Determination of selected N-Nitrosoamino acids in smokeless tobacco products

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1. INTRODUCTION

The IARC monograph 89 [1] summarised historical literature on the presence of carcinogens in smokeless tobacco products (STPs), including a number of nitrosamines such as N-nitrosonornicotine (NNN), 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and four N-nitrosoamino acids (N-nitrososarcosine (NSAR), 3-(N-methylnitrosamino) propionic acid (MNPA), 4-((N-methylnitrosamino) butyric acid (MNBA) and Nitrosoazetidine-4-carboxylic acid (NazCA)) (Figure 1 and Table 1). Several researchers have also reported the presence of other N-nitrosoamino acids in tobacco [2-8]. Over the last 20-30 years a significant number of studies have characterised the contents of NNN and NNK in STPs, and research has shown changes in levels of these compounds over this time period. However, the nitrosoamino acids have recieved significantly less attention. Given the lack of pertinent information in this area, a survey of the levels of 11 N-nitrosoamino acids was considered of interest to more fully characterise the chemistry of currently available STPs.

The analytical methods used historically for the determination of N-nitrosoamino acids were mainly based on GC-TEA analysis, which required the derivatization of N-nitrosoamino acids and often required multiple extraction procedures in order to recover all of the relevant compounds [6, 7, 8]. The most commonly used derivatisation agent, diazomethane, is not recommended to use today due to its carcinogenicity. In addition, one method based on ion-pairing liquid chromatography using mass spectrometric detection of four N-nitrosoamino acids has been published [9]. No method suitable for all eleven N-nitrosoamino acids has been published. In this study an analytical method based on HPLC-MS analysis, has been developed and validated for eleven selected N-nitrosoamino acids in seven types of of commercial STPs.

2. OBJECTIVE:

The objective of this study was to develop an analytical method for the quantification of eleven selected N-nitrosoamino acids in commercial STPs and to demonstrate its fitness for purpose through method validation. A wide range of STPs included in the validation including loose and pouched snus, dry snuff, moist snuff, chewing tobacco, pellets and plugs.

In order to be fit for purpose, the limit of detection of the method should be in the order of 0.01 mg/kg.

Table 1. Summary of compounds included in the method.

Compound Name	Abbrev.	CAS No	MW / g/mol	Quantification[a] m/z	Qualification[a] m/z
N-Nitrososarcosine	NSAR	10478-42-9	118.09	119→88	none
N-nitroso-N-methyl-3-aminopropionic acid	MNPA	61445-55-4	132.12	133→103	133→73
N-nitroso-N-methyl-4-aminobutyric acid	MNBA	61445-55-4	146.15	147→117	147→87
(4-methylnitrosoamino)-4-(3-pyridyl)-butyric acid	Iso-NNAC	123478-84-0[r]	223.23	224→121	224→194
N-nitroso-thiazolidine-4-carboxylic acid	NTCA	88381-44-6	162.17	161[a] →117	161[a] →71
N-nitroso-2-methylthiazolidine-4-carboxylic acid	NMTCA	103659-08-1	176.19	177→75	177→132
N-nitroso-pipecolicacid	NPIPAC	4515-18-8[m]	158.16	159→84	159→128
N-nitroso-azetidine-2-carboxylic acid	NAzCA	30248-47-6[L]	130.10	131→100	131→56
N-nitroso-proline	NPRO	7519-36-0[L]	144.13	145→70	145→114
N-nitroso-hydroxy-proline	NHPRO	30310-80-6[L]	160.13	161→86	161→130
N-nitroso-N-methyl-phenyl alanine	NMPhPA	41867-08-7[m]	208.21	209→103	209→149

[r] Racemic standard
[L] L-form of standard
[m] Mixture of D and L forms of standards
[a] All compounds analyzed by positive electrospray ionisation except NTCA

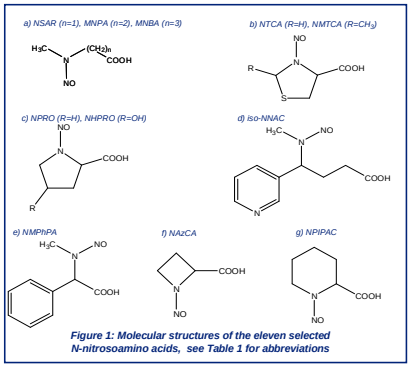

Figure 1: Molecular structures of the eleven selected N-nitrosoamino acids, see Table 1 for abbreviations

3. EXPERIMENTAL

Sample extraction using SPE

1 gram of homogenized tobacco sample was extracted in 15 mL of deionized water for 16 hours. Before extraction, standard additions were made at a concentration of 0.05 – 0.50 mg/kg for samples expected to have a low concentration or in the range 0.05 – 5.00 mg/kg for samples expected to be high.

