A Comparison of Two Methods for the Quantification of Tobacco-Specific Nitrosamines in Human Urine

P.S. Miller, A.M. Dzerk, G.P. Brown, R. Nachi, C.J. Kafonek and K.E. Newland
Celerion, Lincoln, NE, USA

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ABSTRACT

A validated method for the determination of four tobacco-specific nitrosamines (TSNAs) – NNAL, NNN, NAT, and NAB – was compared to a method published in 2009 *Journal of Chromatography B* **877**: 1185-1192, by Kavvadias, Scherer, et al. Single sets of calibration standards and quality control samples, which covered the full analytical range of each analyte in both methods, were utilized. The analytical range for NNN differed the most between the methods, with the Celerion method covering 0.200 – 40.0 pg/mL and the published method covering 2.00 – 250 pg/mL.

In total, 20 urine lots (10 from nonsmokers and 10 from smokers) were assayed with each method for a comparison of the basal concentrations of each TSNA. Selectivity and matrix effect were assessed by comparing the quantitative accuracy of each method when known amounts of the TSNAs were added to the basal concentrations. Basal concentrations of the TSNAs compared within 20% for 8/10 smoker lots for NNAL, 7/10 smoker lots for NAT, 6/10 smoker lots for NAB, but only 2/10 smoker lots for NNN. For some of the smoker urine lots, the differences in the basal concentrations of NNN were substantially different. A summary of the results is presented, including sample chromatography, and possible reasons for differences between the methods are discussed.

INTRODUCTION

Tobacco products contain three major types of nitroso- compounds. Among these are the non-volatile tobacco-specific nitrosamines (TSNAs), which include 4-(*N*-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) – further metabolized to the reduced butanol form, NNAL – along with *N*-nitrosonornicotine (NNN), *N*-nitrosoanatabine (NAT), and *N*-nitrosoanabasine (NAB). TSNAs are found at variable concentrations in processed tobacco products as a result of nitrosation of endogenous tobacco alkaloids – namely nicotine, nornicotine, anatabine, and anabasine. TSNAs are known to induce tumors in the lung, oral cavity, esophagus, pancreas, and liver. As such, there is significant interest in reducing the amount of TSNAs to which users of tobacco products are exposed.

N-glucuronides on the pyridyl ring of NNAL, NNN, NAT, and NAB, along with the *O*-glucuronide of NNAL, are produced by UDP-glucuronyl transferases. β-glucuronidase is used to hydrolyse TSNA glucuronides in human urine into the unconjugated (aglycone) species for extraction and analysis of total TSNAs in urine.

References

- WHO, International Agency for Research on Cancer. "Smokeless Tobacco and Some Tobacco-specific *N*-Nitrosamines", 2007 IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, Volume 89
- G. Chen, R.W. Dellinger, D. Sun, T.E. Spratt, and P. Lazarus. "Glucuronidation of Tobacco-Specific Nitrosamines by UGT2B10", 2008 *Drug Metab Dispos*. **36**(5): 824–830

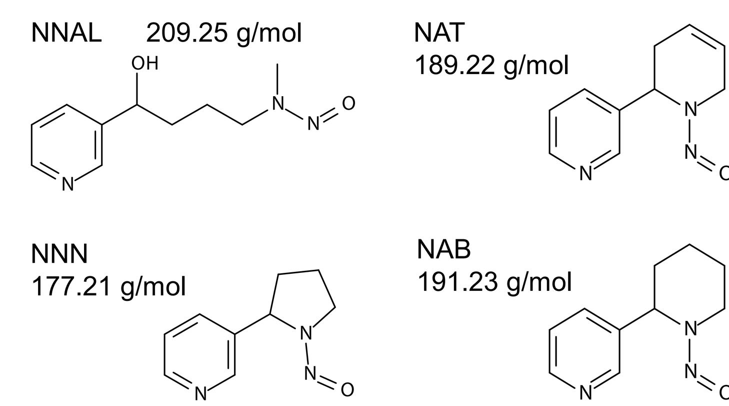

Figure 1. Structures of Tobacco Specific Nitrosamines (TSNAs)

Table 1. Calibration Standard Concentrations (pg/mL)

