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Formation of Tobacco Specific Nitrosamines in Mainstream Cigarette Smoke; Part 1, FTC Smoking*

by

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SUMMARY

This report evaluates the formation of nitrosonornicotine (NNN) and of 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) from nicotine, and of NNN from nornicotine in the mainstream smoke of a burning cigarette. The cigarettes analyzed in the study were Kentucky reference cigarettes 1R4F and 2R4F, and five other cigarettes, three of them having tobaccos with low levels of tobacco specific nitrosamines (TSNAs). All cigarettes had 'tar' levels around 10 mg [where 'tar' is defined as the weight of total wet particulate matter (TPM) minus the weight of nicotine and water]. Cigarettes were smoked according to the U.S. Federal Trade Commission (FTC) puffing regimen, using a 35 mL puff volume, 2 sec puff duration and 60 sec puff intervals. Three separate experiments were performed in this study to evaluate the proportion of TSNAs transferred from preexistent tobacco TSNAs and the proportion formed during smoking (pyrosynthesized). In one experiment, the results were obtained by GC/MS analysis of $^{13}C_3$-TSNAs formed in smoke when $^{13}C_3$-nicotine was intentionally added to cigarettes. Another experiment used GC analysis with chemiluminescence detection of TSNAs from smoke before and after an excess of nornicotine was intentionally added to cigarettes, and another experiment consisted of LC/MS/MS analysis of $^{2}H_4$-TSNAs formed in the smoke when $^{2}H_4$-nicotine and when $^{2}H_4$-nornicotine were intentionally added to cigarettes. The use of different analytical methods for the study of TSNA formation conveyed an additional level of confidence regarding the reliability of the results obtained. It was found that NNK was generated during smoking from nicotine with 3×10^{-5}% to 8×10^{-5}% conversion (0.3 ppm to 0.8 ppm conversion of the nicotine) while the result for NNN generation was not conclusive. One experiment demonstrated the formation of NNN from nicotine between 4×10^{-5}% and 1.5×10^{-4}% (0.4 ppm to 1.5 ppm reported to nicotine), but another experiment did not provide proof of NNN formation, with a limit of quantitation LOQ for NNN corresponding to 5×10^{-5}% (or 0.5 ppm). Nornicotine was proven to generate NNN, and the results for the 2R4F cigarette showed 3.3×10^{-3}% yield (33 ppm reported to nornicotine) in one experiment and 4.6×10^{-3}% (46 ppm reported to nornicotine) in a different experiment, the agreement being very good. Using the results from this study, it was concluded that pyrosynthesis may account for about 5% to 10% of the NNK in mainstream smoke for a filter cigarette with the FTC 'tar' level around 10 mg. Pyrosynthesis may account for higher proportions of smoke TSNAs when the cigarette tobacco is low in TSNAs, since the mainstream smoke TSNAs yield from direct transfer from tobacco is small in this case. The contribution of pyrosynthesis may account for 5% to 25 % of NNN in mainstream cigarette smoke, or potentially an even higher proportion when the tobacco blend is both low in TSNAs and high in nornicotine. Anabasine is typically present at low levels in tobacco and therefore the formation of nitrosoanabasine (NAB) is of less interest. Anatabine is present in different tobaccos in a range similar to that of nornicotine and being a secondary amine has the potential to act similarly to nornicotine. However, the pyrosynthesis of nitrosoanatabine (NAT) from anatabine was not evaluated in the present study. The study indicated that complete elimination of TSNAs from tobacco is unlikely to completely eliminate the TSNAs from cigarette smoke, and that high nornicotine tobaccos should be avoided in order to minimize the levels of NNN in cigarette smoke. [Beitr. Tabakforsch. Int. 23 (2008) 19–31]

ZUSAMMENFASSUNG

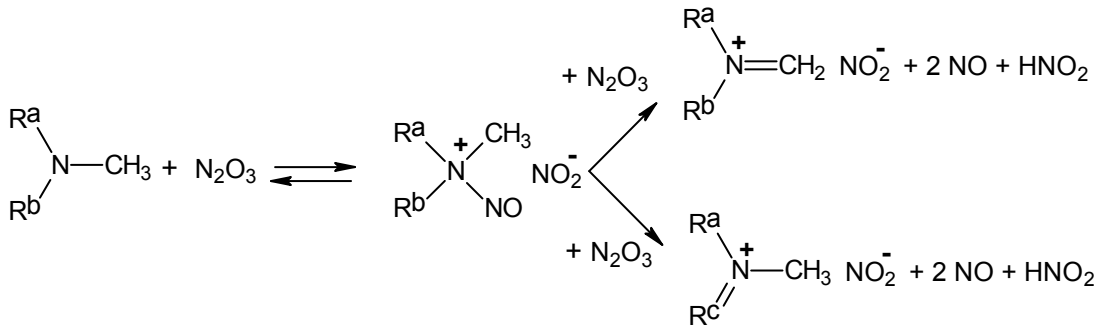
Diese Studie untersucht die Bildung von Nitrosonornikotin (NNN) und 4-(Methyl-nitrosamino)-1-(3-pyridyl)-1-butanon (NNK) aus Nikotin sowie von NNN aus Nornikotin im Hauptstromrauch einer brennenden Zigarette. Bei den in dieser Studie untersuchten Zigaretten handelte es sich um