After addition of the internal standard (IS) solution (80 µL, 25 µg/mL) containing d_3-NSAR, d_3-MNPA and d_3-MNBA, extraction by shaking for 16 hours, and centrifugation (5100 rpm, 5500 G) for 15 minutes, the analytes in the supernatant (20 mL) were collected on a ChemElute diatomaceous earth cartridge. The analytes were eluted with three portions of ethyl formate each containing 2% each of ethanol and 2% acetic acid. The eluate was evaporated until dryness, reconstituted in 0.5 mL of deionized water and filtered (0.2 µm PTFE) prior to HPLC-MS analysis.

3. EXPERIMENTAL continued

HPLC-MS/MS analysis

The HPLC-MS system was comprised of an Agilent 1290 Infinity HPLC system with a C18 column with integral polar groups (ACE 3 AQ, 3 µm, 150 x 3 mm) and an Agilent 6460A triple quadrupole mass spectrometer. Gradient elution was performed using mobile phases containing 1% formic acid in water (mobile phase A) and in methanol (mobile phase B). The column temperature was 50°C, the mobile phase flow rate 0.4 mL/minute and the injection volume 2µL. Mass spectrometric (MS) detection was achieved by electrospray ionization in the positive mode, except for NTCA, which was detected in negative electrospray mode, followed by MRM (multiple reaction monitoring) of one transition per analyte, using another transition for qualification. The ionization was performed at 200°C with gas flow of 8 L/minute, nebuliser gas flow of 45 psi, sheath gas temperature of 300°C and a sheath gas flow of 11 L/minute. In positive mode the capillary voltage was 4 kV and the nozzle voltage was 500 V. The same voltages in negative mode were 3 kV and 500 V, respectively.

Method Validation

The method was validated by investigation of linearity of the standard addition calibration procedure, limit of detection, precision by means of within-laboratory repeatability and reproducibility, reporting limit and selectivity. The accuracy was evaluated by its precision term only as the standard addition calibration procedure should compensate for any bias. These results are reported below. As future data will be obtained through subsequent sample analysis, the validation data will be updated, but are not expected to change significantly.

4. RESULTS

Two of the N-nitrosoaminoacids were found to degrade during analysis, and another substance could not be detected. The remaining eight N-nitrosoamino acids could be analysed with high reliability (Figure 3). NHPRO was measured in standards in solvent but not in spiked samples. NTCA and NAzCA degraded during analysis and could not be detected. The method was validated according to the IUPAC harmonized guidelines [10].

4.1 Linearity

The linearity was evaluated by the R^2 value from standard additions made to the seven matrices at the levels 0, 0.05, 0.05 and 5.0 mg/kg. In Table 2, the lowest R^2 value obtained is reported. The calibration was achieved by correcting for IS (Figure 2). Standard addition was necessary due to matrix effects.

4.2 Limit of Detection

The Limit of Detection (LOD) was determined as the peak corresponding to a signal to noise (S/N) ratio of 3 in matrix. As no blank matrix was available, the LODs were extrapolated from samples spiked with 0.05 mg/kg of each N-nitrosoamino acid.

4.3 Precision

The repeatability (within-run precision) and the within-laboratory reproducibility (between-run precision) was determined. The between-run precision for the mean results obtained on different days are reported in Table 3. In some cases a negative sample result was obtained for the spiked samples. In those cases no between-run precision was reported.

4.4 Reporting limit

The reporting limit was statistically estimated on a worst case basis from highest standard deviation from the within-laboratory reproducibility. With a 95% confidence level, concentrations above 0.20 mg/kg could be quantified. As this measure is based on the worst (highest) standard deviation obtained for all matrices and compounds it is a very conservative estimate.

4.5 Selectivity

The selectivity was evaluated as the concentration level at which the risk of falsely reporting a result is 5%. This was estimated from the reporting limit. At concentrations above 0.20 mg/kg, the risk of false positive results are less than 5%. Again, this is a conservative estimate based on a worst case evaluation.

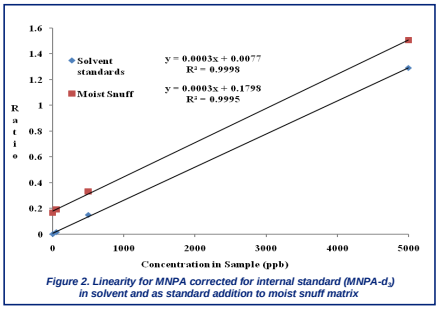

Figure 2. Linearity for MNPA corrected for internal standard (MNPA-d_3) in solvent and as standard addition to moist snuff matrix

Table 2. Linearity expressed as minimum R^2 value for standard addition to the seven matrices in the range 0 – 5.0 mg/kg. The Limit of Detection (LOD in mg/kg) as determined in 4.2 is also shown.

