Variations of TSNA Levels in Tobaccos Upon Heating at Moderate Temperatures *

by

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SUMMARY

Tobacco-specific nitrosamines (TSNAs) including nitrosoanabasine (NAB), nitrosoanatabine (NAT), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and nitroso-nornicotine (NNN) are naturally present at trace levels in tobacco. During tobacco processing, preparation of expanded tobacco, and when tobacco is used in heat-not-burn type cigarettes, the tobacco is exposed to different degrees of heat. Heating of tobacco has been reported in the literature to increase the level of TSNAs. Since the increase of TSNAs in heated tobacco is still not well understood, the present study evaluated TSNA levels in six types of tobacco as a function of moderate heat exposure. These tobaccos included: flue-cured lower stalk, flue-cured upper stalk (US), Burley lower stalk, Burley upper stalk (US), and two Oriental blends (Turkey, Greece, Bulgaria, Northern Republic Macedonia). Heating was performed in sealed glass tubes at oven temperatures of 100 °C, 150 °C, 200 °C, and 250 °C for time intervals of 2 min and 5 min. The temperatures inside the glass tubes were lower than the targets and were monitored separately as a function of glass tube heating. The study showed no meaningful differences within tobacco type (by stalk position) but showed considerable differences in the levels of TSNAs between different tobaccos, with the Burley tobaccos having the highest levels, and the Orientals the lowest. For all tobacco types, TSNAs increase to some extent when temperature increases. For 2-min heating, the increase in TSNAs is relatively small up to about 200 °C, but the levels almost double when the oven temperature increases to 250 °C. For 5-min heating, the increase in TSNAs starts at about 150 °C

with a maximum at 200 °C which can reach more than double the initial TSNA level. Longer heating at 250 °C (5 min) starts to cause TSNAs decomposition and the levels are reduced. [Beitr. Tabakforsch. Int. 29 (2020) 84–96]

KEY WORDS:

TSNAs; tobacco heating; moderate temperatures

ZUSAMMENFASSUNG

Tabakspezifische Nitrosamine (TSNA) einschließlich Nitrosoanabasin (NAB), Nitrosoanatabin (NAT), 4-(Methyl-nitrosamino)-1-(3-pyridyl)-1-butanon (NNK) und Nitroso-nornikotin (NNN) kommen im Tabak natürlicherweise in Spuren vor. Während der Tabakverarbeitung, der Herstellung von expandiertem Tabak und wenn der Tabak in Tabakprodukten verwendet wird, bei denen er erhitzt und nicht verbrannt wird, ist er unterschiedlichen Hitzegraden ausgesetzt. In der Literatur wird berichtet, dass das Erhitzen von Tabak die TSNA-Konzentration erhöht. Da es noch keine hinreichenden Erkenntnisse zum Anstieg des TSNA-Gehalts in erhitztem Tabak gibt, wurde in der vorliegenden Studie die Konzentration an TSNA in Abhängigkeit von moderater Hitzeeinwirkung in sechs Tabaksorten untersucht. Diese Tabaksorten waren: Flue-cured Lower Stalk, Flue-cured Upper Stalk (USA), Burley Lower Stalk, Burley Upper Stalk (USA) sowie zwei Orienttabak-Mischungen (Türkei, Griechenland, Bulgarien, Republik Nordmazedonien). Das Erhitzen erfolgte in versiegelten Glasröhren

bei Ofentemperaturen von 100 °C, 150 °C, 200 °C und 250 °C über einen Zeitraum von 2 min bzw. 5 min. Die Temperaturen in den Glasröhren waren niedriger als die Zieltemperaturen und wurden separat in Abhängigkeit von der Erwärmung der Glasröhre überwacht. Die Studie zeigte innerhalb einer Tabaksorte (bei verschiedenen Blattstufen) keine bedeutsamen Unterschiede, es ergaben sich jedoch beträchtliche Unterschiede bezüglich des TSNA-Gehalts bei verschiedenen Tabaken. Hierbei konnten in den Burley-Tabaken die höchsten Konzentrationen und in den Orient-Tabaken die niedrigsten Konzentrationen nachgewiesen werden. Bei allen Tabaksorten steigt der TSNA-Gehalt zu einem gewissen Grad mit dem Temperaturanstieg. Bei einem zweiminütigen Erhitzen bleibt die TSNA-Konzentration bis zu einer Temperatur von 200 °C relativ niedrig. Sie verdoppelt sich jedoch beinahe, wenn die Ofentemperatur auf 250 °C ansteigt. Beim fünfminütigen Erhitzen beginnt sich die TSNA-Konzentration bei ca. 150 °C zu erhöhen und erreicht ihren Maximalwert bei einer Temperatur von 200 °C. Dieser kann mehr als das Doppelte des ursprünglichen TSNA-Gehalts betragen. Das längere Erhitzen bei 250 °C (5 min) führt zu einer allmählichen Zersetzung der TSNA und damit zu geringeren Konzentrationen. [Beitr. Tabakforsch. Int. 29 (2020) 84–96]

