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Changes in TSNA Contents during Tobacco Storage and the Effect of Temperature and Nitrate Level on TSNA Formation

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ABSTRACT: Samples of burley, sun-cured, and flue-cured tobacco from the main producing areas of relevant tobacco types in China were collected to study the changes in tobacco-specific nitrosamine (TSNA) contents during storage and to investigate the effect of storage temperature and tobacco nitrate level on TSNA formation of cured tobacco. Contents of TSNAs in burley and sun-cured tobacco increased substantially during 1 year under natural storage environment, with total TSNA content increasing about 215% for both tobacco types. The most rapid increase occurred during the high temperature season. Temperature had a significant promoting effect on TSNA formation during storage. Storage temperature as high as 27 °C for 12 days was enough to induce the increase of TSNA formation, while the most significant effect was shown when the temperature was above 30 °C. The increased rate of accumulation became greater as the temperature increased. Total TSNA content in air-cured burley tobacco after the treatment of 60 °C for 24 days was 772% higher than that in the low temperature control. Different types of tobacco showed different results in terms of the response of TSNA formation to high temperature. TSNA formation in flue-cured tobacco did not increase after high-temperature treatment for 36 days, while burley and sun-cured tobacco saw a dramatic increase of TSNA content. This difference could be explained by the fact that burley tobacco and sun-cured tobacco usually had more than 10 times the nitrate content than flue-cured tobacco. As the nitrate nitrogen increased in cured burley tobacco, TSNA formation during leaf storage at high temperature significantly increased. Addition of nitrate onto flue-cured tobacco to the level equivalent to burley tobacco followed by high-temperature treatment increased the TSNA concentration comparable to burley tobacco. The interaction between high temperature and abundant nitrate content in cured tobacco could be responsible for TSNA formation during storage.

KEYWORDS: *tobacco, storage, tobacco-specific nitrosamines, temperature, nitrate*

■ INTRODUCTION

Tobacco leaves and smoke contain a certain number of harmful components, and harm reduction has been the focus of tobacco scientific research for many years. Tobacco-specific nitrosamines are important toxic components in tobacco and maintain their status as important main stream smoke toxicants since the 1960s due to their presence in tobacco and tobacco products.[1] There are mainly four kinds of tobacco-specific nitrosamines, *N*-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N*-nitrosoanatabine (NAT) and *N*-nitrosoanabasine (NAB), of which NNN and NNK have been proved to be strong animal carcinogens[2−5] The precursors of TSNA are alkaloids and nitrite. The secondary alkaloids such as nornicotine, anatabine, and anabasine can directly react with NO_x (nitrite) to form relevant NNN, NAT, and NAB, while nicotine, a tertiary alkaloid, first must undergo oxidation in the 1′, 2′ bond of the pyrrole ring to form pseudo-oxo nicotine (PON) followed by nitrosation to produce NNK.[1,6,7]

In general, TSNAs are undetectable or at a very low level in fresh leaves before harvest, while in air-cured leaves after curing, TSNA contents are readily measurable. In China, air-cured leaves are generally stored on the farm where the tobacco was grown for 2−3 months before selling to tobacco companies. Then, they are stemmed and redried, followed by 18 months of storage in the company warehouses before being processed for cigarette production. TSNAs could be formed during both air-curing and leaf storage. Nevertheless, research has been conducted mainly on TSNA formation during air-curing.[1,8−11] Burton et al [9] studied the changes in TSNA content in the leaves of different maturity under two air-curing environments. At normal conditions, the rapid increase of TSNA concentration occurred during the period from 2 weeks after housing to leaf browning. The study conducted by Cui[8] revealed that during the first 2 weeks of air-curing, the TSNA levels of leaf lamina and midrib were at the low levels, which increased substantially during weeks 4−7 of the curing. During the process of air-curing, water loss and cell exudates increased, indicating that the integrity of cellular membranes was damaged, which provided substrates for microorganism activity to reduce nitrate to nitrite, leading to the increase of nitrite and TSNA formation.

