Determination of TSNAs in tobacco by GC-CI/MS/MS

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ABSTRACT

A method was developed to quantify tobacco specific N-nitrosamines (TSNAs): N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), N-nitrosoanabasine (NAB) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in tobacco leaf. TSNAs were extracted from one gram of leaf tobacco milled to 1 mm with 10 ml of alkaline dichloromethane (10 mL CH_2Cl_2 + 0,5 mL NaOH 10%), the extract was purified with $MgSO_4$ + Na_2SO_4 and then two microliters of this sample were injected directly in the GC 3900/ Varian Saturn 2100 MS (ion trap). The column was a VF-1MS in CI/MS/MS mode using diphenylamine (DFA) as internal standard. The method showed a linear range between 2 and 5000 ng/mL with detection limits from 0,01 to 0,07 µg/g of tobacco. Afterwards 45 different types of tobaccos where analyzed for TSNAs; 34 Virginia from Argentina, Brazil, Italy, Paraguay and Uruguay, and 11 different Burleys from Argentina, Paraguay, regional blends and Italy.

MATERIALS AND METHODS

GC analysis of TSNAs by CI/MS/MS and EI/MS/MS was compared. Although TSNAs could be detected by EI/MS/MS, the specificity and sensitivity by CI/MS/MS was better due to the instability of N-nitroso compounds. Different extraction solvents, different purification media and three internal standards were tested according to the following table:

Ext. solvent	Ext. vol.	Purif. media	In.st.	Results
MeOH	40	none	NGV	Good extraction, bad reproducibility in GC/CI/MS/MS mode
CH_2Cl_2	20	none	NGV	Incomplete extraction of TSNAs
CH_2Cl_2 + NaOH	20	Na_2SO_4+$MgSO_4$	NGV	Good extraction of TSNAs bad extraction of Instd. No retention of TSNA by Na_2SO_4+$MgSO_4$ and cleaner extracts.
CH_2Cl_2 + NaOH	20	Na_2SO_4+$MgSO_4$	NNPA	Bad resolution NNPA/ NNN
CH_2Cl_2 + NaOH	20	Na_2SO_4+$MgSO_4$	DFA	Good extraction and resolution of peaks.
CH_2Cl_2 + NaOH	10	Na_2SO_4+$MgSO_4$	DFA	Good extraction and resolution of peaks.
Et.Ac.	10	Glass wool	DFA	Incomplete extraction
Et.Ac.	20	Glass wool	DFA	Incomplete extraction
Et.Ac. + Na_2SO_4	10	Glass wool+Na_2SO_4	DFA	Incomplete extraction
Et.Ac.+ NaOH	10	Na_2SO_4+$MgSO_4$	DFA	Good extraction but bad reproducibility in GC/CI/MS/MS mode
Buffer pH 4,3	20	SupelMIP	DFA	Incomplete extraction
Buffer pH 4,3	50	SupelMIP	DFA	Good extraction and purification, higher detection limits.

Table 1. Experiments carried out and their results.

The TSNAs extraction from one gram of tobacco leaf, milled to 1 mm, with 10 ml of alkaline dichloromethane (10 mL CH_2Cl_2 + 0,5 mL NaOH 10%), was selected as the best extraction solvent. Followed by a purification through a microcolumn of $MgSO_4$ + Na_2SO_4 and no other treatment before injecting two microliters of the extract in the GC 3900/ Varian Saturn 2100 MS (ion trap). The column was a VF-1MS in CI/MS/MS mode using DFA as an internal standard. The following tables show retention times, ion selection and condition analysis for the GC-CI/MS/MS of TSNAs.

Compound	Ret. time	Ion range (m/z)	MW	Precursor ion (m/z)	Excitation storage level (m/z)	Excitation amplitude (V)	Quantification ion (m/z)
DFA	8,76	85-171	169	170	62	0,60	92
NNN	10,63	110-179	177	178	65	0,40	148
NAT	11,86	100-191	189	190	70	0,40	160
NAB	12,36	125-193	191	192	71	0,48	162
NNK	14,65	100-210	207	208	77	0,45	122

Table 2. Retention times, ion selection and conditions for the GCCI-MS/MS.