Calibration Standard	NNAL	NNN	NAT	NAB
STD K	1500	250	1500	250
STD J	1000	100	1000	200
STD I	800	40	800	160
STD H	400	32	400	120
STD G	200	16	200	80
STD F	100	4	100	40
STD E	50	2	25	25
STD D	20	0.8	10	10
STD C	10	0.4	5	5
STD B	5	0.2	2	2

Published Method only: blue shading
Celerion Method only: red shading

Table 2. Comparison of Method Parameters in Published Method vs Celerion Method

Parameter	Published Method	Celerion Method
Analytes in Sample Preparation (Sample Volume)	1) NNAL, NNN, NAT, NAB (6 mL)	1) NNAL, NNN (2 mL) 2) NAT, NAB (2 mL)
Analytes in Sample Injection (Detector)	1) NNAL, NNN, NAB (API 4000™) 2) NAT * (API 4000™)	1) NNAL (API 4000™) 2) NNN (Triple Quad™ 6500) 3) NAT, NAB (API 5000™/QTRAP® 5500)
Hydrolysis		
pH, Temperature, Time	pH 7.2, 37 °C, 14 hours	pH 4.5 - 5.0, 55 °C, 16 - 21 hours
Enzyme source	*E. coli* Type IX-A	*H. pomatia* Type H-1
Enzyme amount	10,000 U/sample	(1) 15,000 or (2) 7,500 U/sample
Extraction Method	1) SupelMIP® SPE – TSNAs (10 mL, 50 mg), then Oasis® MCX SPE (3 mL, 60 mg)	1, 2) Oasis® MCX SPE (3 mL, 60 mg)
Extraction Time	1) 3 days **	1, 2) 3 days (2 days per extraction) [Day 2 of (1) & Day 1 of (2) can overlap]
Chromatography		
Column	1, 2*) Phenomenex Luna® C18 (250 x 3.0 mm, 3µm)	1) Agilent ZORBAX 300-SCX (50 x 2.1 mm, 5 µm) 2) ACE® *Excel*™ C18-PFP (150 x 2.1 mm, 2 µm) 3) Waters SymmetryShield™ RP_{18} (50 x 2.1 mm, 3.5 µm)
Acquisition Time	1) 17 min 2) 17 min *	1) 2.8 min 2) 8.0 min 3) 6.0 min

* As published. However, due to time and cost constraints, NAT was not reinjected separately for this comparison. An injection volume of 5 µL was used to keep NAT (with a 750-fold analytical range in Published Method) within the detection limits of the instrument. As a result, S/N for NNAL, with half injection volume, for some of the suppressed lots in the Published Method was rather low.
** Time needed to extract batch using published method in Celerion laboratory setting. When done in 2 days, Day 2 was an extended workday.

Table 3. Reproducibility of Internal Standard (Peak Area) Response in Basal Concentration (Duplicate), Low, Mid, and High Spiked Samples

	NNAL Published Method	NNAL Celerion Method	NNN Published Method	NNN Celerion Method	NAT Published Method	NAT Celerion Method	NAB Published Method	NAB Celerion Method
Nonsmoker Lots								
Mean	9748	119528	62590	2387478	1362892	809681	248852	390285
C.V. %	**44.3**	3.4	**25.6**	13.6	10.0	9.4	10.8	9.5
N	50	50	50	50	50	50	50	50
Smoker Lots								
Mean	3395	57689	23403	1495893	447862	743852	89796	326393
C.V. %	**86.6**	3.0	**40.1**	11.8	**45.0**	9.5	**41.0**	9.6
N	40	50	40	50	40	50	40	50

Table 4. Quantification of Basal Levels with Low, Mid, and High Spikes of TSNAs in 10 Lots (1-10) of Nonsmoker Urine and 10 Lots (11-20) of Smoker Urine Using Published Method vs. Celerion Method

