

## Technical Report

# Tobacco-Specific Nitrosamines: Efficient Extraction of Toxic Compounds from Complex Matrices using Molecularly Imprinted Polymers

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### Abstract

***In an effort to develop a simple and highly sensitive assay for the extraction and analysis of TSNA's in urine, MIP Technologies AB has developed a molecularly imprinted polymer phase and validated an SPE method and analytical LC-MS-MS procedure specific for this application. In the validation study, effective limits of detection at low ppt level for all TSNA's (NNK, NAB, NNN, NAT) were achieved. In this report, the high performance of the molecularly imprinted polymer SPE material, the SupelMIP™ SPE-TSNA's, is further demonstrated.***

### Introduction

Tobacco-specific nitrosamines (TSNAs) are created through the burning, curing, and fermentation of tobacco leaf and can be found in chewing, smoking, and snuff tobacco. They are believed to play a significant role as causes of cancer in people who use tobacco products and are found on the US Surgeon General list of carcinogens from 1989 (1). The most carcinogenic of the commonly occurring TSNAs are 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N'-nitrosonornicotine (NNN) (2). Because TSNAs are only found in tobacco products, their characterization is invaluable in the study of tobacco's cancerous nature (2). The monitoring of humans for exposure to tobacco smoke (active or passive) is an important clinical test - NNK is found in tobacco smoke in significant amounts (2)

The extraction and quantitation of TSNAs in urine is a useful biomarker when assessing a subject's exposure to tobacco smoke. TSNAs are not only found in smokers but in non-smokers (via second-hand smoke) as well. Because TSNAs are detected in urine at low ppt levels, a highly specific and sensitive assay is required. Although extraction and analysis protocols have been previously developed, many of them require extensive and time-consuming sample preparation (3).

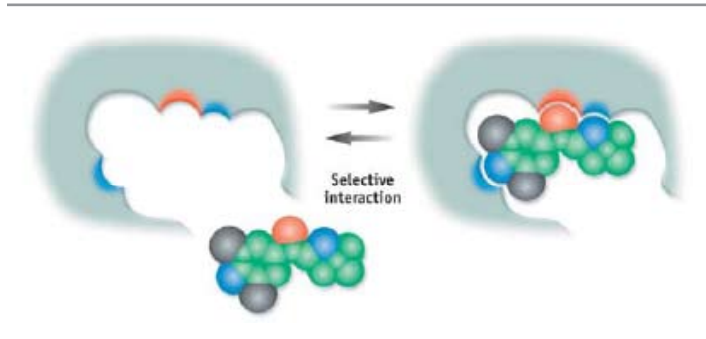
In this article we will show the performance of the extraction of the four different TSNAs from urine samples using a class selective SupelMIP SPE-TSNA.

### Molecularly Imprinted Polymers

Molecularly imprinted polymers (MIPs) are a class of highly cross-linked polymer-based molecular recognition elements engineered to bind one target compound or a class of structurally related compounds with high selectivity.

Selectivity is introduced during MIP synthesis in which a template molecule, designed to mimic the analyte, guides the formation of specific cavities or imprints that are sterically and chemically complementary to the target analyte(s). After complete polymerization, sophisticated wash procedures are used to remove the template from the polymer, leaving the imprints or binding sites accessible to bind the analyte(s) of interest. As a result, multiple interactions (e.g., hydrogen bonding, ionic, Van der Waals, hydrophobic) can take place between the MIP cavity and analyte functional groups. The strong retention offered between a MIP phase and its target analyte(s) allows for the use of exhaustive wash procedures during solid phase extraction that results in superior sample cleanup prior to analysis. This leads to cleaner extracts, lower detection limits and a more efficient sample cleanup process. An illustration of the selective cavity is shown in Figure 1. The SupelMIP TSNA was developed as a class selective MIP material. The binding sites were designed to provide specific recognition of NNN, NNK, NAB and NAT using a non-TSNA template.