Solvent delay	7 min.	Ejection amplitude	20 V
Emission current	50 µA	Isolation window	3.0 m/z
Mass defect	0 mmu/ 100u	Low-edge offset	6 steps
Count threshold	1 counts	High-edge offset	2 steps
Ion preparation technique	CI/MS/MS	High-edge amplitude	30.0 V
Multiplier offset	300 V	Isolation time	2 ms
Manifold temp.	40 ºC	Excitation time	10 ms
Transferline temp.	250 ºC	Waveform	Resonant
Ion Trap Temp.	160 ºC	Modulation range	2 steps
Axial modulating voltage	4,5 V	Number of frequencies	1
Electron multiplier voltage	1850 V	Modulation rate	3000 µs/ step

Table 5. MS parameters

Temp. (ºC)	Rate (ºC/min)	Time (min)	Total time (min)
100		1.00	1.00
200	30.0	0.00	4.33
208	3.0	1.00	8.00
215	1.0	0.00	15.00
280	30.0	5.00	22.17

Table 3. GC temperatures

Column	Varian VF-1ms
L (m) x ID (mm)	30 x 0,32
Split	off
Injector Temp.	250 ºC
Injection volume	1 µL
Flow	1,2 mL/ min
Carrier	Helium
Stabilization Time	5.00 min

Table 4. GC conditions

CI-Auto	
Gas ionization	Methanol
CI Storage level	19 m/z
Ejection Amplitude	15 m/z
Background	55 m/z
Maximum Ionization Time	2000 µs
Maximum Reaction Time	100 ms
Target TIC	5000 counts
Prescan Ionization Time	200 µs

Table 6. CI conditions

A standard curve with eight points for each TSNAs ranging from 2 to 5000 ng/mL with DFA as internal standard was prepared.

The LOD for each compound was estimated as three times the standard deviation of the lowest standard over different days and the LOQ as 10 times the same standard deviation. The precision of the method was evaluated as the relative standard deviation (RSD) of inter-assay measurements of the mid-scale standard. Accuracy of the method was tested by analyzing by triplicate 1R5F tobacco and by spiking five concentrations of TSNAs in a virgina extract of very low TSNAs content. The recovery was determined by calculating each TSNAs content and dividing the nominal amount.
Different types of tobacco produced in five different countries were analyzed by duplicate for TSNAs content. 34 Virginia type from; Argentina, Brazil, Italy, Paraguay and Uruguay, and 11 Burley type from; Argentina, Paraguay, regional blends and Italy. Tobaccos were also analyzed for total reducing sugars (Reduc.sugar %), nitrogen (N2 %) , nicotine (Nic %) and total volatile bases (TVB %) . All results were treated in the Unscrambler 8.0 for a principal component analysis.

RESULTS AND DISCUSION

The method showed a linear range between 2 and 5000 ng/mL with detection limits from 0,01 to 0,07 µg/g of tobacco. Results of TSNA in 1R5F tobacco were between limits found in bibliography (ref.3), except for NAT which was 20 % higher than that bibliography. Recovery results ranged from 85 -114 %.