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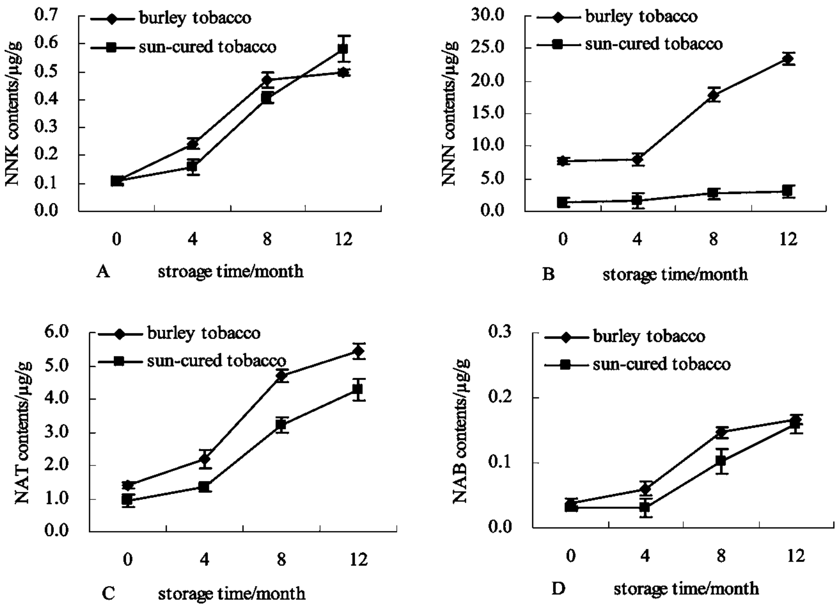

Figure 1. Changes in TSNA contents of burley and sun-cured tobacco during storage in ambient environment at Zhengzhou, Henan Province, PRC.

The period of storage after air-curing is also an important time of TSNA formation; the TSNA level may increase several fold compared with that in the freshly air-cured leaves.[12−14] De Roton et al. reported that in ground samples of burley tobacco stored at ambient environment for 6 months, TSNA concentration increased from the initial 1.3−1.4 to 8.1−9.5 $\mu g\ g^{-1}$, while TSNAs in the samples stored in the refrigerator did not increase.[12] The mechanism of TSNA formation during storage was not elucidated. Saito et al [15] reported that as the storage temperature increased, TSNA and nitrite contents increased. Burley tobacco had much higher levels of nitrate than that of flue-cured tobacco, with the concentration of nitrate in air-cured leaves of burley tobacco ranging from 1500 to 5000 $\mu g\ g^{-1}$, which was 8−10 times higher than that in flue-cured leaves. Nitrate contents were positively correlated with TSNA content among samples from the same variety and same growing area.[16] Nitrate is a chemically unstable compound and may produce gaseous nitrogen oxides which could easily react with alkaloids to form TSNAs by nitrosation. This may be the cause of the TSNA increase during storage under relatively high temperatures. To test this hypothesis, a series of experiments were designed to investigate the changes in TSNA contents during a one year storage and the effect of temperature and nitrate on TSNA formation to elucidate the mechanism of TSNA formation during storage and to provide a theoretical basis for the adoption of environment and chemical control measures to minimize TSNA formation and accumulation after air-curing.

■ MATERIALS AND METHODS

Experimental Materials. All the cured tobacco used in these experiments were produced in 2011 and stripped from the leaf stalk position. Burley tobaccos were TN90 from Yunnan and Dabai 1 from Sichuan. Flue-cured tobaccos were Zhongyan 100 from Henan and Hongda from Yunnan. Sun-cured tobacco, Wanmao 1, was from Sichuan.

Methods. Experiments included storage in ambient and in controlled-environment conditions. Ambient storage experiments used 15 kg of tobacco of the same type and from same producing area. The controlled-environment experiments were designed separately to investigate the effects of temperature and nitrate treatments on TSNA formation. For these experiments, 5 kg of tobacco from each sample was stripped and cut into 0.5 cm squares, mixed thoroughly, and stored at 5 °C until further use.

Storage Experiment. Fifteen kilograms of cured leaves of each type were stripped and cut into 3 cm strips and mixed thoroughly before being packed in a hard paper case for storage under ambient temperature. Containers were prepared for 4 separate samplings—0, 4, 8, and 12 months—starting in mid-December 2011. Sampled leaves were stored −20 °C to await analysis at the same time. After 12 months of storage, all the samples were freeze-dried and ground for quantitative analysis of TSNAs and their precursors.

Controlled Experiments. *Temperature Treatment to Different Types of Tobacco.* Fifty grams of the 0.5 cm squares tobacco samples of burley, sun-cured, and flue-cured were used in the test. The experiment was conducted in temperature- and humidity-controlled chambers at 10 and 45 °C with 60% relative humidity. Samples with water content of 8% were separately stored in capped glass vials. After 36 days of treatment, the samples were freeze-dried and ground for chemical analysis.

Different Storage Temperatures. Five samples of 50 g of the 3 cm strips of each tobacco were stored in controlled environment chambers at temperatures of 10, 27, 30, 45, or 60 °C and a relative humidity of 60%. Each tobacco treatment was sampled on day 12 and 24 of treatment, freeze-dried, and prepared for chemical analysis.

Nitrate Level on TSNA Formation. A Field experiment was conducted in Binchuan of Yunnan on the loam soil with medium fertility. Four nitrogen amounts, 120, 180, 240, 300 kg/km^2, were used in the experiment, with 75% of the nitrogen source as nitrate nitrogen. The experiment was a randomized complete block design with three replicates. Freshly air-cured leaf samples were divided into two parts; one part was stored in the refrigerator as control, and the other part was put in the temperature-controlled chamber storing for 15 d at 45 °C. Tobacco samples before and after storage were tested for nitrate and individual TSNA contents.