RESUME

Les nitrosamines spécifiques du tabac (TSNA), y compris la nitrosoanabasine (NAB), la nitrosoanatabine (NAT), la 4-(méthylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) et la nitrosornicotine (NNN) sont naturellement présentes, à l'état de trace, dans le tabac. Lors du traitement du tabac, de la préparation du tabac expansé et d'une utilisation dans des produits de tabac qui "ne brûlent pas mais chauffent", le tabac est exposé à des degrés variables de chaleur. La littérature indique que le chauffage du tabac augmente les niveaux des TSNA. Sachant que l'augmentation des TSNA dans le tabac chauffé demeurait encore mal comprise, la présente étude évalua les niveaux de TSNA dans six types de tabacs en fonction d'une exposition modérée à la chaleur. Les tabacs retenus pour l'analyse furent: La partie inférieure sur la tige de tabac blond, la partie supérieure sur la tige de tabac blond (USA), la partie inférieure sur la tige de tabac Burley, la partie supérieure sur la tige de tabac Burley (USA) ainsi que deux mélanges de tabac d'Orient (Turquie, Grèce, Bulgarie, Macédoine du Nord). Le chauffage fut effectué dans des tubes de verre scellés dans un four porté à des températures de 100 °C, 150 °C, 200 °C et 250 °C durant des intervalles de 2 et 5 minutes. Les températures à l'intérieur des tubes de verre s'avèrent inférieures aux valeurs-cibles et furent observées séparément en fonction du chauffage des tubes de verre. L'étude ne révéla aucune différence significative pour un même type de tabac (indépendamment de la portion de la tige) mais épinglea des différences considérables entre les divers types de tabac eu égard aux niveaux de TSNA; en l'occurrence, les tabacs Burley présentèrent les niveaux les plus élevés tandis que les tabacs d'Orient livrèrent les niveaux les plus bas. Indépendamment du type de tabac, les TSNA augmentèrent, dans une certaine mesure, à chaque hausse de la température. Pour un temps de chauff-

fage de 2 minutes, l'augmentation des TSNA fut relativement mineure jusqu'à une température d'environ 200 °C mais les niveaux se mirent à presque doubler quand la température du four atteignit 250 °C. Pour un temps de chauffage de 5 minutes, la hausse des TSNA s'enclencha à une température approximative de 150 °C pour atteindre un pic à 200 °C qui provoqua plus qu'un doublement du niveau initial des TSNA. Un chauffage prolongé à 250 °C (5 minutes) déclencha un phénomène de décomposition des TSNA dont les niveaux se mirent à chuter. [Beitr. Tabakforsch. Int. 29 (2020) 84–96]

1. INTRODUCTION

Tobacco-specific nitrosamines including nitrosoanabasine (NAB), nitrosoanatabine (NAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), and nitrosornicotine (NNN) are naturally present at trace levels in tobacco. These levels vary considerably depending on many factors such as the type of tobacco (flue-cured, Burley, Oriental), the location of tobacco production, the leaf position, the leaf processing procedure, the tobacco age, etc. The level of TSNA in tobacco is of considerable interest since these compounds are listed among the harmful and potentially harmful constituents (HPHC) of tobacco products on which FOOD & DRUG ADMINISTRATION (FDA) requires reporting (1). Also, NNK and NNN have been classified as human carcinogens (Class 1) by the INTERNATIONAL AGENCY FOR RESEARCH ON CANCER (IARC) (2). Tobacco heating has been reported to increase the level of TSNA in tobacco (3, 4). In addition to the heating of tobacco behind the burning cone of a common burning cigarette, there are other instances when tobacco is heated, although at lower temperatures. Such cases include processing (e.g., drying), Burley toasting, preparation of expanded tobacco, as well as the use in "heat-not-burn" type cigarettes. Since the increase of TSNA in heated tobacco is still not well understood, this was investigated in the present study. Tobacco-specific nitrosamines (TSNA) can be analyzed by various methods that have been extensively reported in the literature (5–18). These methods were applied on tobacco, cigarette smoke, and several tobacco products such as moist snuff. An adaptation of one of these methods (18) has been used in the present study and was applied to TSNA analysis in tobaccos. The study and the selection of the temperature range in which the tobacco was heated (100, 150, 200, 250 °C) intended to provide information for the process in heat-not-burn type cigarettes and other instances in which tobacco is subject to moderate temperatures.