All compounds showed excellent calibration coefficients and precision (relative standard deviation). Linear models values for calibration curves of each TSNA and estimated LOD, LOQ and RSD are shown in the table 7:

	Interception (ng/mL)	Slope (ng/mL)	R2	LOD (µg/g)	LOQ (µg/g)	%RSD
NNN 148	0,6	339	0,997	0,07	0,2	9,0
NAT 160	0,4	397	0,999	0,04	0,1	8,7
NAB 162	0,3	304	0,996	0,01	0,04	13,3
NNK 122	0,2	472	0,999	0,03	0,1	6,0

Table 7. Calibration parameters, LOD, LOQ and RSD

Results of TSNAs in µg/g of tobacco, total nitrogen, total volatile bases, nicotine and total reducing sugars as percentage are shown in the following table:

Virginia Tobacco /year	Origin	NNN (µg/g)	NAT (µg/g)	NAB (µg/g)	NNK (µg/g)	TOTAL (µg/g)	N2 %	TVB %	NIC %	Reduc. sugar %
V01/09	Argentina	0,2	0,5	0,05	0,3	1,1	3,1	0,7	2,4	7,0
V02/10	Argentina	0,4	1,1	0,08	0,8	2,4	3,0	0,7	2,1	5,0
V03/10	Argentina	1,6	3,0	0,27	6,2	11,0	2,7	0,5	1,2	0,7
V04/10	Argentina	0,4	0,6	0,04	0,5	1,5	2,9	0,7	2,3	6,6
V05/09	Argentina	1,7	3,1	0,43	8,2	13,4	3,2	0,5	1,8	2,2
V06/10	Brasil	0,9	1,8	0,12	0,8	3,6	3,0	0,6	1,5	5,3
V07/10	Brasil	3,4	6,2	0,58	4,4	14,6	3,2	0,6	1,2	1,3
V08/09	Brasil	0,5	0,4	0,07	0,5	1,5	3,2	0,7	3,4	8,0
V09/10	Brasil	0,3	0,0	0,04	0,4	0,8	3,2	0,7	3,4	8,0
V10/09	Brasil	0,3	0,4	0,07	0,3	1,0	3,2	0,7	3,8	12,3
V11/09	Brasil	0,2	0,3	0,05	0,3	0,9	2,9	0,6	3,0	11,8
V12/10	Brasil	0,7	0,8	0,12	0,4	2,0	3,7	0,9	3,8	6,6
V13/09	Brasil	0,4	0,2	0,05	0,3	1,0	2,6	0,6	3,1	16,0
V14/10	Brasil	0,6	0,6	0,12	0,6	2,0	3,3	0,8	3,1	9,0
V15/09	Italy	0,5	0,7	0,08	0,6	1,9	2,6	0,5	1,7	6,9
V16/09	Italy	0,7	0,8	0,09	0,5	2,0	2,7	0,6	1,9	8,4
V17/10	Italy	0,5	0,4	0,06	0,3	1,3	2,8	0,6	1,8	7,5
V18/10	Italy	0,6	0,8	0,08	0,4	1,9	2,6	0,6	1,9	10,5
V19/10	Italy	0,4	0,4	0,07	0,3	1,1	3,0	0,6	1,5	5,5
V20/10	Italy	0,6	0,9	0,11	0,7	2,3	2,7	0,6	1,6	8,9
V21/10	Italy	3,7	7,5	0,55	5,2	17,0	2,6	0,6	1,6	1,6
V22/09	Uruguay	0,4	0,6	0,10	0,4	1,6	3,0	0,7	3,9	8,6
V23/10	Uruguay	0,2	0,3	0,04	0,4	0,9	2,9	0,6	2,3	14,8
V24/10	Uruguay	0,2	0,3	0,04	0,3	0,8	2,9	0,5	1,9	10,7
V25/09	Uruguay	0,3	0,3	0,05	0,2	0,7	2,5	0,6	3,0	7,9
V26/10	Uruguay	0,2	0,3	0,02	0,3	0,8	2,7	0,4	1,0	9,7
V27/10	Uruguay	0,1	0,2	0,02	0,3	0,7	2,4	0,5	1,8	19,9
V28/09	Uruguay	0,4	0,5	0,05	0,4	1,4	3,2	0,8	4,0	9,2
V29/09	Uruguay	0,2	0,3	0,03	0,4	0,9	2,5	0,5	2,4	18,4
V30/09	Uruguay	1,6	1,5	0,25	1,0	4,3	3,3	0,6	3,2	2,8
V31/09	Paraguay	0,2	0,3	0,03	0,3	0,9	3,5	0,7	1,6	4,4
V32/09	Paraguay	1,1	1,2	0,12	0,3	2,8	4,3	1,1	4,2	2,4
V33/09	Paraguay	0,7	0,9	0,07	0,2	1,9	4,5	1,0	3,6	3,2
V34/06	France	0,2	0,3	0,04	0,2	0,8	2,0	0,4	1,7	20,6