Nitrate Addition to Flue-Cured Tobacco. Aqueous solutions of NH_4NO_3, KNO_3, $NaNO_3$, and pure water control were separately sprayed onto each 50 g flue-cured tobacco cuts resulting in tobacco samples with nitrate nitrogen concentration of 4000 $\mu g\ g^{-1}$, which was equivalent to the level in burley tobacco. The sprayed samples were air-dried in ambient environment before they were placed into a controlled temperature of 60 °C and relative humidity of 60% for 10 days.

Measurement of Chemical Contents. *TSNA Measurement.* TSNA determinations were done at the University of Kentucky and the Beijing Cigarette Factory. The analysis conducted at the University of Kentucky was done with the GC-TEA method of Morgan et al.[17] Analysis at the Beijing Cigarette Factory was done via GC-NPD. Extraction and chromatography were similar to standard practices.

Alkaloid Measurement. Methyl tert-butyl ether (MTBE) was used as the extraction agent and quinoline as the internal standard. For each sample, 100 mg of ground tobacco was accurately weighed into a culture tube, and 0.5 mL of 2N NaOH was added to moisten the tobacco sample for 15 min. Then, 5 mL of extraction solution was added into the tube to extract alkaloids. Samples were shaken in a linear shaker for 2 h. After the solvent and sample were separated, the solvent was decanted into a GC vial for alkaloid separation and quantification (Jack and Bush, 2007).[18]

Nitrate and Nitrite Measurement. Nitrate and nitrite contents were determined at the University of Kentucky by the method of Crutchfield and Grove.[19]

RESULTS AND DISCUSSION

Changes in TSNA Content in Storing Tobacco Leaves of Different Types. Air-cured burley tobacco and sun-cured tobacco produced in the 2011 season was put into storage in the cold season after the first sampling, and then samples were taken every 4 months. Individual TSNA in all the collected samples was analyzed after 1 year of storage. The results presented in Figure 1a illustrate that with increased storage time, NNK contents in both burley and sun-cured tobacco increased throughout the year, and that the largest increase occurred during the second 4 month period, which coincided with the high temperature season. Overall, the burley level of NNK increased nearly 3.5 times and for the sun-cured treatment 4.2 times. Both of these increases are highly significant.

For NNN content, there was no significant change occurring during the first 4 month time period (Figure 1b); however, during the second period, which was from middle of April to middle of August when the air temperature was high, NNN content increased very significantly. For the year of storage, NNN in burley and sun-cured tobaccos increased about 2-fold, or much less than the increase for NNK. The much greater increase of NNN content in burley tobacco than sun-cured tobacco may be related to the higher nornicotine level and nitrate levels in the tobacco.[1,16,20]

NAT contents of burley and sun-cured tobacco were greatly increased during 1 year of storage, and the magnitude was the largest in the high temperature season with an increased percentage of 117 and 136%, respectively, compared with the increased percentage of 56 and 49% for the first 4 months and the increasing percentage of 16 and 34% for the last 4 month period (Figure 1c). The NAT content in burley tobacco was always higher than that in sun-cured tobacco, but the increase was nearly parallel as described by the percentages given above.

The NAB content of burley and sun-cured tobacco increased slowly in the first 4 month period, followed by a rapid increase in the second 4 month period (Figure 1d), similar to the NNN response. From the middle of April to the middle of August, NAB content of burley and sun-cured tobacco increased by 0.087 and 0.071 $\mu g\ g^{-1}$, respectively, with the increasing rate of 145 and 227%. In the last 4 month period, the NAB increase was much lower, but the sun-cured tobacco increased much more than in the burley tobacco.

During the 1 year period of storage, the total TSNA contents increased continuously, with the second 4 month period having a greater rate of increase for both burley and sun-cured tobacco. After 1 year of storage, the total TSNA content increased by 20.26 $\mu g\ g^{-1}$ for burley tobacco, which was a 218% increase over the original. For sun-cured tobacco, the net increase of total TSNA in 1 year of storage was 5.49 $\mu g\ g^{-1}$, which was a 217% increase over the original level. The total TSNA content in burley tobacco was consistently higher than that in sun-cured tobacco.

The increase of TSNA content during the 1 year ambient storage of burley and sun-cured tobacco coincided with the changes in temperature. In the first 4 month period, which was the winter season with the average temperature of the whole period being only 5.2 °C, the TSNA content generally did not increase significantly, while in the second 4-month period, which was from the middle of April to the middle of August and with average storage temperature of 26 °C, the TSNA levels increased dramatically; the NNK contents increased 94 and 153% in this period for burley tobacco and sun-cured tobacco, respectively. In the following 4 month period, the ambient temperature decreased gradually, with the average temperature being 15.4 °C for the whole period, and the increased rate of TSNA accumulation was slowed. Apparently, the increase of TSNA content during the 1 year storage time was highly related to the storage temperature, and the higher temperatures were favorable to the accumulation of TSNA.