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die Kentucky-Referenzzigaretten 1R4F und 2R4F sowie fünf weitere Zigaretten, von denen drei niedrige Werte an tabakspezifischen Nitrosaminen (TSNA) aufwiesen. Alle Zigarettensorten hatten Kondensatwerte von ca. 10 mg [wobei Kondensat als das Gewicht der Gesamtpartikelmasse (TPM) abzüglich des Gewichts von Nikotin und Wasser definiert wird]. Die Zigaretten wurden nach den Abrauchnormen der U.S. Federal Trade Commission (FTC) mit einem Zugvolumen von 35 mL, einer Zugdauer von zwei Sekunden und einem Zugintervall von 60 Sekunden abgeraucht. Drei unabhängige Expermerimente wurden in dieser Studie durchgeführt, um den Anteil, der aus den bereits im Tabak enthaltenen TSNAs zu bestimmen, der in den Rauch überging und den während des Rauchens durch Pyrolyse entstandenen TSNA-Anteil zu ermitteln. In einem Experiment wurde durch gaschromatographische-massensprektrometrische (GC/MS) Analyse die Menge an $^{13}C_3$-TSNA, die im Rauch nach Zugabe von ^{13}C-Nikotin zum Zigarettentabak gebildet wurden, ermittelt. In einem anderen Experiment wurde eine GC-Analyse mit Chemiluminiszenz-Detektion der TSNA im Rauch vor und nach erhöhter Nornikotinzugabe zum Zigarettentabak durchgeführt. Bei einem weiteren Experiment wurden $^2H_4$-TSNA, die sich nach Zugabe von $^2H_4$-Nikotin beziehungsweise $^2H_4$-Nornikotin zum Zigarettentabak gebildet hatten, mittels Flüssigkeitschromatographie/Tandem-Massenspektrometrie (LC/MS/MS) analysiert. Die Anwendung verschiedener analytischer Methoden zur Untersuchung der TSNA-Bildung brachte ein zusätzliches Maß an Verlässlichkeit der Ergebnisse. Es wurde herausgefunden, dass NNK während des Rauchvorgangs mit einer Konversionsrate von 3×10^{-5}% bis 8×10^{-5}% (0.3 ppm bis 0.8 ppm Konversion des Nikotins) aus Nikotin gebildet wurde, während die Ergebnisse für die Generierung von NNN nicht schlüssig waren. Ein Experiment zeigte die Bildung von NNN aus Nikotin von zwischen 4×10^{-5}% und $1,5 \times 10^{-4}$% (0,4 ppm bis 1,5 ppm bezogen auf den Nikotingehalt), ein anderes Experiment zeigte jedoch keinen Beweis für die Bildung von NNN, wobei die Bestimmungsgrenze (LOQ) von NNN 5×10^{-5}% (oder 0,5 ppm) betrug. Aus Nornikotin entstand nachweislich NNN und die Ergebnisse für die 2R4F Referenzzigarette zeigten in einem Experiment einen NNN-Gehalt von $3,3 \times 10^{-3}$% (33 ppm bezogen auf Nornikotin) und $4,6 \times 10^{-3}$% (46 ppm bezogen auf Nornikotin) in einem anderen Experiment, die Übereinstimmung war also sehr gut. Auf der Basis der Ergebnisse dieser Untersuchung wurde die Schlussfolgerung gezogen, dass zwischen 5% und 10% des NNK-Gehalts im Hauptstromrauch einer Filterzigarette mit einem FTC-Kondensatgehalt von ungefähr 10 mg auf die NNK-Synthese während der Pyrolyse zurückzuführen ist. Die während der Pyrolyse stattfindende Synthese könnte zu einem höheren Anteil der TSNAs im Rauch führen, wenn der TSNA-Gehalt im Zigarettentabak niedrig ist, weil der TSNA Gehalt im Hauptstromrauch, der direkt aus dem Tabak übergeht, in diesem Fall gering ist. Die Pyrosynthese kann 5–25% zum NNN-Gehalt im Hauptstromrauch beitragen, oder möglicherweise sogar zu einem höheren Anteil, wenn die Tabakmischung sowohl einen niedrigen TSNA-Gehalt als auch einen hohen Nornikotingehalt aufweist. Anabasine ist typischerweise zu einem niedrigen Anteil im Tabak enthalten und aus diesem Grund für die Bildung von Nitrosoanabasine (NAB) unbedeutend. Anatabine ist in den verschiedenen Tabaken in vergleichbaren Mengen wie Nornikotin enthalten und hat als sekundäres Amin vergleichbare Eigenschaften wie Nornikotin. Die Pyrosynthese von Nitrosoanatabine (NAT) wurde jedoch in der vorliegenden Studie nicht untersucht. Die vorliegende Untersuchung weist darauf hin, dass es unwahrscheinlich ist, dass die vollständige Eliminierung der TSNAs aus dem Tabak zu einer vollständigen Eliminierung der TSNAs aus dem Zigarettenrauch führt und dass Tabake mit einem hohen Gehalt an Nornikotin vermieden werden sollten, um den Gehalt von NNN im Zigarettenrauch so gering wie möglich zu halten. [Beitr. Tabakforsch. Int. 23 (2008) 19–31]

RESUME

Cette étude examine la formation de nitrosonornicotine (NNN) et de 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) à partir de la nicotine et de NNN à partir de la nornicotine dans la fumée principale d'une cigarette en combustion. Dans cette étude, les cigarettes de référence Kentucky 1R4F et 2R4F ont été analysées, de même que cinq autres cigarettes dont trois ayant une faible teneurs en nitrosamines spécifiques du tabac (TSNA). Toutes les cigarettes ont des rendement en goudron autour de 10 mg (le goudron étant défini comme la matière particulaire totale exempte de nicotine et l'eau). Les cigarettes ont été fumées sous les conditions normalisées de la Federal Trade Commission (FTC) avec un volume de bouffée de 35 mL, une durée de bouffée de 2 sec et une fréquence de bouffée de 60 sec. Dans cette étude trois essais ont été réalisés séparément pour évaluer le taux des TSNAs transférées dans la fumée à partir des TSNAs présentes dans le tabac et le taux des TSNA formées par pyrosynthèse. Dans un essai, les résultats ont été obtenus par chromatographie en phase gazeuse/spectrométrie de masse (GC/MS) des $^{13}C_3$-TSNAs formées dans la fumée après l'apport de $^{13}C_3$-nicotine aux cigarettes. Dans une étude différente les TSNAs dans la fumée ont été dosées par GC avec détection par chimioluminescence avant et après l'apport intentionnel d'un excès de nornicotine aux cigarettes. Une autre étude a examiné par chromatographie en phase liquide avec spectrométrie de masse (LC/MS/MS) les $^2H_4$-TSNAs formées dans la fumée après l'apport de $^2H_4$-nicotine et $^2H_4$-nornicotine aux cigarettes. L'utilisation des méthodes analytiques différentes a amélioré la fiabilité des résultats obtenues. Il a été trouvé que la NNK est formée au cours du fumage à partir de la nicotine avec une conversion de 3×10^{-5}% à 8×10^{-5}% (0,3 ppm à 0,8 ppm conversion de la nicotine), tandis que les résultats obtenus pour la formation de la NNN ne sont pas probants. Un essai a démontré la formation de la NNN à partir de la nicotine avec un taux de conversion entre 4×10^{-5}% et $1,5 \times 10^{-4}$% (0,4 ppm à 1,5 ppm conversion de la nicotine), tandis qu'un autre essai n'a pas apporté la preuve pour la formation de NNN, avec une limite de dosage pour la NNN de 5×10^{-5}% (ou 0,5 ppm). Les résultats ont montré que la NNN est formée à partir de la nornicotine, pour la cigarette 2R4F le rendement est de $3,3 \times 10^{-3}$% (33 ppm conversion de la nornicotine) lors d'un essais et $4,6 \times 10^{-3}$ (46 ppm conversion de la nornicotine) dans un autre essai, les résultats étant en bon



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accord. Les résultats de cette étude révèlent que la pyrosynthèse rend compte de 5% à 10% de la NNK dans la fumée du courant principal pour une cigarette filtre avec un taux de goudron de près de 10 mg. La pyrosynthèse pourrait rendre compte d'une proportion plus élevée des TSNAs si le tabac des cigarettes est pauvre en TSNAs, lorsque le rendement en TSNAs par transfert direct est faible dans ce cas. La pyrosynthèse pourrait rendre compte de 5% à 25% des NNNs dans la fumée principale de cigarettes ou même d'un taux plus élevé, si le mélange de tabac est pauvre en TSNAs et riche en nornicotine. La teneur en anabasine dans le tabac est faible et ainsi la formation de nitrosoanabasine (NAB) est de moindre importance. La teneur en anatabine dans des tabac différents est comparable avec celle de la nornicotine et étant une amine secondaire la nornicotine peut réagir comme la nornicotine. Cependant, la pyrosynthèse de nitrosoanatabine (NAT) à partir de l'anatabine n'a pas été évaluée dans cette étude. L'étude montre que l'élimination complète des TSNAs du tabac n'est pas susceptible d'éliminer complètement les TSNAs de la fumée de cigarette et que les tabacs riches en nornicotine devraient être évités pour minimiser les taux de NNN dans la fumée de cigarette. [Beitr. Tabakforsch. Int. 23 (2008) 19–31]

INTRODUCTION

Tobacco specific nitrosamines (TSNAs) include nitrosonornicotine (NNN), 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), nitrosoanabasine (NAB) and nitrosoanatabine (NAT). From this group of compounds, NNN, NNK and NAB are considered biologically active agents in the mainstream smoke of cigarettes, as listed in a study published in 1998 by D. HOFFMANN and I. HOFFMANN (1). Also, a number of other papers have identified TSNAs as being potentially associated with increased risk of illness (2–5). For these reasons, a multitude of studies related to TSNA formation (see e.g. (6–11)) have been performed, and effort is being made to reduce these compounds in both tobacco and cigarette smoke (8).