Table 8. Virginia results

Analyzing data by a PCA the following diagrams were obtained, where it shows that tobaccos with lower content of reducing sugars presented higher levels of total TSNAs.

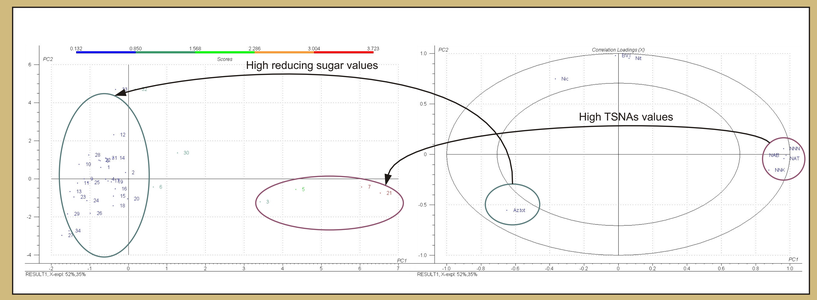

Figure 1. PCA results for Virginia tobaccos

Burley TSNAs results are shown in figure 2:

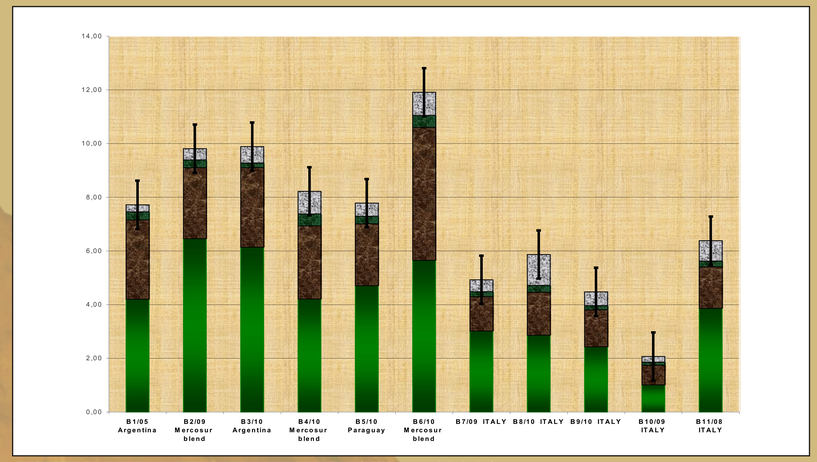

Figure 2. TSNAs content in burley leaf

CONCLUSIONS

A simple, selective and sensitive method was developed for the determination of TSNAs in tobacco leaf using GC/ion trap CI/MS/MS. The simplicity of the extraction and no need of concentrate the sample makes it easy and simple to perform.
Total content of TSNAs was almost the double in Burley than in Virginia, having a range from 2 to 12 µg/g of tobacco, being the highest values the corresponding to the NNN and NAT, while in Virginia the total TSNAs range was from 1 to 4 µg/g of tobacco (except for four tobaccos) with very similar content for each TSNAs a-part from the NAB which was the smaller. Burley tobacco from Argentina, Paraguay and regional blends showed almost double content of TSNAs than Burley tobacco from Italy.
Analyzing the loadings graph for Virginias it can be seen that TSNAs and total sugars have high loadings along the same axis, witch means that the two variables are inversely related (when one variable increases the other decreases).

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