2. EXPERIMENTAL

2.1. Materials

Several chemicals including ammonium acetate, ammonium formate, formic acid, and acetonitrile were obtained from Sigma/Aldrich (St. Louis, MO, USA). Nicotine, nitrosoanabasine (NAB), nitrosoanatabine (NAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), nitrosornicotine (NNN), NAB-d₄, NAT-d₄, NNK-d₄ and

NNN-d₄ were obtained from Toronto Research Chemicals Inc. (TRC) (North York, ON, Canada). Pure water (18.2 MΩ/cm) was obtained from a Barnstead water purification unit (Thermo Fisher Scientific, Waltham, MA, USA). The GC vials were 2 mL with screw top caps with septa. For filtration 0.45-μm PVDF filters were used (Whatman Autovial, GE Healthcare, Little Chalfort, UK).

2.2. Instrumentation

The instruments used for the analysis consisted of an Agilent 1290 HPLC binary system with a binary pump, an autosampler with cooling capability, and a column thermostatted compartment. The HPLC chromatographic separation was achieved on a Luna® 3 μm C18 150 × 3 mm from Phenomenex (Torrance, CA, USA). The MS/MS system was an API-6500 triple quadrupole mass spectrometer (AB Sciex, Framingham, MA, USA). The LC-MS/MS system was controlled using Analyst 1.6.2 software, and the peak integration was performed with MultiQuant 3.0.1 software. The heating was performed in a Thermoline Furnace 62700 (Thermo Fisher, Waltham, MA, USA). Tobacco moisture was measured with a halogen moisture analyzer HE53 (Mettler Toledo, Greifensee, Switzerland).

2.3. TSNA standards preparation

An extraction solution of 100 mM ammonium acetate in water was prepared by dissolving 7.7 g of ammonium acetate into 1 L of purified water. The standard solutions were prepared from a stock solution of 162.65 μg/mL N-nitrosornicotine (NNN), 164.036 μg/mL N'-nitrosoanatabine (NAT), 41.508 μg/mL N-nitrosoanabasine (NAB) and 161.10 μg/mL nicotine-derived nitrosamine ketone (NNK). Standard 1 was prepared by 1/1000 dilution of the stock solution with the extraction solution and used to prepare Standards 2–7, 1000 μmL each. To each 1 mL standard was added 20 μL of the internal standard solution. The internal standard solution contained 1.6 μg/mL NNN-d₄, 1.6 μg/mL NAT-d₄, 1.33 μg/mL NAB-d₄ and 1.6 μg/mL NNK-d₄. The concentration of internal standard (I.S.) in the final solution is indicated in Table 1.

Table 1. Concentrations of deuterated TSNA in the samples containing internal standard solution.

NNN-d ₄ (ng/mL)	NAT-d ₄ (ng/mL)	NAB-d ₄ (ng/mL)	NNK-d ₄ (ng/mL)
40.663	41.009	10.377	40.275

The concentrations of the utilized standards are given in Table 2.

2.4. Sample heat processing

Heating of the tobacco was performed in sealed Pyrex glass tubes. This was done to avoid potential loss of material by evaporation when the tobacco was heated. Glass tube dimensions were 8 mm i.d. and a length of 15 cm with a

Table 2. Concentrations of TSNA in the standard solutions.

Standard	NNN (ng/mL)	NAT (ng/mL)	NAB (ng/mL)	NNK (ng/mL)
1	162.65	164.04	41.51	161.10
2	81.33	82.02	20.75	80.55
3	40.66	41.01	10.38	40.28
4	20.33	20.50	5.19	20.14
5	10.17	10.25	2.59	10.07
6	5.08	5.13	1.30	5.03
7	2.54	2.56	0.65	2.52

1.0-mm wall thickness. About 600 mg tobacco (± 20 mg) were weighed into each tube. The tubes were sealed at one end and were subjected to a mild vacuum prior to sealing the other end. The purpose of applying a vacuum was to prevent the tubes from breaking when exposed to heat due to the expansion of gases in the tube. Figure 1 shows several sealed tubes containing powdered tobacco (Burley lower stalk) before heating.

Four different temperatures were used for heating the tubes: 100 °C, 150 °C, 200 °C, and 250 °C. Two time intervals were selected for heating: 2 min and 5 min. The actual temperature of the tobacco was not measured during the experiments performed for TSNA measurement. However, in a published study (3) the heating inside of a tobacco rod was evaluated, when exposed from exterior to 100 °C or 200 °C. The study (3) indicated a delay in the temperature reached by the middle of a tobacco rod as compared to the applied external temperature. In the present study, an additional issue was that upon removing the glass tube with the tobacco from the oven, the cooling of the material was not instantaneous and it took 3–4 min for the tube to reach room temperature. For this reason, the temperature inside a sample (of Burley) was measured separately as a function of heating time. For this purpose a thermocouple was inserted into the sample during the heating of the glass tube containing the sample. At 5 min the heating was stopped by removing the glass tube from the oven and exposing it to room temperature. The temperature variation inside the sample is shown in Figure 2.