**Figure 1. Illustration of a Selective MIP Cavity**

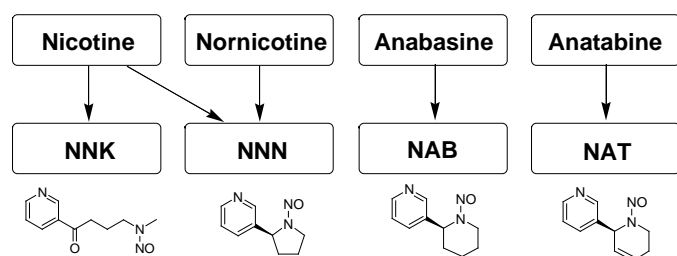


Trace level determination of compounds from complex matrices often requires a sample cleanup step such as liquid-liquid extraction (LLE) and/or solid-phase extraction (SPE) prior to analysis in order to clean and pre-concentrate the sample. LLE is time-consuming and need high solvents consumption. SPE using non-polar, polar, ionic or mixed-mode (e.g. non-polar and ion exchange groups) sorbents is more rapid, simpler and more economical than traditional LLE. Even so, the hydrophobic, ionic and polar sorbents most widely used typically do not provide a high enough selectivity resulting in tedious sample preparation methods and low throughput. The use of MIP based SPE methods has been growing rapidly in recent years. For example, applications have been published for banned veterinary drugs beta-agonists (4), beta-blockers (5), chloramphenicol (6-7), environmental contaminants such as triazines (8), and cancer related biomarkers and carcinogens such as NNAL, cotinine, and nicotine (9-13).

## The Class Selective SupelMIP SPE - TSNAs

The analysis of the four TSNA's; NNK, NNN, NAB and NAT is challenging due to the detection limits required, especially in the monitoring of non-smoker's exposure to second-hand tobacco smoke. In the case of non-smokers, detection limits at low ppt levels are required. Such low detection limits are not attainable using conventional phase SPE cleanup even with MS/MS detection (14). A summary of the pathway of formation of TSNA's from tobacco is outlined in Figure 2. Since there are several TSNA's formed from precursors present in tobacco, an analytical method that is 'class selective' for TSNA's is appealing. The SupelMIP SPE-TSNA is designed to offer class-selective binding sites for the NNK, NNN, NAB and NAT molecules. The SupelMIP template was designed to trigger specific interactions with the functionality and stereochemistry of the TSNA molecules, providing multiple interaction binding sites that permit strong differentiation between the TSNA's and related interferents in the matrix.

**Figure 2. Extraction and Analysis of TSNA's (NNN, NNK, NAB and NAT) Using SupelMIP SPE-TSNAs**



In this study, NNN, NNK, NAB and NAT were extracted from urine using SupelMIP SPE-TSNAs via the extraction procedure described in Table 1. Analysis of the resulting eluate was conducted by LC-MS-MS using the procedure (15) described in Table 2.

**Table 1. SupelMIP Extraction Procedure for TSNA's**

### Sample Pre-Treatment:

Adjust sample pH to 5.5 with acetic acid. Add 1 ng/mL NNK d<sub>3</sub> internal standard.

**SPE Procedure:** SupelMIP SPE-TSNAs, 25 mg/3 mL (LRC) (Cat. No.53222-U)

1. Condition and equilibrate MIP phase with 1 mL methanol, 1 mL DI water (do not allow the cartridge to dry during conditioning).
2. Load 1 mL pre-treated urine sample.
3. Wash (elute interferences) using the following wash scheme:
  - 1 mL 10 mM ammonium acetate, pH 5.5
  - Apply full vacuum through cartridge for 10 min. to remove residual moisture from cartridge.
  - 1 mL heptane (selective removal of hydrophobic interferences)
  - Apply full vacuum through cartridge for 5 min. to remove residual solvent.
4. Elute TSNA's with 2 x 1 mL 10% methanol in dichloromethane. Apply a gentle vacuum between each fraction. Evaporate and reconstitute with 100 µL LC mobile phase prior to analysis.

