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# Fast and sensitive assay of tobacco specific nitrosamines by UHPLC-MS/MS

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## 1. Introduction

Tobacco specific nitrosamines (TSNA) are volatile compounds formed from nicotine and related alkaloids found in tobacco and tobacco products. The TSNAs are created during fermentation, curing and burning of the tobacco leaf. Due to their carcinogenic properties, the four major TSNA are assayed to control tobacco quality and to control cigarette smoke or public atmospheres.

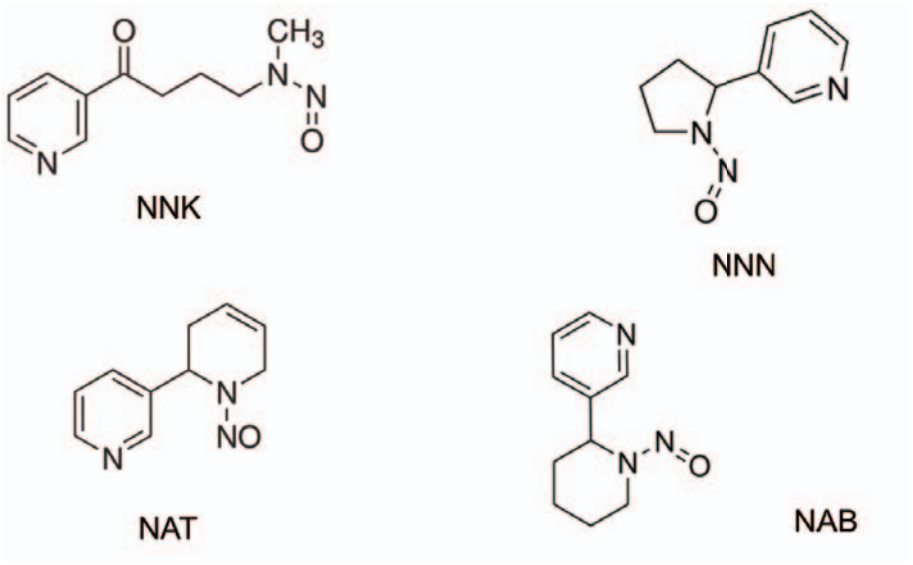


Fig. 1 Structure of the studied tobacco nitrosamines

## 2. Methods and Materials

The four major TSNA, i.e. N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), were assayed by UHPLC-MS/MS. Specific solid phase extraction was performed using selective molecularly imprinted polymers (Biotage). Deuterium labeled internal standards are used to improve assay ruggedness and accuracy.

### Solid Phase Extraction with Molecularly Imprinted Polymers

Column: Biotage AFFINILUTE™ MIP – TSNAs 50 mg/3 mL

Sample preparation: Accurately weighted 250 mg of tobacco were transferred in a 30 mL glass tube with 10 mL of Ammonium acetate buffer (10 mM pH 5.5) and internal standards solution (NNN-D4, NNK-D4, NAT-D4, final concentration of 10 ng/mL). After 1hr of sonication a 2 mL aliquot was centrifuged.

Extraction: Column was conditioned with 1 mL of Methanol and 1 mL of water. 1 mL of sample was applied to the column. Interferences were washed out with 1 mL of ammonium acetate (10 mM pH 5.5) and 1 mL of hexane. Elution was performed with 2 × 1 mL of methanol/dichloromethane 1/9. After complete evaporation, the dry residue was reconstituted with 1 mL of mobile phase mixture (A/B 9/1).

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## UHPLC conditions (Nexera MP system)

Column : Phenomenex Synergi Fusion-RP C18 50 × 2 mm 2.5 µm
Mobile phase A : 5 mM Ammonium bicarbonate in water
B : 5 mM Ammonium bicarbonate in methanol
Flow rate : 0.4 mL/min
Time program : B conc. 30%(0 min) -90%(0.35 min) - 30%(0.36-1.5 m min)
Injection vol. : 5 µL (with 1 µL air gaps)
Needle wash : external wash only with methanol (rinse pump 2 sec)
Column temperature : 30°C

## MS conditions (LCMS-8040)

Ionization : ESI, Positive MRM mode
Ion source temperatures: Desolvation line : 250°C
Heater Block : 400°C
Gases: Nebulization : 2 L/min
Drying : 15 L/min

MRM Transitions

| Compound (msec) | MRM | Dwell time (msec) |
|---|---|---|
| NAB | 192.10>162.30 (Quan) | 12 |
| | 192.10>106.25 (Qual) | 12 |
| NAT | 190.10>79.20 (Quan) | 12 |
| | 190.10>160.25 (Qual) | 12 |
| NAT-D4 | 194.20>164.10 | 25 |
| NNK | 208.10>122.25 (Quan) | 12 |
| | 208.10>79.20 (Qual) | 12 |
| NNK-D4 | 212.10>126.00 | 25 |
| NNN | 178.10>148.25 (Qual) | 12 |
| | 178.10>120.25 (Quan) | 12 |
| NNN-D4 | 182.10>152.20 | 25 |

Pause time: : 3 msec
Loop time: : 0.2 sec (minimum 15 points per peak for each MRM)

# 3. Results

## Mobile Phase Selection

Mobile phase solvent and additives were selected using a rational approach described elsewhere (60th ASMS, ThP512). Results are presented in Fig. 2.
Bicarbonate ammonium 5 mM and methanol were selected to obtain the best ionization efficiency.

