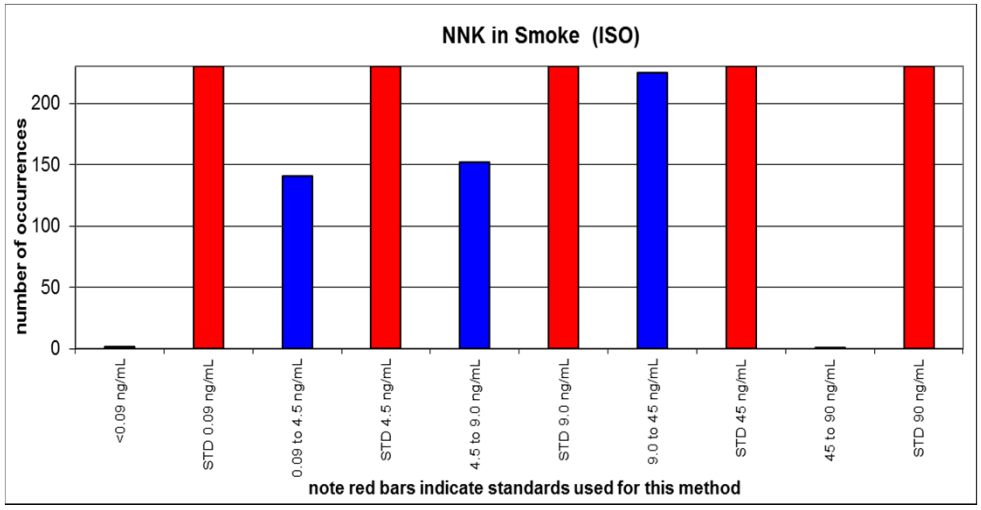
Tobacco Filler = 3.6 – 3600 ng/g

Smoke = 3.6 – 3600 ng/cig

Calibration standards (red) fully bracket samples (blue)

Calibration range appropriate





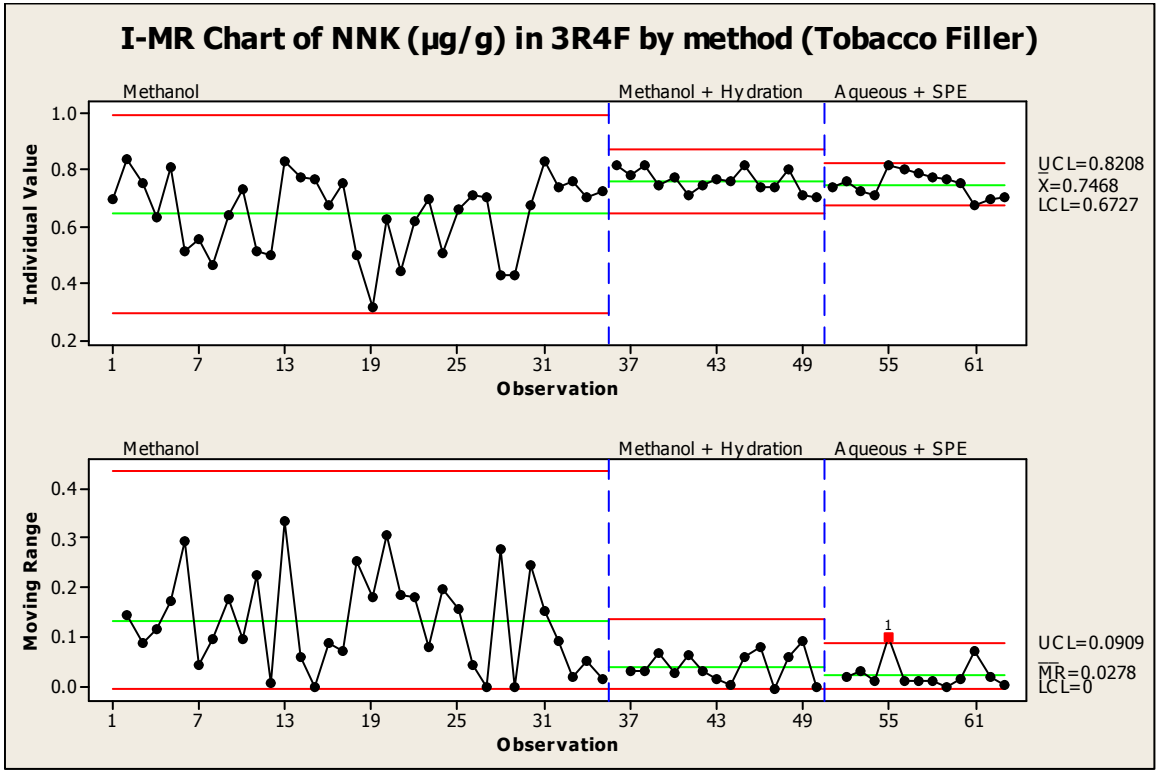
TSNAs in Tobacco Filler and Cigarette Smoke

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

Solvent choice influences recoveries and uncertainty of the method

Aqueous extraction + SPE produces comparable results to methanol with hydration

NB Accuracy cannot be adequately assessed – no certified reference materials / standards.