NNAL

Lot#	Mean Basal pg/mL	%Diff Published vs. Celerion	Low Spike 10.0 pg/mL % Dev.	Mid Spike 50.0 pg/mL % Dev.	High Spike N: 800 S: 200 pg/mL % Dev.
1	*3.20*		+1.5	+8.8	+0.5
	1.10		+0.9	+2.0	+0.9
2	--		-5.9	+8.2	-1.4
	--		+0.0	+3.6	+1.4
3	--		**+61.0**	+13.8	-1.8
	--		+10.0	+3.4	+1.6
4	--		**+22.0**	**+20.6**	+1.8
	0.797		+2.8	+3.5	+1.0
5	--		+15.0	+12.0	+2.8
	--		-1.7	+0.6	+2.1
6	--		+7.0	**+24.6**	+7.7
	--		+12.0	+5.8	+2.4
7	--		**+16.0**	+1.2	+4.8
	--		+5.0	+5.4	+0.9
8	--		-14.2	+13.6	-0.1
	--		+4.0	+2.6	+2.0
9	--		**+29.0**	**+29.6**	+10.1
	--		+5.0	+4.4	+3.0
10	--		**+51.0**	**+41.4**	-4.0
	0.975		+4.5	-0.4	+1.2
11	111	***23.3***	-3.3	+0.0	+3.9
	90.0		+0.0	+0.7	+1.7
12	58.5	***23.7***	+1.5	-13.9	+3.1
	47.3		+0.2	+2.5	+0.8
13	65.5	11.8	-6.8	+1.7	+7.5
	58.6		-0.6	-1.8	-0.4
14	103	6.3	+8.8	-0.7	-4.0
	96.9		+5.6	-0.7	+0.0
15	19.3	*9.7*	+9.2	**-15.9**	-7.8
	17.6		-1.8	+5.0	-2.8
16	83.6	-9.8	+9.0	+6.0	+1.1
	92.7		+1.0	+0.0	+0.3
17	142	-4.1	-3.9	+5.7	-0.6
	148		+1.3	+1.5	+0.9
18	59.1	-13.5	+2.9	-1.8	-2.7
	68.3		-3.3	-1.7	-0.4
19	189	-1.6	+1.0	+5.4	-11.3
	192		-0.5	+0.8	-0.8
20	46.3	7.9	**-17.1**	-7.3	+11.8
	42.9		-0.9	+0.0	+0.4

NNN

Lot#	Mean Basal pg/mL	%Diff Published vs. Celerion	Low Spike 0.400 pg/mL % Dev.	Mid Spike 2.00 pg/mL % Dev.	High Spike N: 40.0 S: 16.0 pg/mL % Dev.
1	--			**+24.0**	+2.3
	--		+0.3	+2.5	+2.8
2	*0.420*			-19.4	-2.2
	--		+5.7	+1.5	+3.3
3	--			+1.5	-0.3
	0.0161		+4.1	+5.4	+9.5
4	--			**-26.0**	-3.0
	--		+9.3	+5.0	+5.5
5	--			-8.5	-3.3
	--		+0.5	+0.5	+1.5
6	--			-18.5	-7.0
	--		+3.5	+2.5	+3.5
7	--			-10.5	-2.5
	--		+5.0	+2.0	+4.0
8	--			-4.5	+2.0
	--		-0.3	+2.0	+4.7
9	--			+10.5	+2.0
	--		+8.3	+6.5	+4.0
10	--			+8.5	+7.5
	0.0644		**+19.2**	+12.6	+14.7
11	9.72	-9.2		+0.9	+12.1
	10.7		-1.8	-0.8	+5.2
12	6.23	***26.1***		+14.8	-1.4
	4.94		-1.9	+1.0	+3.3
13	8.12	***41.2***		-1.1	+0.0
	5.75		-1.6	+1.0	+5.0
14	4.22	***81.1***		**+23.8**	+7.9
	2.33		-0.7	+0.9	+2.2
15	3.14	***492.5***		-16.3	+3.1
	0.530		+2.6	+3.6	+4.2
16	5.83	-1.0		+10.7	+4.6
	5.89		+1.9	+2.7	+0.0
17	5.67	***37.3***		-19.3	-5.1
	4.13		+0.0	+0.8	-0.5
18	*0.0266*	***-98.4***		+4.4	**-16.3**
	1.68		-1.9	-5.2	-6.2
19	2.20	***-29.0***		-1.0	-12.1
	3.10		+0.0	+3.1	+4.7
20	6.70	***26.2***		+0.9	+3.1
	5.31		+0.0	-0.4	+2.3