As indicated in Figure 2, the temperature to which the glass tube with sample is exposed is different from the actual temperature of the sample. Assuming a linear idealized temperature profile to which the tobacco is exposed, the temperature variation in the tobacco can be described by the following formula:

$$T(t) = T_{\text{init.}} + b_1 t \quad (0 < t < t_{\text{max}}) \quad [1]$$

$$T(t) = T_{\text{init.}} + b_1 t_{\text{max}} - b_2 t \quad (t_{\text{max}} < t < t_{\text{final}})$$

where $T(t)$ is the temperature of the sample as a function of time t , $T_{\text{init.}}$ is the initial temperature (about 22 °C), t_{max} is the length of time the sample is heated, t_{final} is the time of cooling to room temperature, and b_1 and b_2 are heating and cooling rates, respectively. The fixed oven temperatures 100 °C, 150 °C, 200 °C, and 250 °C for which the results are indicated in this study, are in fact described correctly by sample temperature variations as shown in Figure 2 and approximated by equation [1]. However, the linear approxi-

Figure 1. Sealed tubes containing powdered tobacco (Burley lower stalk) before heating.

Figure 2. Temperature variation inside a tobacco sample placed in a glass tube as a function of heating time of the tube. Heating was stopped at 5 min and the samples were exposed to room temperature.

mation holds only for the first minute of heating. Afterwards the higher temperatures showed an endothermic leveling when passing 100 °C and the rates for all temperatures decreased as the difference in temperature between the tobacco and the outside of the tube decreased. The initial slopes (°C/s) were proportional to the oven temperature and were equal to $[0.0076 \times \text{oven temperature} - 0.494]$ with an $R^2 = 0.999$. The initial rate of cooling was also linear and equal to $[-0.0062 \times \text{oven temperature} + 0.433]$ with an $R^2 = 0.939$. The cooling rate was slightly lower than the heating rate because the initial temperature difference was lower between the inside and the outside of the tube. The fixed temperatures of the oven, to which the glass

tubes were exposed, were precisely controlled (within 1–2 °C) while the true temperature of the samples was subject to variations depending on the exact mass of the glass tube, the mass of the tobacco sample, and on the distribution of the tobacco inside the glass tube during heating, all of which affected parameters b_1 and b_2 . The use of sealed tubes as containers for the heated tobacco avoided potential loss of material by evaporation but at the same time did not allow a precise measurement of the temperature in the tobacco sample. This temperature can be inferred only by comparing the temperature of the heating of the glass tube with that inside the sample shown in the graph of Figure 2.

2.5. TSNA analysis

Prior to the analysis, the moisture of the samples was measured using a halogen moisture analyzer. For the analysis, samples of 250 ± 1.0 mg of tobacco (as is) were weighed directly into 20-mL scintillation vials. To each vial 10 mL of the extraction solution were added, then the vial was capped and placed into a shaker for 30 min. The solution was then filtered through a syringe filter into a test tube. 1000 μ L of the filtered solution was transferred to a 2-mL GC vial and 20 μ L of the TSNA internal standard was added to the sample.

The HPLC separation of TSNA was performed using a gradient program. The mobile phase A was 100 mM ammonium formate pH 4.9 aqueous solution with 5% acetonitrile and mobile phase B was 0.2% formic acid in acetonitrile. The timetable for the gradient is shown in Table 3.

Table 3. Solvent gradient employed for the HPLC-MS/MS analysis of TSNA.

Time	% A	% B	Flow rate (mL/min)
0.0	100.0	0.0	0.6
1.7	100.0	0.0	0.6
3.5	70.0	30.0	0.6
4.5	60.0	40.0	0.6
8.0	50.0	50.0	0.6
10.0	100.0	0.0	0.6

The parameters for the MS/MS detection included: curtain gas CUR = 20 mL/min, collision gas CAD = 4 mL/min, ion spray voltage IS = 4500 V, temperature TEM = 500 °C, ion source gas 1 GS1 = 40 mL/min, ion source gas 2 GS2 = 50 mL/min, entrance potential EP = 10 V, target scan time 0.11 s, scheduled MRM detection window 40 s. Other parameters including the analyzed ions are shown in Table 4.

Extracted ion chromatograms for the eight compounds measured in the study are shown in Figure 3 for a flue-cured tobacco (upper stalk) heated at 200 °C for 5 min. Both NNN and NNN-d₄ show two peaks in the previously described separation conditions. Only the higher of the two peaks was used for the quantitation. Peak splitting for TSNA can be attributed to the presence of a *syn-anti* iso-

mers of the nitrosamine group.

For the quantitation of TSNA, calibration curves were constructed by plotting the concentrations of the standard solutions as a function of the peak areas normalized to the area of the corresponding deuterated internal standards. All calibrations were linear and they were expressed using the formula:

$$Y(\text{conc.}) = a(\text{Std. Area/I.S. Area}) + b \quad [2]$$

Parameters *a* and *b* as well as correlation coefficient *R*² for the calibration lines are shown in Table 5.