It is well established that TSNAs are transferred into cigarette smoke from preexistent tobacco TSNAs (2, 9, 10). However, other potential sources for TSNAs in smoke are possible, such as pyrosynthesis from alkaloid precursors including nicotine, nornicotine, anabasine, and anatabine, which are present in the tobacco blend. The generation of TSNAs from alkaloids in tobacco may follow various paths, involving the reaction between secondary or tertiary amines with HNO_2 as shown in an early paper by KLUS and KUHN (11). However, HNO_2 is not a thermally stable compound and during cigarette burning it is not a likely agent for the formation of TSNAs in the burning zone. During smoking, HNO_2 present in tobacco probably undergoes the following reaction (**1**):

$$2\ HNO_2 \rightarrow NO + NO_2 + H_2O$$

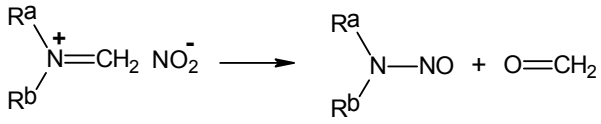
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The presence of NO and NO_2 in cigarette smoke, not necessarily generated from HNO_2 decomposition, can lead to the formation of N_2O_3 following equilibrium reactions of the types shown by reactions (**2**):

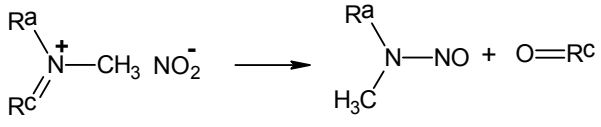
$$NO + NO_2 \rightleftharpoons N_2O_3 \quad \text{and} \quad 2\ NO + \tfrac{1}{2}\ O_2 \rightleftharpoons N_2O_3$$

2

Both secondary and tertiary amines can react with N_2O_3. The tertiary amines with a methyl group connected to the nitrogen probably follow the mechanism shown above in reaction (**3**), where R^a, and R^b are organic monodentate radicals, and R^c is a bidentate radical derived from R^b. The intermediate compounds will further react to generate nitrosamines as follows in reactions (**4**) and (**5**):

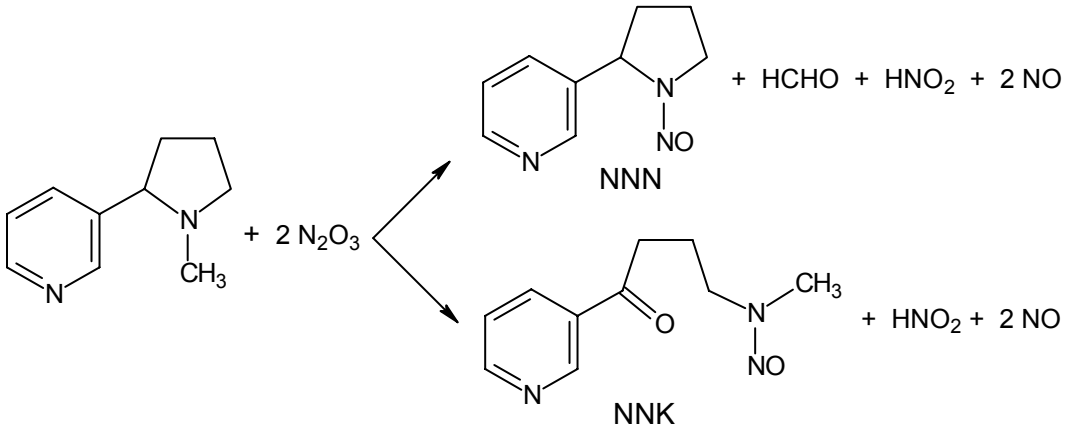


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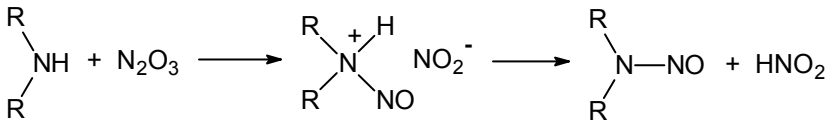


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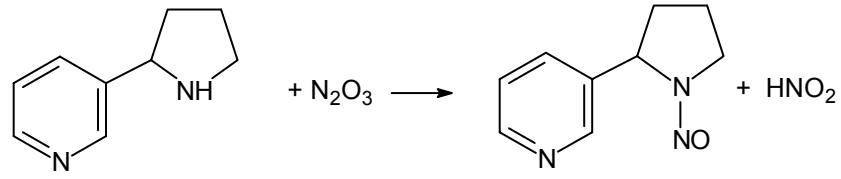
This type of mechanism can explain the formation of NNN and NNK from nicotine by pyrosynthesis during smoking (and during different curing processes that expose tobacco to NO_x). The overall result in this case can be written as follows in reactions (**6**):



6



7



8

The reaction of N_2O_3 with secondary amines, and in particular with nornicotine probably takes place as shown above in reactions (**7**) and (**8**).

Besides pyrosynthesis in the burning zone, a third potential source of TSNAs in mainstream cigarette smoke is their formation as a result of the reaction of NO_x formed in smoke, with the water and the tobacco alkaloids present in the unsmoked part of the cigarette rod. The types of reactions leading to TSNAs formation by this mechanism, would be identical with those described as taking place between tobacco alkaloids and HNO_2 (11). This study did not attempt to differentiate between high temperature pyrosyntheis in the burning zone and formation of TSNAs at lower temperatures as smoke passes through the tobacco column. The formation of TSNAs during cigarette smoking, regardless of the formation mechanism, is indicated throughout this paper as pyrosynthesis.

A number of studies have evaluated the contributions of direct transfer and formation during burning to the presence of TSNAs in cigarette smoke (9, 10, 13, 15). In general, these reports are not in good agreement regarding the extent of TSNAs formation during burning. For example, a paper published in *Cancer Letters* in 1983 by J. D. ADAMS *et al.* (9) indicated up to 74% of NNK as being generated by pyrosynthesis, while a paper by S. FISCHER *et al.* (10) published in *Carcinogenesis* in 1990 indicated that, with few exceptions, the TSNAs present in smoke derive predominantly from preexistent TSNAs transferred from tobacco. Preliminary results regarding the effort to clarify the discrepancies between different literature publications was previously presented (16, 17) and is further elaborated in this paper. The smoking of the cigarettes in this study was performed using the U.S. Federal Trade Commission (FTC) puffing regimen, with a 35 mL puff volume, 2 sec puff duration and 60 sec puff intervals. Further work is planed to investigate the role of pyrosynthesis when more aggressive smoking regimens are applied.

The role of minor alkaloids such as anabasine and anatabine as TSNA precursors during pyrosynthesis was not evaluated in the present study. Anabasine is typically found at low level in tobacco (18) and therefore is of lower interest. Anatabine is present in different tobaccos in a range similar to that of nornicotine (18), and being a secondary amine has the potential to act similarly to nornicotine. However, NAT is not considered an important biologically active compound (1).