NAT

Lot#	Mean Basal pg/mL	%Diff Published vs. Celerion	Low Spike 5.00 pg/mL % Dev.	Mid Spike 25.0 pg/mL % Dev.	High Spike N: 800 S: 200 pg/mL % Dev.
1	*0.909*		-10.8	-2.3	-1.4
	0.377		-1.1	-2.4	-1.0
2	*0.449*		-10.5	+0.0	-3.1
	1.62		-4.5	-1.1	+2.4
3	*0.149*		-0.4	+1.2	-0.3
	--		-0.2	-2.8	+1.3
4	*0.293*		-2.5	-9.1	-2.4
	4.95		-13.0	-2.3	+4.5
5	--		-9.8	-7.6	-1.3
	--		+0.2	+0.4	-1.8
6	--		-12.2	-4.0	-0.8
	--		+9.8	+2.0	-4.5
7	--		-7.8	-1.2	-1.6
	--		+0.8	+2.8	+0.4
8	--		-5.8	-2.8	-1.6
	--		-3.2	+6.0	+0.8
9	--		-14.8	-3.2	-1.9
	--		+5.4	+0.8	-1.9
10	*0.477*		-7.7	+0.4	-2.5
	0.951		+0.7	+4.6	+1.1
11	151	-17.9	-0.6	-2.3	-1.7
	184		+0.5	+0.5	+3.1
12	89.5	10.0	+0.2	+5.2	**+17.9**
	81.4		-2.7	+2.8	+1.1
13	128	12.3	+0.8	+5.2	**+18.3**
	114		-3.4	-0.7	+4.1
14	74.9	-15.0	-3.9	-3.6	-0.4
	88.1		-2.4	+1.8	+2.1
15	15.8	*-14.6*	-4.3	-2.0	-0.9
	18.5		-3.0	-0.9	+0.5
16	80.0	***-23.8***	+1.1	-2.9	-5.4
	105		+4.5	-3.1	+3.3
17	49.8	***-24.2***	-0.5	-1.9	-4.4
	65.7		-5.2	+0.3	+3.8
18	33.1	***-38.0***	-2.1	-9.0	**-21.9**
	53.4		-2.1	-1.0	+3.2
19	153	-13.1	+0.0	+0.0	+0.6
	176		+0.6	+4.0	+1.3
20	61.6	5.8	-1.4	+11.5	**+18.7**
	58.2		+0.8	+3.2	+4.7

NAB

Lot#	Mean Basal pg/mL	%Diff Published vs. Celerion	Low Spike 5.00 pg/mL % Dev.	Mid Spike 25.0 pg/mL % Dev.	High Spike N: 160 S: 80.0 pg/mL % Dev.
1	--		-4.4	+0.8	-1.3
	--		+2.6	+2.8	+1.9
2	--		+11.0	+0.4	-1.3
	--		-2.2	+1.2	+2.5
3	--		+15.4	-4.4	-1.3
	--		-1.6	+1.6	-0.6
4	--		**+23.0**	+1.6	-3.1
	--		-6.4	-0.4	-0.6
5	--		-0.4	+1.6	+3.1
	--		-3.4	+0.4	+1.9
6	--		+1.2	-4.0	-1.9
	--		-3.8	+2.4	+2.5
7	--		+10.4	+2.8	-3.1
	--		-4.4	+1.2	+0.0
8	--		-8.6	+5.6	-1.9
	--		-7.8	+1.6	+1.9
9	--		+10.4	+0.8	+1.3
	0.259		-0.2	-0.8	+3.1
10	--		+19.6	+5.2	-4.4
	--		+5.2	+2.0	+4.4
11	29.2	-11.5	+0.0	+0.0	+0.9
	33.0		+2.1	+3.4	+1.8
12	18.7	***27.2***	+0.4	**+19.5**	**+35.8**
	14.7		+4.1	+4.3	+1.2
13	23.9	***29.9***	-2.8	**+29.7**	**+29.8**
	18.4		+2.1	-2.5	+1.6
14	7.94	-4.5	+1.6	-3.6	-0.8
	8.31		+6.8	+0.6	-0.9
15	3.39	*-10.6*	+2.6	-2.5	-3.8
	3.79		-6.4	+1.0	-1.0
16	12.0	-12.4	-1.8	-0.5	-3.4
	13.7		-1.6	+0.8	+0.1
17	8.45	18.8	-18.5	-4.2	+0.7
	7.11		-1.7	+1.6	-2.0
18	5.09	***21.8***	-1.5	**-21.9**	**-24.7**
	4.18		+1.2	+0.3	-3.4
19	13.5	***-30.4***	-14.6	**-19.5**	**-18.8**
	19.4		-2.9	+1.6	-1.4
20	11.2	3.7	+6.2	+7.2	+2.3
	10.8		+5.7	+2.0	+0.7

italics = extrapolated basal concentration below lower limit of quantification for the method, added to spiked concentration for expected concentration
-- = no basal concentration determined (S/N < 2 or extrapolated concentration < 0)
Bold = Measured concentration >±15.0% (or >±20.0% for LLOQ) of expected concentration
Bold italics = >20% difference in basal concentration
red = extraction or chromatography issue