The overall validation of the analytical method for the analysis of TSNA has been previously reported (18) and no further validation has been performed for the method.

3. RESULTS

3.1. Sample description

The samples analyzed in this study are listed in Table 6, together with the measured moisture % total alkaloid level as well as the time intervals and temperatures to which they were exposed.

Some samples were processed in duplicate or even triplicate, and other samples were analyzed only once. The samples were randomly analyzed, and some duplicate or triplicate evaluations were made to verify the repeatability of the results. Once the sample was heated and extracted, the LC/MS/MS measurement was performed in duplicate.

3.2. Average TSNA levels for the starting tobacco

The results of average TSNA levels in ng/g for the starting tobaccos (analyzed before starting the heating experiments) are shown in Table 7. All results are reported for the tobacco “as is,” a conversion to “dry basis” being possible using the moisture data from Table 6.

As indicated in Table 7, the levels of TSNA did not differ meaningfully within tobacco type (by stalk position), but among the various tobacco types they differed considerably. Burley tobaccos have a very high level of TSNA even before heating. Significantly lower levels compared to the Burleys are present in flue-cured tobaccos. The lowest TSNA levels are present in the Oriental tobaccos.

Table 4. Other parameters for the MS/MS detection.

Compound	Ion for Q1	Ion for Q3	Retention time (min)	DP ¹ (V)	CE ² (V)	CXP ³ (V)
NNN	178.1	148.1	4.73	30	15	9
NNN-d ₄	182.1	152.1	4.70	30	16	9
NAT	190.1	160.1	5.35	28	15.5	10
NAT-d ₄	194.1	164.1	5.31	28	16	10
NAB	192.1	162.1	5.40	26	18	11
NAB-d ₄	196.1	166.1	5.37	26	17	10
NNK	208.1	122.1	5.16	30	17.6	13
NNK-d ₄	212.1	126.1	5.15	30	16	12

¹ DP = Declustering potential; ² CE = Collision energy; ³ CXP = Collision cell exit potential

Figure 3. Extracted ion chromatograms for NNN, NAT, NAB and NNK and their d₄-labeled compounds measured for a flue-cured tobacco (upper stalk) heated at 200 °C for 5 min.

Table 5. Parameters *a*, *b* and *R*² for the MS/MS quantitation.

Analyte	<i>a</i>	<i>b</i>	<i>R</i> ²
NNN	33.5151	0.1798	0.9997
NAT	40.8507	0.0479	0.9998
NAB	35.1528	0.0768	0.9992
NNK	35.7655	0.0889	0.9996

Table 7. Initial TSNA levels in the tobacco samples in ng/g (tobacco as is).

Sample acronym	NNN (ng/g)	NAT (ng/g)	NAB (ng/g)	NNK (ng/g)
FC-Lo	365.2	566.5	33.2	283.1
FC-Up	354.1	458.3	32.6	227.4
Bu-Lo	4688.3	4616.8	199.7	861.0
Bu-Up	4780.3	4662.1	183.2	773.3
Or (1)	156.6	54.8	6.14	43.0
Or (2)	126.4	33.5	4.79	32.6

Table 6. Tobacco samples, moisture (%), alkaloid level (%), and the conditions to which the samples were exposed.

Sample acronym	Tobacco description	Moisture (%)	Total alkaloids (%)	Temperatures (°C)				Time of exposure (min)	
FC-Lo	Lower stalk flue-cured	6.44	2.07	100	150	200	250	2	5
FC-Up	Upper stalk flue-cured	6.71	2.85	100	150	200	250	2	5
Bu-Lo	Lower stalk Burley	6.36	2.51	100	150	200	250	2	5
Bu-Up	Upper stalk Burley	7.11	3.46	100	150	200	250	2	5
Or (1)	Oriental blend (1)	6.64	1.13	100	150	200	250	2	5
Or (2)	Oriental blend (2)	6.23	1.02	100	150	200	250	2	5

Table 8. Variation of TSNA in flue-cured tobaccos upon heating (in ng/g).