EXPERIMENTAL

Three different sets of experiments were conducted in this study. The first experiment (Experiment 1) was based on the results of the GC/MS analysis of $^{13}C_3$-TSNAs formed in smoke when $^{13}C_3$-nicotine was intentionally added to cigarettes. The second set (Experiment 2) consisted of analysis of TSNAs in smoke using GC/chemiluminescence before and after an excess of nornicotine was intentionally added to cigarettes, and the third set (Experiment 3)

Table 1. Description of tested cigarettes in Experiment 1

Descriptor	1R4F Kentucky reference	Cigarette A	Cigarette B
FTC 'tar' (mg/cig)	9.4	10.0	10.6
Cigarette length (mm)	84	83	83
Tobacco weight mg	775	635	623
Filter length (mm)	27.2	27.0	27.0
Filter ventilation (%)	28.4	19.0	29.0
Blend type	American	American	American
Tobacco nicotine (mg/cig)	16.97	13.97	11.40
Nitrate (mg/cig)	0.83	0.34	0.22
Tobacco NNN (µg/cig)	1.94	1.59	0.63
Tobacco NNK (µg/cig)	0.98	0.64	0.32
Smoke nicotine (mg/cig)	0.80	0.80	0.97
CO (mg/cig)	12.3	11.0	8.1

Table 2. Gas chromatographic conditions for the analysis of $^{13}C_3$-NNN and $^{13}C_3$-NNK

Parameter	Setting
Injector temperature	270 ˚C
Injection type	splitless
Injection volume	1 µL
Purge valve off	0.5 min
Initial oven temperature	50 ˚C
Initial hold time	10 min
First temperature rate	5 ˚C/min
Final oven temperature (first ramp)	225 ˚C
Hold time	0.2 min
Second temperature rate	25 ˚C/min
Final oven temperature (sec. ramp)	260 ˚C
Carrier gas at constant flow	helium
Gas flow (at 50 ˚C)	2.2 mL/min
Transfer line temperature	250 ˚C

consisted of a LC/MS/MS analysis of $^2H_4$-TSNAs formed in smoke when $^2H_4$-nicotine and when $^2H_4$-nornicotine were intentionally added to cigarettes. The use of different analytical methods for the study of the same process brought an additional level of confidence regarding the reliability of the results obtained.

Experiment 1. GC/MS analysis of $^{13}C_3$-TSNAs formed in the smoke when $^{13}C_3$-nicotine was intentionally added to cigarettes

In this experiment the cigarettes used included a Kentucky reference 1R4F, a common commercial cigarette (Cigarette A) and a cigarette with tobacco low in TSNA (Cigarette B). Some specifications for these cigarettes are given in Table 1. The cigarettes were first conditioned for 24 hours at 24 ˚C and 60% RH and then manually injected with 20 µL ethanol solution containing 0.5 g/mL $^{13}C_3$-nicotine, to achieve 10 mg of $^{13}C_3$-nicotine (pyrrolidine-3',4',5'-$^{13}C_3$-DL nicotine) per cigarette. The injection was done using a 10 µL syringe (with a 5 cm needle from Hamilton, Reno, NV 89502), that was inserted in the middle of the tobacco column and slowly pulled out while dispensing the solution along the entire cigarette. Special care was taken to discard the cigarettes that generated paper spots during this operation. The $^{13}C_3$-nicotine was obtained from Cambridge Isotope Laboratories, Inc. Andover, MA 01810, USA. Following the drying for about 2 hours of the ethanol used for the injection, five cigarettes for each sample were smoked on a Borgwaldt RM 20/CS rotary smoking machine using the FTC puffing regimen (19) (35 mL puff, 2 sec puff duration, 60 sec puff interval). The particulate phase smoke was collected on a 92 mm diameter Cambridge pad, which had been pretreated with a solution of ascorbic acid in methanol in order to avoid artifact formation during sample processing (20). After smoke collection, 100 µL of 10 µg/mL 2,4,5,6-$^2H_4$-NNN internal standard solution (2,4,5,6-$^2H_4$-NNN purchased from C/D/N/ Isotopes Inc., Pointe-Claire, QU, H9R 1H1, Canada) was added to the pad. The pad was extracted for 25 min with 50 mL of methylene chloride (purchased from VWR Scientific Products, www.vwrsp.com) on a wrist action shaker.

Typical nitrosamine analysis using a chemiluminescence detector, as reported in literature [see e.g. (21)], was not applicable for this study since it cannot differentiate the ^{13}C labeled compounds from those with natural isotopes. A GC/MS analysis was necessary for the quantitative measurement of $^{13}C_3$-NNN and $^{13}C_3$-NNK levels. The analysis required a cleanup step since the low levels of labeled TSNA could not be accurately measured with a high interfering background generated by the smoke condensate matrix. The cleanup was done by passing a 25 mL aliquot of the methylene chloride solution of the pad extract through a Mega Bond Elut Si cartridge (Varian part no. 1225-6026), which had been primed with 5 mL methylene chloride. The cartridge containing the sample was rinsed with 25 mL of methylene chloride and then washed with 7 mL of ethyl acetate (VWR Scientific Products, www.vwrsp.com) to remove matrix components present in the smoke condensate. The TSNAs were eluted with 10 mL of acetone (VWR Scientific Products, www.vwrsp.com). The eluent was concentrated to approximately 0.4 mL under a stream of nitrogen using mild heating with a Pierce Reacti-Therm heating module. The solution was transferred to a 2 mL autosampler vial for analysis by GC/MS working in positive CI mode. An Agilent 6890 GC interfaced to an Agilent 5973 MSD (Agilent, Wilmington, DE 19808, USA) was used for the analysis. The GC was equipped with a SGE BPX-70 column, 15 m long, 0.25 mm i.d., and 0.25 µm film thickness (SGE Inc., Austin TX, 78758, USA). The chromatographic conditions for the separation are given in Table 2.

Table 3. Mass spectrometer conditions for the analysis of $^{13}C_3$-NNN and $^{13}C_3$-NNK

Parameter	Setting	Parameter ion monitored for
Ionization mode	CI+	NNN *m/z* = 178
Reagent gas	isobutane	NNK *m/z* = 208
Gas flow	1 mL/min	$^{13}C_3$-NNN *m/z* = 181
Source temp.	200 ˚C	$^{13}C_3$-NNK *m/z* = 211
Detection mode	SIM	$^{2}H_4$-NNN *m/z* = 182

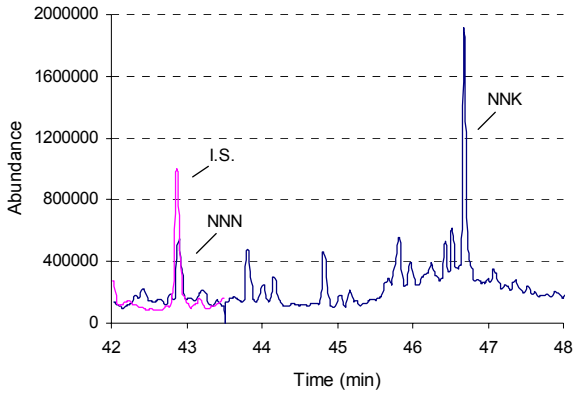


Figure 1. Chromatogram generated in SIM mode for ions *m/z* = 178 (NNN), *m/z* = 182 (I.S.), and *m/z* = 208 (NNK) for cigarette A

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The separation of NNN from NNK was very good when the previous conditions were used for the GC, but as expected the labeled compound cannot be separated from the corresponding unlabeled one. The MS detection with selected ion monitoring allowed the measurements of all compounds of interest. The MS detection settings are given in Table 3. A typical chromatogram obtained under these conditions for Cigarette A (no added labeled compound) is shown in Figure 1.