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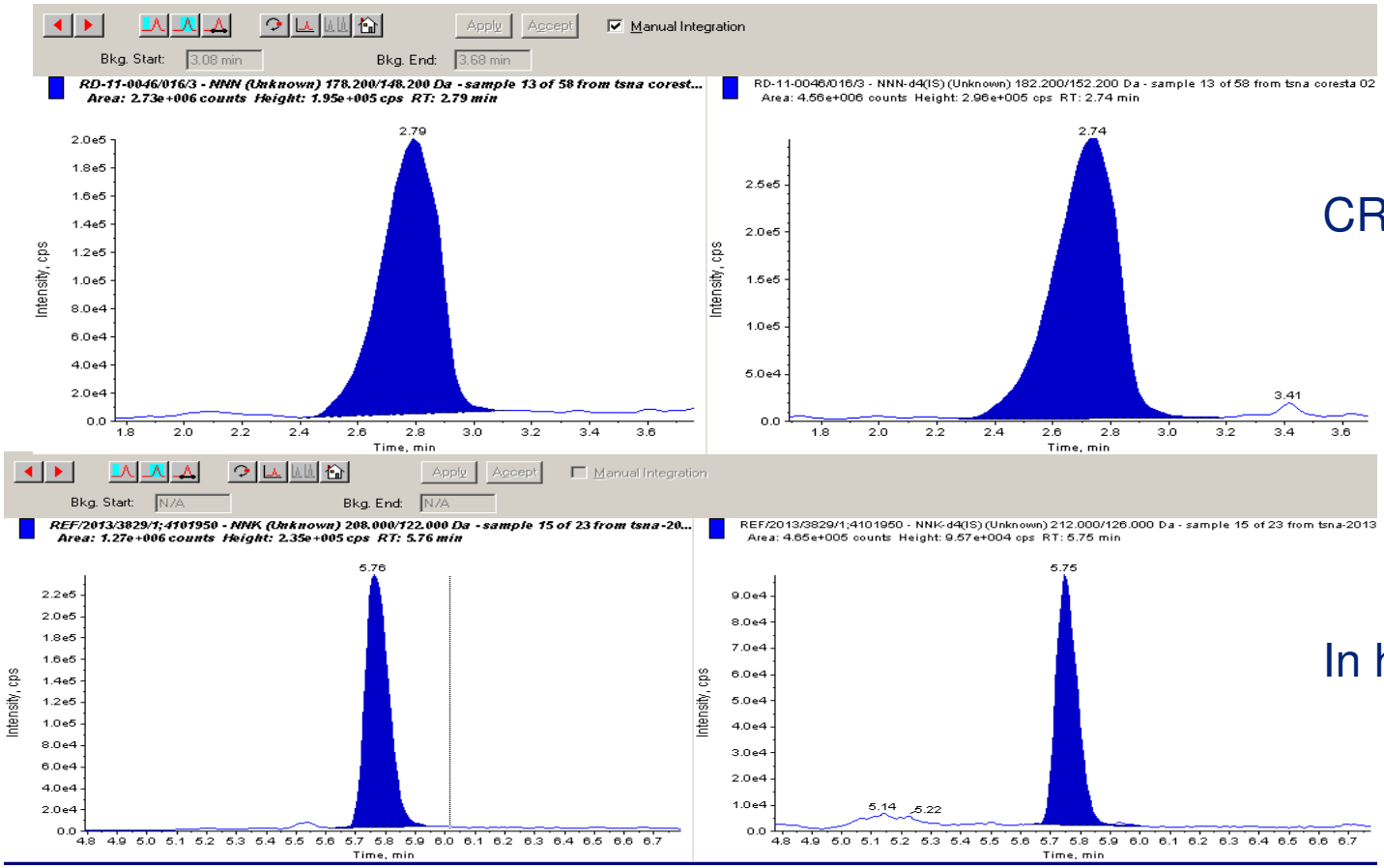
G. Discuss specific method challenges and limitations when testing NNN and NNK.