N = nonsmoker high spiking concentration
S = smoker high spiking concentration

Top line (white background) = Published Method
Bottom Line (gray background) = Celerion Method

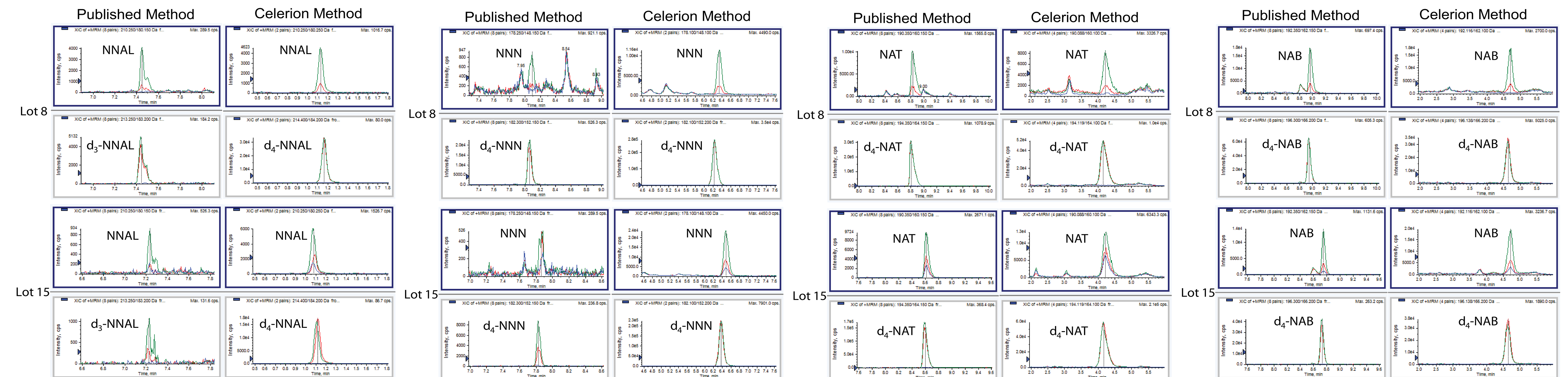

Figure 2. Chromatography of TSNAs for Blank, Low Spike, and Mid Spike in nonsmoker urine Lot 8 and for Blank (basal analytes), Low Spike, and Mid Spike in smoker urine Lot 15 using Published Method vs. Celerion Method

COMPLICATIONS ENCOUNTERED FOR PUBLISHED METHOD

- UV-light irradiation of solvents at 254 nm could not be performed
- Centrifugation of samples before loading on SupelMIP® cartridges was not in published description, therefore not performed. Extensive periods of 15 psi vacuum were required to get (especially aqueous) volumes (including samples) through SupelMIP cartridges. For Lots 11-20, some lots had severe flow problems requiring partial volume of some sample and/or wash solution(s) to be removed from SPE cartridge in order to proceed.
- Baseline subtraction of calibration standards, as described in published method, was not found to be needed. Used $1/X^2$ weighting.
- An autosampler error occurred during injection of samples for Lots 18 and 19, and self-resolved for Lot 20. However peak shape for the Lot 18, 19 samples was extremely broad when reinjected after storage for ~7 days.

DISCUSSION AND CONCLUSIONS

Comparing independent analytical methods is a valuable tool for confirming method performance, particularly with respect to accuracy and selectivity. The published method was performed as written, with minor adaptations for specific sample extraction equipment and chromatographic/mass spectrometric instrumentation available at Celerion.

Despite the number of samples analyzed by the Published Method that were affected by extraction or instrumentation issues, concentrations of NNAL, NAT, and NAB measured by the 2 methods were comparable. The quantification of NNN was not as comparable between the 2 methods, which may have been partially attributable to the large difference in the analytical ranges.

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