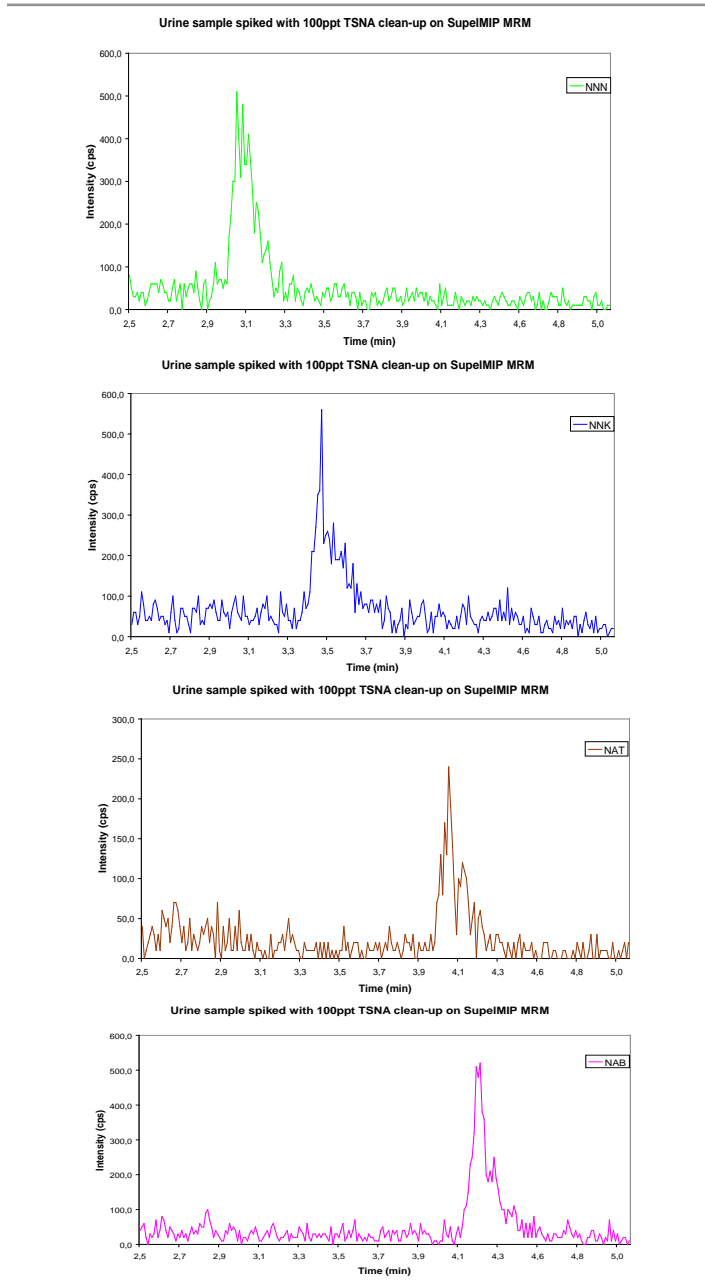
**Table 2. LC-MS-MS Conditions for TSNA's**

column:	Ascentis C18, 50 cm x 3 mm I.D., 3 µm particles (581307-U)									
instrument:	API 3200 MS/MS									
mobile phase A:	10 mM ammonium formate, pH 6.1									
mobile phase B:	acetonitrile									
flow rate:	0.5 mL/min.									
temp.:	25 °C									
injection:	5 µL									
gradient:	<b>Min</b>	<b>%A</b>	<b>%B</b>							
	0.0	90	10							
	1.0	90	10							
	4.0	60	40							
	5.0	30	70							
	6.0	30	70							
	6.1	90	10							
	9.0	90	10							
det.:	<b>Analyte</b>	<b>Rt (min)</b>	<b>Time (ms)</b>	<b>Q1</b>	<b>Q3</b>	<b>DP</b>	<b>EP</b>	<b>CEP</b>	<b>CE</b>	<b>CXP</b>
(MS-MS):	NNN	2.9	178.20	148.10	100	22	5.0	10.0	14	5.0
	NNK	3.5	208.10	122.0	100	25	5.0	10.0	18	5.0
	NAT	4.0	190.10	160.20	100	20	5.0	10.0	14	5.0
	NAB	4.2	192.20	162.20	100	30	5.0	10.0	17	5.0
	NNK-d <sub>3</sub>	3.5	211.30	122.10	100	25	5.0	10.0	18	5.0
	NAB-d <sub>4</sub>	4.2	196	166	50	60	20	10.0	18	12.0
	NAT-d <sub>4</sub>	4.0	194	164	50	60	20	10.0	16	11.0
	NNN-d <sub>4</sub>	2.9	182	152	50	60	20	10.0	16	10.0
dwel time (MS):	100									
ion mode:	Positive									
ion source:	Turbospray									
ion spray voltage:	5500 V									
source temp.:	500 °C									
curtain gas:	30 psi									
collision gas:	5 psi									
gas 1:	40 psi									
gas 2:	30 psi									