For the quantitation of NNN and NNK, response factors (RF) for each compound were obtained by analyzing standard solutions containing 0.1 µg/mL of each TSNA. The response factors were calculated by dividing the area of each analyte peak ($Area_{TSNA}$) by the area of the internal standard peak ($Area_{IS}$) at equal concentrations:

$$RF = Area_{TSNA} \; I \; Area_{IS}$$

The chromatographic peaks were generated using SIM detection for the characteristic ions as shown in Table 3. The concentration of each unknown analyte in ng/cig, was calculated (for samples of 5 cigarettes) from the peak area of the compound with unknown concentration ($Area_{UNK}$) using the following formula:

$$C_{ng/cig.} = \{[(Area_{UNK} \; I \; Area_{IS}) \; I \; RF] \; I \; 5\}$$

Experiment 2. GC/chemiluminescence analysis of TSNAs from smoke before and after an excess of nornicotine was intentionally added to cigarettes

In this experiment, the cigarettes used included a Kentucky reference 2R4F, a common commercial cigarette (Cigarette C) and a commercial cigarette with tobacco low in TSNA (Cigarette D). Some specifications for these cigarettes are found in Table 4.

A procedure similar to standard addition was applied. Nornicotine and NNN were initially measured in the tobacco of the three sample cigarettes. Also, NNN in smoke was analyzed. Following these analyses, incremental levels of nornicotine were added to cigarettes, and the level of NNN in smoke was measured. From the dependence of the increased NNN in smoke to the amount of added nornicotine, the formation of NNN by pyrosynthesis was evaluated. The addition of nornicotine (Toronto Research Chemicals Inc., North York, Canada) on the cigarettes was done using a 10% solution in ethanol. Appropriate volumes were injected in conditioned cigarettes, for achieving a specific added amount (e.g. 5 µL, for 0.5 mg nornicotine added). The injection of cigarettes was done manually, using the same procedure as for the $^{13}C_3$-nicotine solution injection. For amounts larger than 1 mg/cig the injection was done in two stages, allowing 4 hours for the ethanol from the first injection to dry.

The analysis of nornicotine was performed following a method developed for a collaborative study on minor alkaloids in tobacco (18). NNN analysis followed a procedure which used the addition of 1 mL of a 1N NaOH aqueous solution to 1 g tobacco, followed by extraction with CH_2Cl_2 and cleanup with Celite 545 (Fischer Scientific, Catalog No. C212-500) (9,10). The separation was performed with an Agilent 6890 GC equipped with a DB5-MS 30 m × 0.53 mm column with 1.5 µm film thickness(J&W Scientific/

Table 4. Description of tested cigarettes in Experiment 2

Descriptor	2R4F Kentucky reference	Cigarette C	Cigarette D
FTC 'tar' (mg/cig)	8.9	9.0	10.5
Cigarette length (mm)	84	83	83
Tobacco weight (mg)	852	663	614
Filter length (mm)	27.0	27.0	27.0
Filter ventilation (%)	28.0	20.0	29.0
Blend type	American	American	American
Tobacco nicotine (mg/cig)	16.75	13.50	11.70
Nitrate (mg/cig)	0.77	0.60	0.29
Tobacco NNN (µg/cig)	2.58	1.49	0.24
Tobacco NNK (µg/cig)	1.39	1.25	0.16
Smoke nicotine (µg/cig)	0.75	0.67	0.94
CO (mg/cig)	12.0	11.0	8.4

Table 5. Description of cigarette E used in Experiment 3

Descriptor	Cigarette E
FTC 'tar' (mg/cig)	10.6
Cigarette length (mm)	86
Tobacco weight (mg)	619
Filter length (mm)	27.0
Filter ventilation (%)	27.8
Blend type	American
Tobacco nicotine (mg/cig)	14.24
Nitrate (mg/cig)	0.24
Tobacco NNN (µg/cig)	0.21
Tobacco NNK (µg/cig)	0.15
Smoke nicotine (mg/cig)	0.97
CO (mg/cig)	8.1

Agilent, Wilmington, DE 19804, USA). The detection was done using an Antek 705E Thermal Energy Analyzer (Antek, Houston TX, 77064, USA).

The cigarettes (with no nornicotine addition or with different levels of added nornicotine) were smoked using FTC puffing regimen (19). The analysis of NNN in smoke was performed following a routine procedure, using supercritical fluid extraction of smoke pads, followed by GC with chemiluminescence detection (22–24).

Experiment 3. LC/MS/MS analysis of $^2H_4$-TSNAs formed in the smoke when $^2H_4$-nicotine and when $^2H_4$-nornicotine was intentionally added to cigarettes

All the LC/MS/MS analyses were performed at Arista Labs (Arista Laboratories – North America, Richmond, VA 23237). In this experiment, the cigarettes used included a Kentucky reference 2R4F and a cigarette with low tobacco TSNA (Cigarette E). The specifications for the 2R4F Kentucky reference cigarette are given in Table 4, and those of Cigarette E are given in Table 5.

In Experiment 3, potential pyrosynthesis of TSNAs from nicotine was evaluated independently from that of nornicotine. For the evaluation of the pyrosynthesis of TSNAs from nicotine, the cigarettes were conditioned for 24 hours at 24 ˚C and 60% RH, and then injected with two levels of $^2H_4$-nicotine (C/D/N/ Isotopes Inc., Pointe-Claire, QU, H9R 1H1, Canada). The first inclusion level was 4.83 mg/cig. and the second was 10.0 mg/cig. The $^2H_4$-nicotine was added by injecting each cigarette with 30 µL of a methanol solution with the concentration selected to achieve the desired inclusion level. The injection was done using a similar procedure as for the injection of cigarettes in the previously described experiments, except that the moving of the syringe was achieved using a mechanical device (two low delivery syringe pumps, Harvard Apparatus. Holliston, MA 01746).

For the evaluation of the pyrosynthesis of TSNAs from nornicotine, the cigarettes were conditioned for 24 hours at 24 ˚C and 60% RH, and then injected with two levels of $^2H_4$-nornicotine (C/D/N/ Isotopes Inc., Pointe-Claire, QU, H9R 1H1, Canada). The first inclusion level was 0.33 mg/cig. and the second was 1.04 mg/cig. The $^2H_4$-nornicotine was added by injecting each cigarette with 30 µL of a methanol solution with the concentration selected to achieve the desired inclusion level. The true level of $^2H_4$-nicotine or of $^2H_4$-nornicotine achieved on cigarettes was further measured on tobacco.