Temperature (°C)	Flue-cured lower stalk, FC-Lo		Flue-cured upper stalk, FC-Up	
	2 min	5 min	2 min	5 min
NNN (ng/g)				
23	372.5 ± 2.1	372.5 ± 2.1	358.3 ± 3.9	358.3 ± 3.9
23	357.9 ± 0.5	357.9 ± 0.5	342.9 ± 1.7	342.9 ± 1.7
23	—	—	361.0 ± 2.9	361.0 ± 2.9
100	397.9 ± 6.9	400.8 ± 0.2	378.9 ± 9.1	389.5 ± 5.3
100	—	—	388.8 ± 2.6	380.5 ± 3.5
150	420.5 ± 1.7	465.3 ± 1.4	392.7 ± 10.4	422.6 ± 4.7
150	—	—	391.7 ± 14.3	—
200	479.8 ± 1.7	697.4 ± 5.2	457.5 ± 10.2	540.5 ± 9.3
200	—	—	461.3 ± 4.7	546.0 ± 3.3
250	496.7 ± 9.2	109.1 ± 0.4	595.8 ± 42.4	102.4 ± 0.6
NAT (ng/g)				
23	566.7 ± 7.2	566.7 ± 7.2	469.1 ± 4.7	469.1 ± 4.7
23	566.4 ± 4.8	566.4 ± 4.8	447.9 ± 7.4	447.9 ± 7.4
23	—	—	457.9 ± 2.1	457.9 ± 2.1
100	602.8 ± 18.0	645.0 ± 4.6	520.0 ± 12.7	492.6 ± 1.0
100	—	—	517.5 ± 22.5	483.6 ± 3.1
150	693.2 ± 3.2	836.3 ± 10.9	529.3 ± 8.4	618.3 ± 1.6
150	—	—	520.9 ± 4.1	—
200	909.8 ± 5.0	1366.4 ± 5.6	649.3 ± 11.7	871.2 ± 1.7
200	—	—	657.5 ± 1.4	885.8 ± 6.6
250	945.6 ± 5.8	17.1 ± 0.9	1039.2 ± 30.6	10.9 ± 0.2
NAB (ng/g)				
23	31.9 ± 0.1	31.9 ± 0.1	34.8 ± 3.1	34.8 ± 3.1
23	34.5 ± 0.2	34.5 ± 0.2	30.4 ± 0.2	30.4 ± 0.2
23	—	—	32.7 ± 0.2	32.7 ± 0.2
100	40.3 ± 0.5	38.0 ± 0.8	36.5 ± 0.5	36.9 ± 0.4
100	—	—	38.4 ± 0.4	37.1 ± 0.5
150	39.6 ± 0.2	46.4 ± 1.2	37.0 ± 0.2	43.8 ± 0.2
150	—	—	38.2 ± 0.8	—
200	51.1 ± 0.5	101.0 ± 0.3	45.7 ± 2.4	72.3 ± 0.6
200	—	—	48.6 ± 0.7	74.2 ± 0.6
250	72.4 ± 0.6	41.1 ± 0.2	99.7 ± 5.7	43.5 ± 1.3
NNK (ng/g)				
23	270.1 ± 1.5	270.1 ± 1.5	225.3 ± 4.9	225.3 ± 4.9
23	296.1 ± 2.4	296.1 ± 2.4	218.0 ± 1.7	218.0 ± 1.7
23	—	—	239.0 ± 1.4	239.0 ± 1.4
100	318.5 ± 7.4	314.5 ± 1.9	242.8 ± 5.7	235.0 ± 1.5
100	—	—	236.2 ± 8.1	225.6 ± 3.3
150	341.0 ± 1.33	343.9 ± 3.9	240.6 ± 5.5	225.4 ± 0.1
150	—	—	231.4 ± 0.1	—
200	352.7 ± 4.3	658.7 ± 9.4	249.1 ± 4.9	349.1 ± 6.8
200	—	—	259.0 ± 0.2	340.3 ± 0.4
250	640.0 ± 13.1	269.7 ± 0.5	430.6 ± 32.1	216.4 ± 2.7

3.3. TSNA levels in the heated tobaccos

The experimental results for levels of TSNA in the six evaluated tobaccos for the two heating intervals (2 min and 5 min) are indicated in Tables 8 to 10. In these tables, the listed and repeated temperatures (for a specific TSNA in each tobacco) indicate results from repeated whole experi-

ments (including heating of the tobacco and LC/MS/MS measurement).

Each LC/MS/MS measurement for every experiment was performed in duplicate and the RSD% values are reported between repeated LC/MS/MS measurements.

The results of TSNA levels in heated flue-cured tobaccos FC-Lo and FC-Up are indicated in Table 8.

Table 9. Variation of TSNA levels in lower stalk Burley tobaccos upon heating (in ng/g).