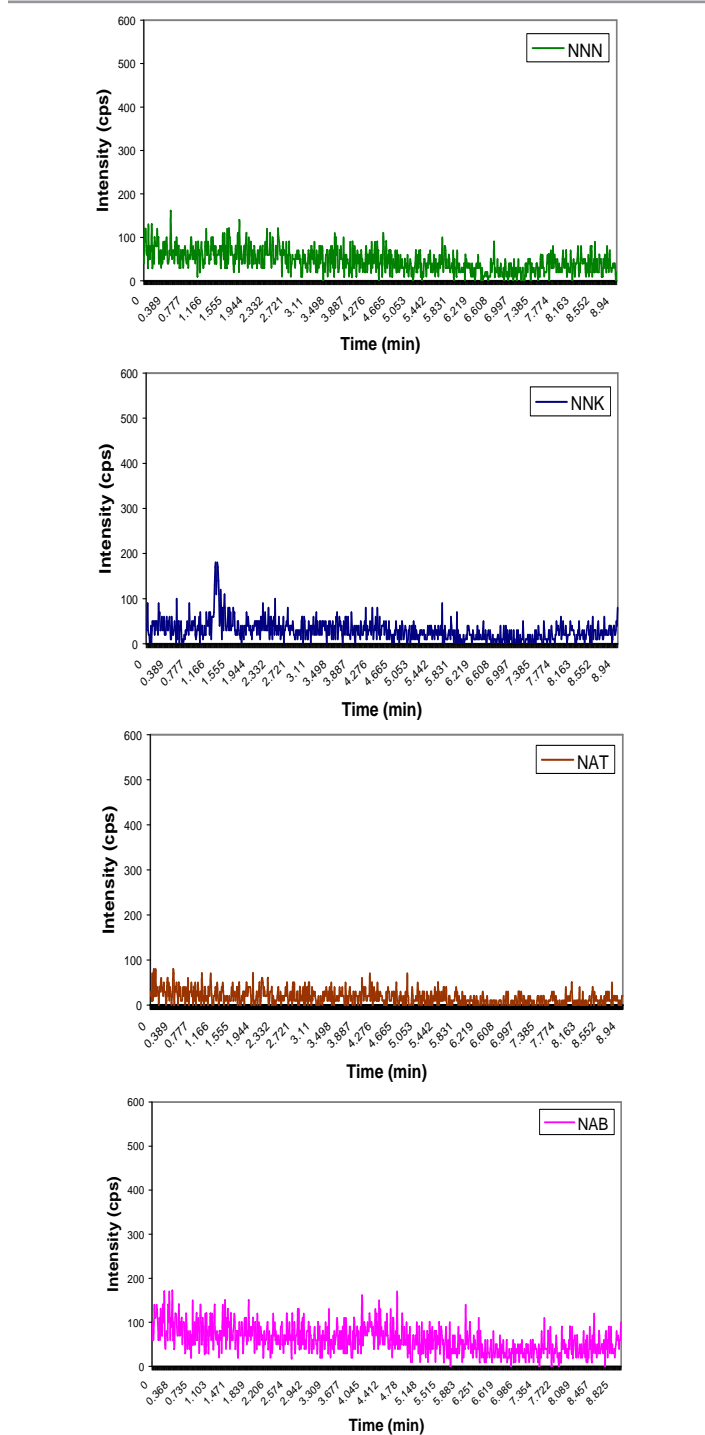
## Lower Limits of Quantitation in Urine

Using the procedure described, trace levels of TSNA were determined in spiked urine, and lower limits of quantitation (LLOQ) values were determined by measuring the signal-to-noise ratio for each analyte response. Using the SupelMIP SPE protocol and LC-MS-MS conditions described in this report for analyzing urine samples, the limits of detection were 5.1 pg/mL for NNN, 3.8 pg/mL for NNK, 4.7 pg/mL for NAB and 6.2 pg/mL for NAT. The limits of quantification were 16.8 pg/mL for NNN, 12.7 pg/mL for NNK, 15.8 pg/mL for NAB and 20.8 pg/mL for NAT. A typical chromatogram of an extracted urine sample spiked with 100 pg/mL of each TSNA is depicted in Figure 3. In Figure 4 the chromatograms for the corresponding blank urine samples are shown.

**Figure 3. MRM Chromatogram of Extracted Urine Sample Spiked with 100 pg/mL of NNN (green), NNK (blue), NAT (brown) and NAB (pink)**



**Figure 4. MRM Chromatogram of Extracted Blank Urine Sample, NNN (green), NNK (blue), NAT (brown) and NAB (pink)**



## Recovery

Eight different urine samples were spiked to a level of 100 pg/mL with TSNA. These samples were extracted by the procedure described in Table 1. The intermediate precision was established by testing the performance of the method using three different urine samples, three different analysts, three separate lots/batches of SupelMIP TSNA and on two different LC-MS-MS instruments.