For the tobacco analysis of $^2H_4$-nicotine and $^2H_4$-nornicotine, after the cigarette injection and solvent drying, the tobacco was carefully removed and weighed in a 40 mL amber bottle. The tobacco was extracted with 20 mL of a solution of 10 mM aqueous ammonium acetate (pH ~8.5) using mechanical shaking for 30 min. The extract was centrifuged for approximately 5 min. A 4 mL aliquot of extract was transferred to a 10 mL volumetric flask and made to volume with 10 mM aqueous ammonium acetate (pH ~8.5). The diluted tobacco extract was decanted into a disposable syringe barrel (5 mL capacity) attached to a 0.45 µm nylon syringe filter. The first few mLs from the filtered solution were discarded and approximately 1 mL was collected in 2 mL amber colored autosampler vial with a Teflon snap-cap. All calibration standards were prepared using a 4 mL aliquot of a non-spiked 2R4F tobacco extract and diluted to 10 mL with 10 mM aqueous ammonium acetate (pH ~8.5). Each calibration curve consisted of at least 5 standards and ranged in concentration from 25–1000 µg/mL. The analyses were done using the LC/MS/MS instrumentation. The separations were done using a Waters XTerra 50 × 2.1 mm ID, 3 µm particle size analytical column maintained at a temperature of 65 ˚C. Gradient elution was performed using an aqueous ammonium acetate and methanol mobile phase at a flow rate of 200 µL/min. Nicotine and nornicotine eluted at approximately 3.3 and 2.1 min, respectively. The volume of sample injected was 3 µL. The ions monitored for quantitation are described in Table 6.

The LC/MS/MS instrumentation used for all measurements consisted of a Waters HPLC system (Milford, MA) equipped with a binary pump, in-line vacuum degasser and column oven, a CTC Analytics HTC Pal autosampler (Zwingen, Switzerland) used to perform automated injections, and a Waters Ultima triple quadrupole mass spectrometer equipped with either an electrospray (ESI) source (for TSNA analysis) or an atmospheric pressure chemical ionization (APCI) source (for nicotine/nornicotine analysis), both of which were operated in the positive ion mode. Nitrogen was used for desolvation and as a nebulizing gas; argon was used as the collision gas.

Following the analysis of $^2H_4$-nicotine, or with of $^2H_4$-nornicotine, further analysis was performed on the tobacco to

Table 6. LC/MS/MS conditions for the analysis of nicotine and nornicotine

Analyte	Precursor ion	Product ion	CV (V) [a]	CE (eV) [a]	Dwell time (s)
$^2H_4$-nicotine	166.9	120.9	25	25	0.2
$^2H_4$-nornicotine	153.0	136.0	20	20	0.2

[a] CV = Cone voltage, CE = Collision energy

Table 7. LC/MS/MS conditions for the analysis of TSNAs

Analyte	Precursor ion	Product ion	CV (V)	CE (eV)	Dwell time (s)
$^{2}H_4$-NAB	196.2	166.0	35	10	0.1
$^{2}H_4$-NAT	194.1	164.0	35	10	0.1
$^{2}H_4$-NNK	212.2	126.1	35	10	0.1
$^{2}H_4$-NNN	182.3	152.0	35	10	0.1

Table 8. The levels of $^{13}C_3$-NNK and $^{13}C_3$-NNN for the cigarettes with 10 mg/cig $^{13}C_3$-nicotine analyzed by GC/MS (five replicates) in Experiment 1

	$^{13}C_3$-NNK		$^{13}C_3$-NNN	
Cigarette	Average ng/cig	RSD%	Average ng/cig	RSD%
Kentucky reference 1R4F	8.0	13.0	15.0	7.0
Cigarette A	6.0	17.0	4.0	3.0
Cigarette B	5.0	20.0	14.0	14.0

verify that no TSNAs are formed after the addition of the deuterated compound and then transferred to smoke, rather than pyrosynthesized. For the tobacco analysis of TSNAs, the samples were processed very similar to those used for nicotine and nornicotine analysis. The tobacco from one cigarette was removed and weighed in a 40 mL amber bottle, followed by extraction with 20 mL of 100 mM aqueous ammonium acetate solution using mechanical shaking for 30 min. The extract solution was centrifuged for approximately 5 min. A 4 mL aliquot of extract solution was transferred to a 10 mL volumetric flask and made to volume with 100 mM aqueous ammonium acetate solution. The diluted tobacco extract was decanted into a disposable syringe barrel (5 mL capacity) attached to a 0.45 μm nylon syringe filter. The first few milliliters of filtered solution were discarded. Approximately 1 mL of filtered extract was collected in a 2 mL amber colored autosampler vial with a Teflon snap-cap. All calibration standards were prepared using a 4 mL aliquot of a non-spiked tobacco extract and diluted to 10 mL with 100 mM aqueous ammonium acetate solution. Each calibration curve consisted of at least 5 standards and ranged in concentration from 0.1–50 ng/mL of the specific deuterated TSNAs. The deuterated standards, ($^{2}H_4$-nitrosonornicotine ($^{2}H_4$-NNN), $^{2}H_4$-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone ($^{2}H_4$-NNK), $^{2}H_4$-nitroso-anabasine ($^{2}H_4$-NAB) and $^{2}H_4$-nitroso-anatabine ($^{2}H_4$-NAT)) were obtained from Toronto Research Chemicals, North York, ON, M3J 2J8, Canada. The HPLC separations were carried out using the same Waters XTerra 50 × 2.1 mm i.d., 3 μm particle size analytical column, which was maintained at a temperature of 60 ˚C. Gradient elution was performed using an aqueous acetic acid and methanol mobile phase at a flow rate of 200 μL/min. The four deuterated TSNAs eluted between 2.2–3.6 min, respectively. The volume of sample injected was 5 μL. The ions monitored for quantitation are described in Table 7. The recoveries of deuterated TSNAs added on tobacco was very good, ranging from 82% to 101%.

For the analysis of TSNAs in smoke, following the injection and solvent drying five cigarettes for each sample were smoked on a Borgwaldt RM 20/CS rotary smoking machine using FTC puffing regimen (19). The particulate phase smoke was collected on a 44 mm diameter Cambridge smoke pad. Each Cambridge pad was placed in a 40 mL amber bottle and extracted with 20 mL of 100 mM aqueous ammonium acetate solution on a mechanical shaker, for 30 min. The solution was further treated exactly as the tobacco extract, and also analyzed in the same way.

The instrument precision for deuterated TSNAs in mainstream smoke was evaluated by analyzing four smoke matrix matched standards containing all four deuterated TSNAs at 1 ng/mL, 2 ng/mL, 8 ng/mL and 20 ng/mL. The highest RSD% for the low sample was found to be 5.9% (for $^{2}H_4$-NNK) all the other RSD% values being lower. Also, the recovery from matrix matched standards for both 2R4F and for cigarette E were verified with added deuterated TSNAs (8 ng/mL). These samples were each analyzed four times showing recoveries between 85% to 106%. The limit of quantitation (LOQ) for $^{2}H_4$-NNN was found 5.0 ng/cig. and for $^{2}H_4$-NNK was found 2.0 ng/cig.

RESULTS AND DISCUSSION

Formation of TSNAs by pyrosynthesis from nicotine

The formation of $^{13}C_3$-TSNAs from $^{13}C_3$-nicotine, as obtained from the first experiment using GC/MS for the analysis of TSNAs, was evaluated on five smoke replicates for 1R4F Kentucky reference, for Cigarette A, and for Cigarette B, with the results shown in Table 8. Assuming that each cigarette was loaded with exactly 10 mg of $^{13}C_3$-nicotine, the results from Table 8 indicate that $^{13}C_3$-NNK is formed in smoke from $^{13}C_3$-nicotine at a rate between 5×10^{-5}% and 8×10^{-5}% (0.5 and 0.8 ppm reported to nicotine). At the same time, 10 mg of $^{13}C_3$-nicotine per cigarette generated $^{13}C_3$-NNN at a rate between 4×10^{-5}% and 15×10^{-5}% (0.4 and 1.5 ppm reported to nicotine).