Temperature (°C)	Burley lower stalk, Bu-Lo		Burley upper stalk, Bu-Up	
	2 min	5 min	2 min	5 min
NNN (ng/g)				
23	4678.4 ± 262.0	4678.4 ± 262.0	4666.7 ± 65.8	4666.7 ± 65.8
23	4698.2 ± 40.4	4698.2 ± 40.4	4893.9 ± 15.2	4893.9 ± 15.2
100	4715.8 ± 98.1	4701.7 ± 44.7	5458.9 ± 21.3	5753.9 ± 51.8
150	4903.5 ± 219.2	5968.8 ± 69.2	6288.3 ± 4.4	7508.4 ± 7.5
200	5930.8 ± 215.3	8541.1 ± 160.6	6953.1 ± 47.3	10030.8 ± 68.2
200	—	—	6947 ± 42.4	—
250	8961.2 ± 49.3	9383.9 ± 122.0	8525.8 ± 148.3	7077.4 ± 38.9
NAT (ng/g)				
23	4611 ± 29.5	4611 ± 29.5	4430.1 ± 28.8	4430.1 ± 28.8
23	4622.5 ± 268.1	4622.5 ± 268.1	4894.2 ± 5.4	4894.2 ± 5.4
100	4627.4 ± 5.1	4660.9 ± 7.9	5585.9 ± 6.1	5893.8 ± 86.0
150	4812.6 ± 169.9	6485.5 ± 81.7	6386.6 ± 44.1	8067.5 ± 49.2
200	6327.2 ± 160.1	10063.8 ± 169.1	10005.5 ± 0.0	10677.6 ± 59.8
200	—	—	9543.5 ± 40.1	—
250	10655.9 ± 86.3	4775.7 ± 27.2	8994.5 ± 161.9	1356.7 ± 3.7
NAB (ng/g)				
23	204.7 ± 10.8	204.7 ± 10.8	176.4 ± 2.1	176.4 ± 2.1
23	194.8 ± 0.8	194.8 ± 0.8	190.1 ± 0.8	190.1 ± 0.8
100	199.2 ± 1.4	196.0 ± 1.2	223.8 ± 0.1	224.5 ± 1.7
150	207.6 ± 9.3	290.2 ± 1.6	249.1 ± 2.7	340.8 ± 0.5
200	282.6 ± 7.1	629.9 ± 7.1	423.4 ± 2.4	582.1 ± 2.9
200	—	—	396.8 ± 3.1	—
250	637.3 ± 2.5	1683.8 ± 11.1	474.9 ± 6.5	1734.9 ± 15.4
NNK (ng/g)				
23	855.6 ± 32.3	855.6 ± 32.3	791.7 ± 1.7	791.7 ± 1.7
23	866.4 ± 0.4	866.4 ± 0.4	754.8 ± 0.0	754.8 ± 0.0
100	844.9 ± 9.5	866.9 ± 6.7	858.8 ± 13.7	884.9 ± 1.8
150	898.8 ± 29.3	1039.6 ± 11.1	963.6 ± 3.1	1145.3 ± 1.3
200	1040.9 ± 38.6	2551.8 ± 14.5	1298.4 ± 2.9	2065.1 ± 14.2
200	—	—	1216.6 ± 13.5	—
250	3517.8 ± 1.8	4749 ± 2.4	1711.0 ± 27.5	2916.5 ± 3.8

The results of TSNA levels in heated Burley tobaccos Bu-Lo and Bu-Up are indicated in Table 9. The results of TSNA levels in heated Oriental tobaccos are indicated in Table 10.

4. DISCUSSION

A typical level variation of TSNA levels with oven temperatures as described in Tables 8 to 10, is shown in Figure 4 for NNN in FC-Up samples for 2-min heating intervals, and for 5-min heating intervals in Figure 5.

For other types of tobacco and other TSNA levels it was common to obtain similar curves of variations although there were also some exceptions when the drop of the TSNA level for 5-min heating at 250 °C was less obvious. This can be explained by possible non-homogeneities in the heating of the specific sample. The typical increase in TSNA levels described in Tables 8 to 10 can be visualized

by comparing Figure 6, which shows the initial TSNA levels with Figure 7, which shows the levels after 2-min heating at 200 °C.

Also, as expected, the heating of tobacco for 5 min led to increased formation of TSNA levels, as shown by comparing the results illustrated in Figure 7 with those illustrated in Figure 8 for heating at 200 °C for 5 min (all data taken from Tables 8 to 10).

The heating of tobacco for 2 min at 250 °C continues in most cases to increase the TSNA level, as is illustrated by comparing Figure 7 with the data from Figure 9 which shows the results for heating the tobaccos for 2 min at 250 °C. However, by further heating the tobacco for 5 min at 250 °C, the levels of TSNA were decreasing in most cases, as can be seen by comparing the results from Figure 9 with those from Figure 10 which shows the results for heating the samples for 5 min at 250 °C.

The results of this study indicate that moderate temperatures and extended heating time are factors leading to an

Table 10. Variation of TSNA's in Oriental tobaccos upon heating (in ng/g).