Tobacco	n	NSAR	MNPA	MNBA	Iso-NNAC	NMTCA	NPIPAC	NPRO	NMPhPA
LOD (mg/kg)		0.040	0.010	0.010	0.010	0.020	0.020	0.010	0.020
Loose Snus	2	1.000	1.000	1.000	0.999	1.000	1.000	0.966	0.999
Pouched Snus	8	0.958	0.964	0.990	0.968	0.998	0.998	0.992	0.999
Chewing Tobacco	4	0.998	0.971	0.998	0.990	0.992	0.999	0.736	0.995
Moist Snuff	6	0.999	0.999	0.999	0.999	0.999	0.999	0.949	0.999
Dry Snuff	2	0.999	0.892	1.000	0.993	0.999	0.999	1.000	0.991
Plug	8	0.993	0.956	0.928	0.996	0.998	0.997	0.853	0.968
Pellet	6	0.995	0.971	0.999	0.968	0.996	0.995	0.930	0.989

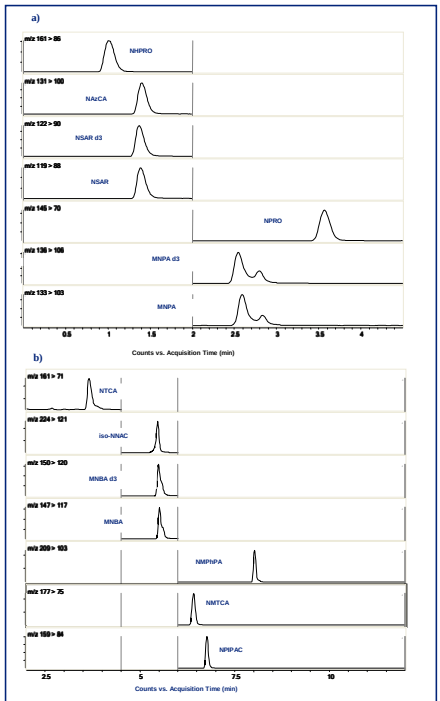

Figure 3. Chromatograms of the eleven N-nitrosoamino acids and the three internal standards in solvent. Note the different time scales in figure a (0 - 4.5 minutes) and b (2.0 – 12 minutes).

5. DISCUSSION

Although two compounds, NTCA and NAzCA, degraded during analysis by the method used in this work, they have been analyzed previously [6]. It is possible that the derivatisation process used in the earlier methods stabilized these two compounds. Moreover, the limit of detection achieved in this study was in the expected range, i.e. 0.01 – 0.04 mg/kg, which is similar to the limit of detection obtained by earlier GC-TEA methods, i.e. 0.001 – 0.05 mg/kg [6, 7, 8]. A previous HPLC-MS method was reported in the literature, this was not suitable for the STPs as the LODs were 1 - 80 mg/kg [9].

6. CONCLUSION

A new HPLC-MS/MS method was developed for the analysis of eleven N-nitrosoamino acids (Figure 1 and Table 1) in a wide range of commercial STPs. The method is based on a simple aqueous sample extraction and liquid partition cleanup on diatomaceous earth with analysis by reverse phase high performance liquid chromatography and detection by MS/MS.

Due to instability issues, the method was validated and shown to be fit for purpose for eight of the eleven N-nitrosoamino acids according to international protocol [10]. The range for limits of detection was 0.01 – 0.04 mg/kg (10 - 40 ng/g), while the reporting limit was 0.20 mg/kg (20 ng/g).

Table 3. Within laboratory between run precision expressed as the relative standard deviation (RSD in %) of the mean of analyses performed on at least three different days.

Tobacco	NSAR	MNPA	MNBA	Iso-NNAC	NMTCA	NPIPAC	NPRO	NMPhPA
Loose Snus	NE	NE	NE	NE	NE	NE	NE	NE
Pouched Snus	87*	4.7	8.6	2.8	53*	23*	8.8	35*
Chew. Tobacco	32*	7.9	6.8	14	550*	26*	52	73*
Moist Snuff	93*	14	30	34	NR	NR	10	NR
Dry Snuff	NE	NE	NE	NE	NE	NE	NE	NE
Plug	36*	13	6.8	12	76*	64*	42	92*
Pellet	107*	15	29	22	71*	96*	13	330*

NE = Not Evaluated due to low sample number (n = 2)
NR = Not Reported due to negative mean value
* = high relative standard deviations due to results below the LODs

7. DISCLOSURE

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8. ACKNOWLEDGEMENTS

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