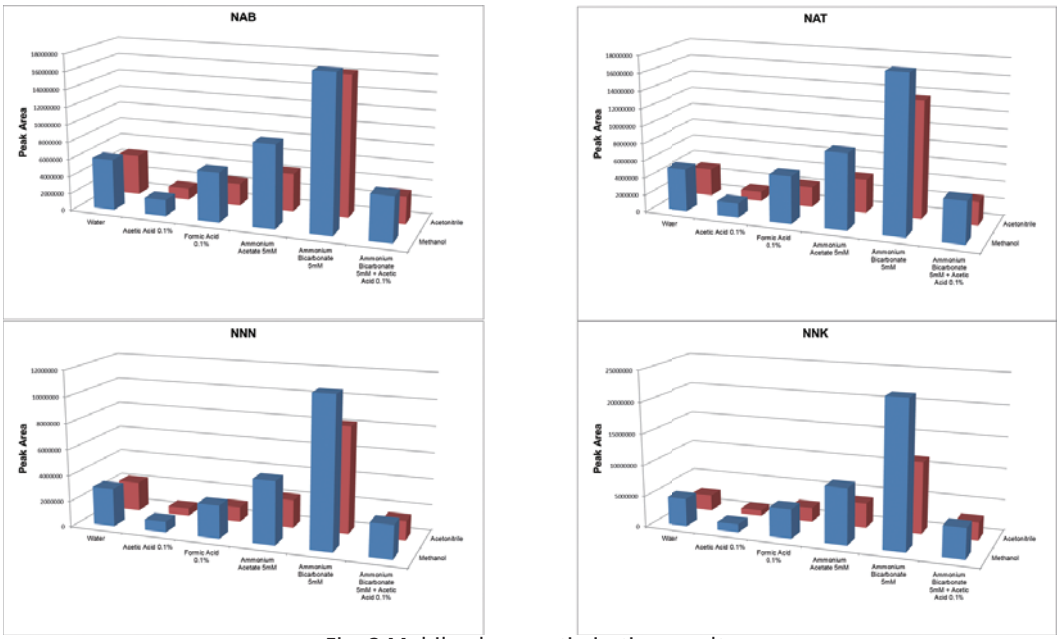


Fig. 2 Mobile phase optimisation results

## Extraction Recovery and Matrix Effect

Due to their presence in all tobacco samples tested, tobacco extracts were spiked at 50 ng/mL with all TSNAs. Spiking was performed before and after extraction to measure extraction recovery. A neat solution at 50 ng/mL was compared to the extracts spiked after extraction to calculate matrix effect.

| Sample | Extraction recovery | Ionization recovery |
|---|---|---|
| Cigarette tobacco brand A | NNN : 70%<br>NNK : 93%<br>NAT : 96%<br>NAB : 72% | NNN : 99%<br>NNK : 102%<br>NAT : 97%<br>NAB : 95% |
| Cigarette tobacco brand B | NNN : 75%<br>NNK : 92%<br>NAT : 93%<br>NAB : 77% | NNN : 105%<br>NNK : 93%<br>NAT : 99%<br>NAB : 99% |
| Cigarette tobacco brand C | NNN : 75%<br>NNK : 96%<br>NAT : 90%<br>NAB : 74% | NNN : 100%<br>NNK : 104%<br>NAT : 101%<br>NAB : 107% |

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## Calibration Curves

Calibration in neat solutions were prepared. For all TSNAs, calibration range was of 0.1 ng/mL to 50 ng/mL. This corresponds to 500 fg to 250 pg of each TSNA injected; and this corresponds to a quantity of 0.004 µg/g to 2 µg/g in tobacco products. All calibration levels were injected in 5 replicates. Intra-level %RSD were inferior to 5% at all levels for all compounds. Fig. 3 presents the calculated calibration curves. Fig. 4 shows the LOQ chromatograms.