Temperature (°C)	Oriental (1), Or(1)		Oriental (2), Or(2)	
	2 min	5 min	2 min	5 min
NNN (ng/g)				
23	156.9 ± 1.3	156.9 ± 1.3	130.2 ± 0.5	130.2 ± 0.5
23	156.3 ± 1.3	156.3 ± 1.3	127.0 ± 0.3	127.0 ± 0.3
23	—	—	122.0 ± 2.7	122.0 ± 2.7
100	203.7 ± 1.9	218.0 ± 1.8	146.2 ± 0.1	147.6 ± 1.5
150	211.4 ± 1.6	273.4 ± 1.5	194.1 ± 0.2	225.3 ± 1.5
200	282.1 ± 3.4	489.2 ± 13.0	205 ± 0.8	325.3 ± 2.6
200	307.0 ± 1.0	—	—	319.5 ± 5.8
250	386.7 ± 11.5	146.2 ± 1.0	289.1 ± 5.2	—
250	—	—	298.3 ± 0.5	—
NAT (ng/g)				
23	55.0 ± 0.9	55.0 ± 0.9	33.3 ± 0.1	33.3 ± 0.1
23	54.6 ± 0.7	54.6 ± 0.7	33.8 ± 1.3	33.8 ± 1.3
100	69.7 ± 0.4	77.7 ± 1.2	39.0 ± 1.0	39.1 ± 0.7
150	73.2 ± 1.5	122.1 ± 0.2	53.5 ± 0.8	67.7 ± 2.0
200	130.8 ± 1.5	284.4 ± 4.5	64.7 ± 1.0	121.7 ± 2.1
200	139.3 ± 0.9	—	—	118.6 ± 0.5
250	207.3 ± 3.8	12.9 ± 0.4	105.6 ± 2.7	—
250	—	—	108.8 ± 1.7	—
NAB (ng/g)				
23	6.2 ± 0.3	6.2 ± 0.3	4.9 ± 0.1	4.9 ± 0.1
23	6.1 ± 0.3	6.1 ± 0.3	4.7 ± 0.1	4.7 ± 0.1
100	6.7 ± 0.3	7.1 ± 0.6	4.8 ± 0.1	5.1 ± 0.0
150	6.9 ± 0.5	10.3 ± 0.1	5.7 ± 0.3	6.7 ± 0.0
200	10.3 ± 0.2	28.0 ± 0.2	7.0 ± 0.0	18.1 ± 0.4
200	11.2 ± 0.4	—	—	17.6 ± 0.0
250	18.4 ± 0.4	25.6 ± 0.1	13.9 ± 0.7	—
250	—	—	15.3 ± 0.1	—
NNK (ng/g)				
23	41.3 ± 0.6	41.3 ± 0.6	32.9 ± 0.4	32.9 ± 0.4
23	44.7 ± 1.2	44.7 ± 1.2	32.4 ± 0.4	32.4 ± 0.4
100	54.2 ± 0.8	54.7 ± 0.4	38.2 ± 2.0	38.2 ± 0.4
150	55.2 ± 1.4	57.5 ± 0.4	45.2 ± 0.8	49.0 ± 1.5
200	56.8 ± 2.0	119.8 ± 0.4	48.4 ± 0.6	76.8 ± 0.1
200	59.8 ± 0.7	—	—	76.8 ± 0.3
250	85.5 ± 1.4	91.5 ± 2.8	62.0 ± 0.3	—
250	—	—	65.9 ± 0.2	—

increase in the TSNA levels in tobacco. They also show that this increase takes place up to a point as the temperature increases, and beyond this point, very likely, some TSNA's decomposition starts to take place. In order to keep the TSNA levels as low as possible, unnecessary heating of tobacco at moderate temperatures must be avoided. However, tobacco heating cannot be avoided in many devices such as for example in the heat-not-burn type cigarettes, where certain temperatures are a precondition for the release of flavors and nicotine as well as humectants added to tobacco. As a result, the increase in TSNA's cannot be avoided. By selecting specific tobaccos already low in TSNA's, an excessive increase of TSNA's due to heating can be diminished. The decomposition of TSNA's that occurs

when tobacco is heated beyond a specific temperature and length of time could probably be used for lowering the TSNA levels. Nevertheless a careful evaluation of this process must be performed with additional examination and control of the decomposition of other tobacco constituents such as sugars.

5. CONCLUSIONS

Six commonly used tobaccos, including two flue-cured, two Burleys and two Oriental tobaccos were analyzed for their TSNA levels. These tobaccos were heated in sealed glass containers at oven temperatures of 100, 150, 200 and 250 °C

Figure 4. Variation of NNN in FC-Up sample with 2 min heating at different oven temperatures.

Figure 5. Variation of NNN in FC-Up sample with 5 min heating at different oven temperatures.

Figure 6. Initial levels of TSNAs (data taken from Table 7).

Figure 7. Levels of TSNAs after heating for 2 min at 200 °C (data taken from Tables 8 to 10).

Figure 8. Levels of TSNAs after heating for 5 min at 200 °C (data taken from Tables 8 to 10).

Figure 9. Levels of TSNAs after heating for 2 min at 250 °C (data taken from Tables 8 to 10).

Figure 10. Levels of TSNAs after heating for 5 min at 250 °C (data taken from Tables 8 to 10).

for intervals of 2 min or 5 min. The TSNA levels were found to increase up to a point during tobacco heating, reaching levels as high as double from the initial level. However, the TSNAs increase did not continue when further heating was performed due to some TSNA's decomposition probably taking place beyond a specific point of heating.

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