The recoveries achieved were calculated and are shown in Table 3. Recoveries using the SupelMIP cleanup are high. The variations are also represented by the relative standard deviation (%RSD) of the recovery values from the eight different samples with different operators, urine samples and lot numbers of the SupelMIP TSNA. It should be noted that during the analysis only the NNK-d<sub>3</sub> internal standard was available in the lab. The response from NNK-d<sub>3</sub> was used for the remaining three TSNA also. Further improvements are possible for NNN, NAB and NAT if the equivalent internal standard is used.

**Table 3. Summary of Performance from Intermediate Precision Testing**

	Operator	Batch Number	Urine	NNK	NNN	NAB	NAT
Recovery Sample 1	Analyst 1	MG03-0610	Sample A	105.6	108.8	107.0	102.4
Recovery Sample 2	Analyst 1	MG03-0610	Sample A	104.7	103.4	103.1	101.3
Recovery Sample 3	Analyst 2	MG03-0610	Sample A	104.3	105.5	104.4	102.3
Recovery Sample 4	Analyst 2	MG03-0610	Sample B	105.9	105.6	107.2	102.1
Recovery Sample 5	Analyst 2	MG07-0610A	Sample B	89.1	107.3	124.2	138.3
Recovery Sample 6	Analyst 3	MG07-0610A	Sample C	89.1	108.0	121.7	137.9
Recovery Sample 7	Analyst 3	MG02-0532	Sample C	93.1	94.6	91.5	87.2
Recovery Sample 8	Analyst 3	MG02-0532	Sample C	90.3	91.8	89.8	87.6
			<b>Average</b>	<b>97.8</b>	<b>103.1</b>	<b>106.1</b>	<b>107.4</b>
			<b>SD</b>	8.0	6.4	12.3	20.0
			<b>%RSD</b>	8.1	6.2	11.6	18.6

## Ion Suppression

Eight blank urine samples were also cleaned up according to the SPE procedure for SupelMIP SPE TSNA. The extracted samples from the SupelMIP SPE cartridges were spiked with TSNA before evaporation. Standards were prepared in the elution solvent. Samples and standard solutions were evaporated and redissolved in mobile phase prior to analysis. The recoveries were then calculated and related to the sample data outlined in Table 3. Effects due to matrix are summarized in Table 4. Effects due to the matrix are low from three different urine samples demonstrating the accuracy of the method.

**Table 4. Summary of Performance from Intermediate Precision Testing**

	Operator	Batch Number	Urine	NNK	NNN	NAB	NAT
Recovery Sample 1	Analyst 1	MG03-0610	Sample A	11.5	14.2	14.6	11.4
Recovery Sample 2	Analyst 1	MG03-0610	Sample A	15.8	8.5	13.4	14.8
Recovery Sample 3	Analyst 2	MG03-0610	Sample A	10.2	9.1	10.2	10.3
Recovery Sample 4	Analyst 2	MG03-0610	Sample B	15.6	12.4	18.1	14.8
Recovery Sample 5	Analyst 2	MG07-0610A	Sample B	10.0	19.0	16.4	22.0
Recovery Sample 6	Analyst 3	MG07-0610A	Sample C	5.6	11.2	10.7	12.7
Recovery Sample 7	Analyst 3	MG02-0532	Sample C	2.3	6.8	7.7	9.7
Recovery Sample 8	Analyst 3	MG02-0532	Sample C	3.4	2.6	5.4	7.3
			<b>Average Suppression due to matrix (%)</b>	<b>9.3</b>	<b>10.5</b>	<b>12.1</b>	<b>12.9</b>

## Conclusions

In this article, we have described a novel method for the extraction of TSNA's (NNN, NNK, NAT and NAB) from urine using SupelMIP SPE-TSNA. The SupelMIP SPE-TSNA assay described in this report took less than two hours to complete and offered the selectivity necessary to achieve detection limits in urine at the low ppt level. Recovery values for each TSNA are above 90% and ion suppression due to matrix effects are low. This performance allows for accurate determination with the required precision for measurement of TSNA's in urine using a simple, one-step SPE cleanup step.

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