The formation of $^{2}H_4$-TSNAs from $^{2}H_4$-nicotine added to cigarettes, as obtained from the third experiment that used LC/MS/MS for the TSNAs determination are shown in Table 9. Reported relative to the initial $^{2}H_4$-nicotine level, the results from Table 9 indicate that $^{2}H_4$-NNK is formed in smoke from $^{2}H_4$-nicotine at a rate of 3.1×10^{-5}% (0.31 ppm reported to nicotine) for the 2R4F cigarette and 3.8×10^{-5}% (0.38 ppm reported to nicotine) for cigarette E. At the same time no $^{2}H_4$-NNN was found in smoke. The result for NNK is in reasonable agreement with the finding from the first

Table 9. The levels of $^2H_4$-NNK and $^2H_4$-NNN for the cigarettes with two levels of added $^2H_4$-nicotine analyzed by LC/MS/MS (three replicates) in Experiment 3

Cigarette	$^2H_4$-nicotine mg	$^2H_4$-NNK		$^2H_4$-NNN	
		Average (ng/cig)	RSD%	Average (ng/cig)	RSD%
Kentucky reference 2R4F	4.83	<LOQ	—	<LOQ	—
Kentucky reference 2R4F	10.0	3.1	12.7	<LOQ	—
Cigarette E	4.83	<LOQ	—	<LOQ	—
Cigarette E	10.0	3.8	7.5	<LOQ	—

Table 10. The levels of NNN in smoke of 2R4F cigarettes with no or various levels of nornicotine intentionally added to the tobacco in Experiment 2

Added nornicotine (mg)	Nornicotine		NNN	
	Average (µg/cig)	RSD%	Average (ng/cig)	RSD%
0.00	874	2.3	142	7.0
0.50	1374	—	164	12.5
0.76	1634	—	178	8.0
1.00	1874	—	200	4.0
1.50	2374	—	209	0.3
2.00	2874	—	236	7.1

Table 11. The levels of NNN in smoke of cigarettes C and D without and with intentionally added nornicotine on tobacco (Experiment 2)

Cigarette	Added nornicotine (mg)	Nornicotine		NNN	
		Average (µg/cig)	RSD%	Average (ng/cig)	RSD%
Cigarette C	0.0	980	1.5	118	2.8
Cigarette C	1.0	1980	—	171	0.2
Cigarette D	0.0	324	2.3	28	13.8
Cigarette D	1.0	1324	-—	49	12.3

experiment. The fact that the cigarettes were different, may explain, in part, the somewhat different percentages of NNK formation found using LC/MS/MS analysis compared with those obtained by the GC/MS analysis of 1R4F, Cigarette A, and Cigarette B.

The absence of $^2H_4$-NNN as detected by the LC/MS/MS analysis may be explained by the higher LOQ for this analyte (5 ng/cig.) and also by the difference in the cigarettes. The results from the GC/MS study may also be affected by some error that showed somewhat inflated levels of $^{13}C_3$-NNN. However, the similarity in the mechanism of NNK and NNN formation by pyrosynthesis from nicotine suggests that as long as NNK is formed, some NNN will likely be formed from nicotine [see reaction (**6**)]. A rate of formation about equal with that of NNK would lead to a rate of 3.0×10^{-5}% (0.3 ppm reported to nicotine) which was below the LOQ for NNN in the third experiment using LC/MS/MS.

Formation of TSNAs by pyrosynthesis from nornicotine

NNN is the only TSNA potentially generated by pyrosynthesis from nornicotine, and the second experiment was focused on NNN. The evaluations were based on the measurement of initial nornicotine and NNN in tobacco and of NNN in smoke, followed by the addition of different amounts of nornicotine to the cigarettes followed by the measurement of NNN in smoke, as explained in Experiment 2. The results obtained for NNN in smoke as a function of the addition of different amounts of nornicotine to the 2R4F cigarettes are given in Table 10. (The nornicotine levels were measured only in the initial sample and for the other samples the level was calculated as a sum of initial value and the added nornicotine). The data demonstrated a linear dependence ($R^2 = 0.8686$) between the level of NNN in smoke and the level of nornicotine on the cigarette. Similar measurements as shown in Table 10 were obtained for Cigarettes C and D, and are given in Table 11.

The results from Tables 10 and 11 generated the equations given in Table 12 for the dependence between the nornicotine in the cigarette (in mg/cig.) and the level of NNN in smoke (in ng/cig.). From the dependences shown in Table 12, the theoretical level of NNN in smoke extrapolated to zero nornicotine in the cigarette can be calculated. This represents the NNN generated in smoke exclusively by transfer from the preexistent tobacco NNN, and the difference in levels from the measured NNN in smoke can be concluded to have been generated by pyrosynthesis. The results of these calculations are given in Table 13. The results from Table 13 also indicate the rate of pyrosynthesis of NNN from nornicotine for the three evaluated cigarettes.

The formation of $^2H_4$-TSNAs from $^2H_4$-nornicotine, as obtained from the third experiment using LC/MS/MS, was evaluated on three smoke replicates for each cigarette at two addition levels of $^2H_4$-nornicotine (0.33 mg/cig. and 1.04 mg/cig.) and the results are shown in Table 14. Since the 2R4F cigarette was analyzed in both Experiment 2 and 3, the results from Table 13 and from Table 14 can be

Table 12. Dependence between the level of NNN in smoke (in ng/cig) and the nornicotine in the cigarette (in mg/cig) (Experiment 2)

Cigarette	Equation	R^2	% Std. error of result [a]
Kentucky reference 2R4F	$Y = 46.754\ X + 102.7$	0.8686	6.7
Cigarette C	$Y = 53.0\ X + 66.06$	0.9948	1.6
Cigarette D	$Y = 21.0\ X + 21.196$	0.8658	13.2

[a]Note: No error was assumed in the measurement of nornicotine level.

Table 13. Total, transferred, and pyrosynthesized NNN from nornicotine in cigarette smoke (Experiment 2)

Cigarette	Nornic. in tobacco (mg/cig)	Total NNN in smoke (ng/cig)	NNN in smoke transfer (ng/cig)	NNN in smoke from pyrosynthesis (ng/cig)	NNN pyrosynthesis from nornicotine (%)
Kentucky reference 2R4F	874	142	102	40	4.58×10^{-3}
Cigarette C	980	118	66	52	5.31×10^{-3}
Cigarette D	324	28	21	7	2.16×10^{-3}

Table 14. The levels of $^2H_4$-NNN for the cigarettes with two levels of added $^2H_4$-nornicotine analyzed by LC/MS/MS (three replicates) in Experiment 3

Cigarette	$^2H_4$-nornicotine (mg)	$^2H_4$-NNN		NNN pyrosynthesis from nornicotine (%)
		Average (ng/cig)	RSD%	
Kentucky reference 2R4F	0.33	10.8	3.3	3.27×10^{-3}
Kentucky reference 2R4F	1.04	31.9	13.4	3.07×10^{-3}
Cigarette E	0.33	18.1 [a]	—	5.48×10^{-3}
Cigarette E	1.04	17.9	20.3	1.72×10^{-3}

Table 15. Summary of pyrosynthesis of NNK and NNN from nicotine and nornicotine [a]