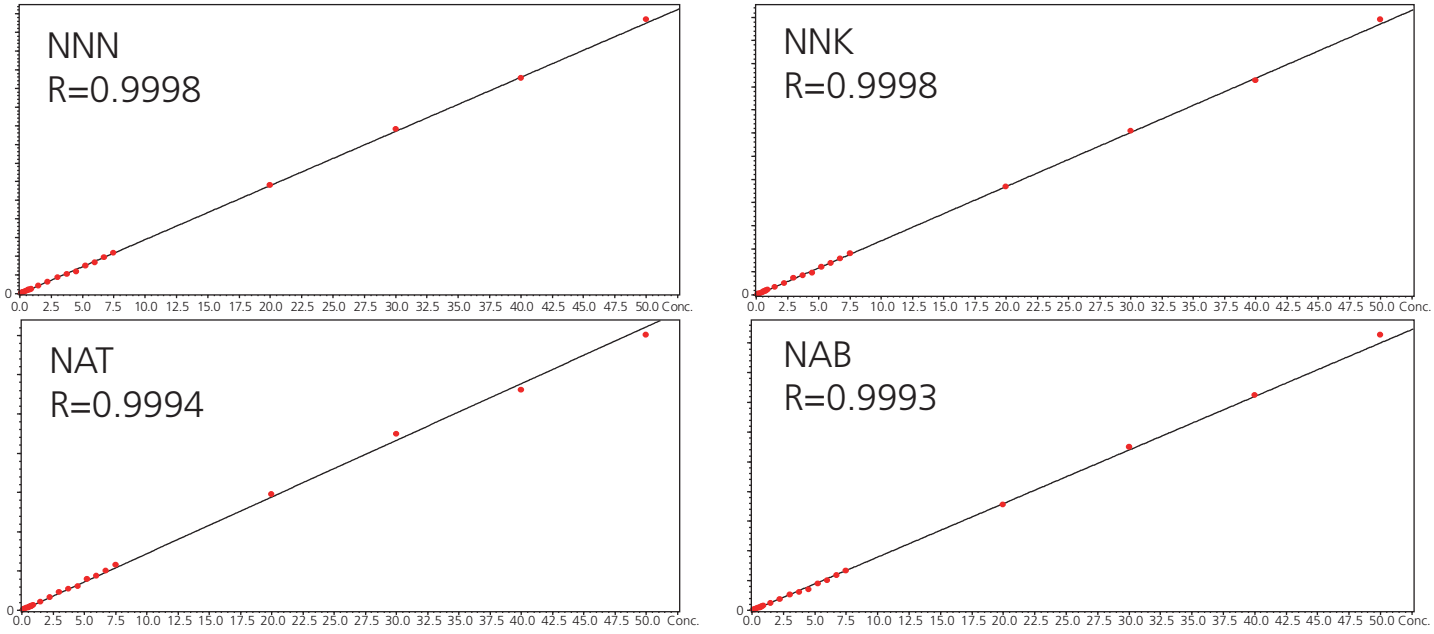


Fig. 3 Calibration curves

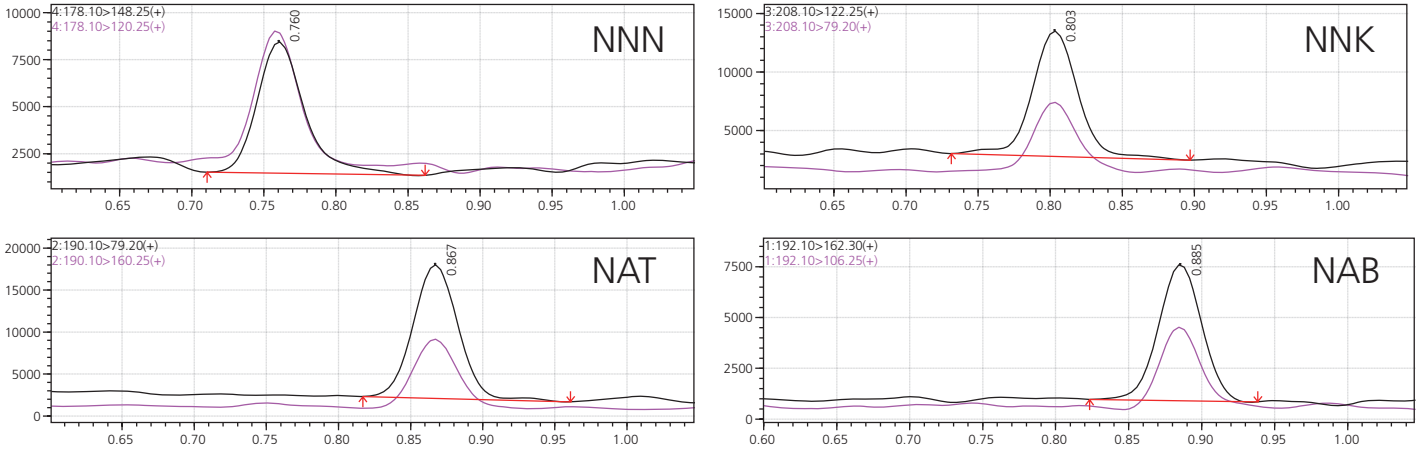


Fig. 4 Lowest calibration standard (100 pg/mL)

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# 4. Conclusions

- Highly selective extraction sorbents enable fast LC-MS/MS without suffering from matrix effect (i.e. ion suppression),
- Therefore, multiple samples can be assayed and quantified against the same calibration curve made in neat solutions,
- Very fast and rugged LC-MS/MS analysis lead to high throughput result generation to test many tobacco samples in quality control,
- The method is sensitive enough to foresee analysis of tobacco smokes or new tobacco products.

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