- **Matrix suppression greater for NNN and NNK than NAT and NAB**
- **Clean up step in the method may be beneficial for smoke extracts**
- **Poor peak shape for NNN when extracted with aqueous buffer (CRM 75)**



CRM 75

In house method

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H. Describe the differences in separation, detection, and limits of detection/quantitation when comparing liquid chromatography/mass spectrometry (LC-MS) and gas chromatography/thermal energy analyzer (GC-TEA) for TSNA analysis.

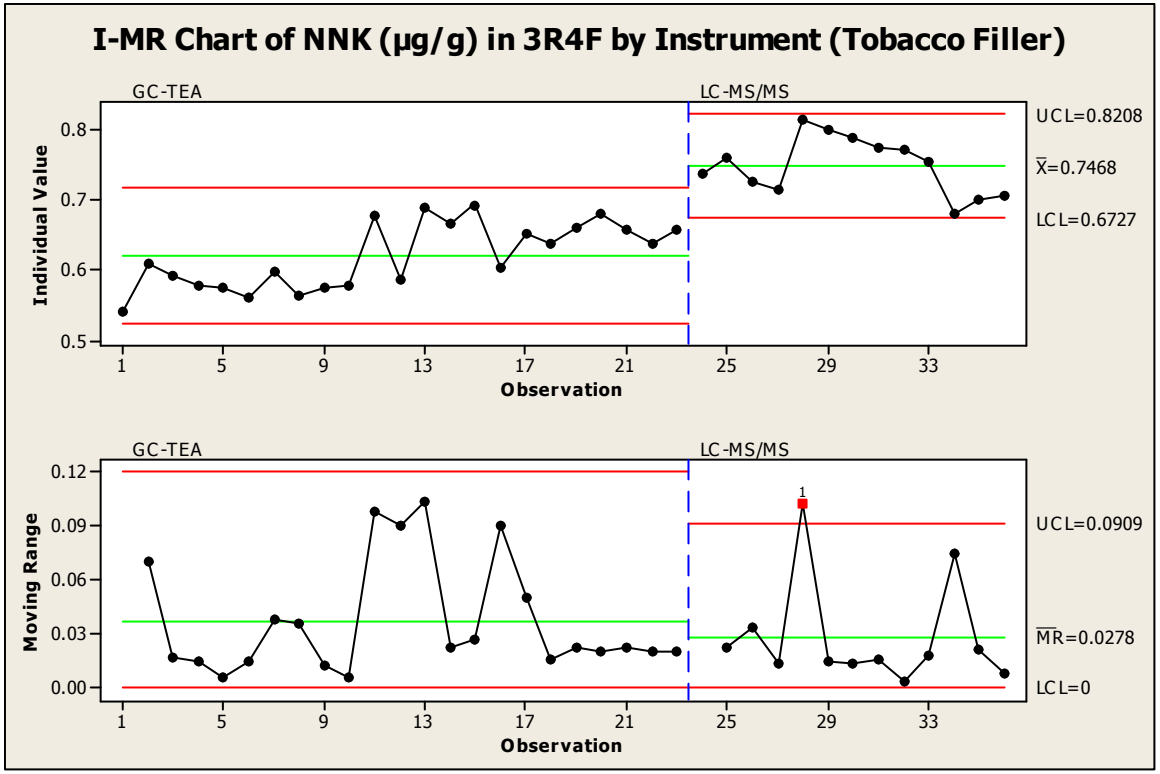
Reporting limits for NNN, NNK (based on lowest calibration point)

Tobacco Filler:

- **LC-MS/MS ≈ 3.6 ng/g**
- **GC-TEA ≈ 200 ng/g**

Cigarette Smoke:

- **LC-MS/MS ≈ 3.6 ng/cig**
- **GC-TEA ≈ 10 ng/cig**



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Main observations

Internal Standards

- **D_4 internal standards for each analyte compensate for matrix suppression, recovery; precision increases with number of IS**

Extraction

- **Use either aqueous buffer or methanol after hydration to obtain highest extraction with greater precision for tobacco filler and STPs**

Matrix reduction

- **Inclusion of SPE (HLB or MCX) reduces matrix artefacts and minimises suppression of instrument response for tobacco filler, STP and cigarette smoke samples**

Instrumentation

- **LC-MS/MS achieves better sensitivity and greater selectivity**
- **Capacity of >500 tests per week on a single instrument**