Cigarette	NNK from nicotine		NNN from nicotine		NNN from nornicotine	
	Average %	RSD%	Average %	RSD%	Average %	RSD% [b]
1R4F	8×10^{-5}	13.0	15×10^{-5}	7.0	not measured	—
2R4F Experiment 2	not measured	—	n. m.	—	4.58×10^{-3}	~6.7
2R4F Experiment 3	3.1×10^{-5}	12.7	—	—	3.27×10^{-3}	9.0
Cigarette A	6×10^{-5}	17.0	4×10^{-5}	3.0	not measured	
Cigarette B	5×10^{-5}	20.0	14×10^{-5}	14.0	not measured	
Cigarette C	not measured	—	not measured	—	5.3×10^{-3}	~1.6
Cigarette D	not measured	—	not measured	—	2.1×10^{-3}	~13.2
Cigarette E	3.8×10^{-5}	7.5	—	—	5.48×10^{-3}	20.3

[a] The number of significant digits in the table follows those of reported results in each study.
[b] The calculation of RSD% values for the NNN levels from Experiment 2 was made using the assumption that all calculated values have the same proportional error.

compared and they are in very good agreement. The results for the formation of NNN via pyrosynthesis from nornicotine in the two studies are 4.58×10^{-3} and 3.27×10^{-3}, respectively. It should be noted again that the two studies used very different experimental approaches, different instrumentation, and unlabeled nornicotine for Experiment 2 versus labeled nornicotine in Experiment 3. The other results are more difficult to compare due to the differences in the analyzed cigarettes.

The contribution of pyrosynthesis to the presence of TSNAs in mainstream cigarette smoke

The results regarding the pyrosynthesis of NNK and NNN from nicotine and nornicotine as measured in the three studies are summarized in Table 15. The ratio of the level of pyrosynthesized TSNAs in smoke relative to the level of its precursor in tobacco can be further used for the evaluation of the relative contribution of pyrosynthesis (and that of transfer) for the formation of TSNAs in cigarette smoke. The levels of nicotine, nornicotine, and smoke TSNAs for some of the analyzed cigarettes are given in Table 16 [see also (25)]. The calculation of the levels of NNK and NNN due to pyrosynthesis can be easily performed based on the values from Tables 15 and 16. The calculated results are given in Table 17.

The values from Table 17 compared to the reported TSNAs levels in smoke allowed the estimation of the proportion of pyrosynthesis in smoke of each cigarette. However, the lack of complete data did not allow the calculation of the values for all cigarettes. Also, only partial data for NNN resulting either from nicotine or nornicotine were available. Considering all these restrictions, the estimated results are summarized in Table 18.

The results given in Table 18 show the relative proportion of pyrosynthesis to the total NNK or NNN in smoke. Being

Table 16. The levels of nicotine, nornicotine, and smoke TSNAs for the evaluated cigarettes

Cigarette	Tobacco nicotine (mg/cig)	Tobacco nornicotine (µg/cig)	Smoke NNK (ng/cig)	Smoke NNN (ng/cig)
1R4F	17.0	830	105	107
2R4F	19.6	874	116	142
Cigarette A	14.0	not measured	95	119
Cigarette B	11.4	not measured	10	29
Cigarette C	not measured	980	not measured	118
Cigarette D	not measured	324	not measured	28
Cigarette E	12.0	392	20	25

Table 17. Calculated levels of pyrosynthesized smoke TSNAs

	NNK from nicotine		NNN from nicotine		NNN from nornicotine	
Cigarette	Average (ng/cig)	RSD%	Average (ng/cig)	RSD%	Average (ng/cig)	RSD%
1R4F	13.6	13.0	25.5	7.0	not measured	—
2R4F Experiment 2	not measured	—	not measured	—	40.0	~6.7
2R4F Experiment 3	6.1	12.7	—	—	28.6	9.0
Cigarette A	8.4	17.0	5.6	3.0	not measured	—
Cigarette B	5.7	20.0	16.0	14.0	not measured	—
Cigarette C	not measured	—	not measured	—	51.9	~1.6
Cigarette D	not measured	—	not measured	—	6.8	~13.2
Cigarette E	4.6	7.5	—	—	21.5	20.3

Table 18. Estimated contribution of pyrosynthesis to the generation of TSNAs in smoke

Cigarette	NNK in smoke (ng/cig)	NNK % from pyrosynthesis	NNN in smoke (ng/cig)	NNN % from pyrosynthesis
1R4F	105	13	130	20
2R4F Experiment 2	116	not measured	142	28
2R4F Experiment 3	116	5	142	24
Cigarette A	95	9	119	5
Cigarette B	10	57	29	55
Cigarette C	not measured	not measured	118	44
Cigarette D	not measured	not measured	28	24
Cigarette E	20	23	18	high

a relative value, the proportion of pyrosynthesis depends not only on the level of pyrosynthesized compound, but also, on the total NNK or NNN in smoke. Therefore, since a tobacco blend that is low in TSNA yields very low levels of NNK or NNN in smoke, the contribution of pyrosynthesized TSNAs (expressed as a percentage of the total found in mainstream smoke) is greater than that found for the other cigarettes studied, even though the absolute amount of pyrosynthesized TSNAs is smaller. For NNK, pyrosynthesis may account for approx. 5% to 10% of the compound in smoke for typical cigarettes that yield around 10 mg 'tar' by the FTC smoking protocol, but can be a much higher percentage for low in TSNA tobacco cigarettes when the NNK and NNN levels are very low in smoke. For NNN, the contribution of pyrosynthesis may account for 5% to 25% of the compound in smoke, or potentially even higher levels for cigarettes with a high level of nornicotine (such as Cigarette C). The percent contribution of pyrosynthsis to the formation of TSNAs as calculated in Table 18 is affected by a number of errors including those in the measurement of the pyrosynthesis yield, of the level of nicotine and nornicotine in the cigarettes, and those of the level of TSNAs in smoke for each cigarette. Also, the level of nitrate in the tobacco blend may have an influence on the yields. Therefore, the values found in Table 18 are only rough estimations of the true values. The results show that a complete elimination of TSNAs from tobacco is unlikely to completely eliminate the TSNAs from cigarette smoke, and further suggest that high nornicotine tobaccos should be avoided in order to minimize the levels of NNN in cigarette smoke.

CONCLUSIONS

The variety of cigarettes and of experimental procedures applied in this study lead to a reliable view on the formation of TSNAs via pyrosynthesis. Collectively, the three studies summarized in this report show between 3×10^{-5}% and 8×10^{-5}% of the nicotine present in the tobacco blend is chemically transformed to NNK by this mechanism, while the result for NNN generation from nicotine is not conclusive. One of the three studies showed formation of NNN between 4×10^{-5}% and 15×10^{-5}% from nicotine,

while another did not prove its formation (with a LOQ corresponding to 5×10^{-5}%). Nornicotine was proven to generate NNN, and the results for the 2R4F cigarette showed 3.27×10^{-3}% yield when using addition of $^{2}H_{4}$-nornicotine and LC/MS/MS analysis and 4.58×10^{-3}% when using addition of nornicotine and GC/ chemiluminescence detection. Using the results from this study it can be calculated that pyrosynthesis may account for approx. 5% to 10% of the NNK in smoke for typical cigarettes that yield around 10 mg 'tar' by the FTC smoking protocol, but can be a much higher percentage for cigarettes with tobacco low in TSNA (since the mainstream smoke TSNAs yield from direct transfer from tobacco is small). The contribution of pyrosynthesis when cigarettes are smoked according to the FTC smoking protocol may account for 5% to 25% of NNN in mainstream cigarette smoke, or for considerable higher proportion when the tobacco blend is both low in TSNAs and high in nornicotine. Other smoking conditions as well as different tobacco blends (e.g. Virginia blend) may generate different results as compared to those reported in this study.

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