Response of TSNA Formation to High Temperature Treatment for Different Tobacco Types. Samples of burley, flue-cured, and sun-cured tobacco were put in temperature-controlled chambers for 36 days at 45 and 10 °C. Flue-cured tobacco had very low levels of NNK content and remained constant in both temperature treatments (Figure 2a). In contrast, burley and sun-cured tobacco had much higher levels of NNK contents than flue-cured tobacco and showed a dramatic increase when the samples were stored at high temperature (45 °C) compared with that stored at 10 °C. The actual increase was greater in the sun-cured tobacco than for the burley tobacco, and the observation that sun-cured tobacco in storage contained more NNK than burley is in agreement with the results from the ambient storage conditions. These results suggest that NNK increases more in sun-cured tobacco than burley tobacco when a high-temperature storage period is included.

NNN content in flue-cured tobacco was initially much lower level than that in burley and sun-cured tobacco and did not change with storage at either temperature treatment (Figure 2b). NNN of burley and sun-cured tobacco increased with storage at both temperatures, but the increase was greater in burley tobacco. Burley tobacco had the highest NNN levels of the three tobaccos, and this was consistent with the nornicotine levels in each tobacco type.

The content of NAT in flue-cured tobacco was the lowest among the three tobacco types, and there was no significant

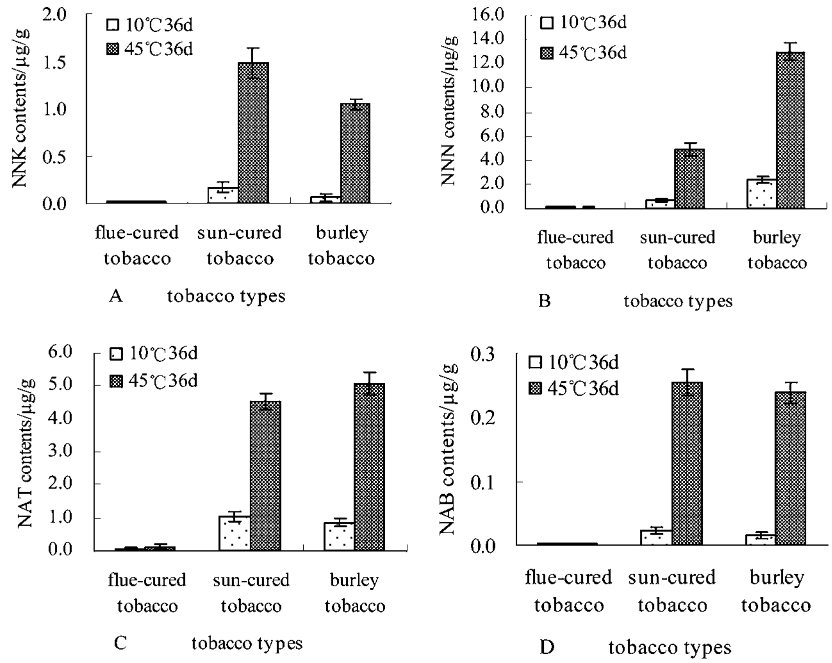

Figure 2. Effect of temperature on TSNA formation in flue-cured, burley, and sun-cured tobacco during 36 days of storage.

Table 1. Alkaloid, Nitrate, and Nitrite Content among Tobacco Types and the Response to Temperature

		flue-cured		burley		sun-cured	
		10 °C	45 °C	10 °C	45 °C	10 °C	45 °C
alkaloids	nicotine/mg g^{-1}	19.7	21.1	38.8	38.7	43.9	40.9
	nornicotine/mg g^{-1}	0.5	0.5	3.4	3.8	1.0	1.4
	anabasine/mg g^{-1}	0.1	0.1	0.2	0.2	0.2	0.2
	anatabine/mg g^{-1}	1.0	1.1	2.9	2.9	2.2	2.1
	percent nicotine conversion[a](%)	2.52	2.45	8.01	9.00	2.27	3.31
NOx	NO_3–N /μg g^{-1}	57.6	60.1	1703.1	1686.1	1157.2	874.3
	NO_2–N /μg g^{-1}	1.23	1.20	1.49	2.24	1.64	3.95

[a]Percent nicotine conversion was expressed as the percentage of nornicotine content in the total amount of nicotine plus nornicotine.

difference between the two storage temperatures (Figure 2c). NAT contents in burley and sun-cured tobacco not only had much higher level but also had a strong response to high-temperature treatment. NAT content in high- temperature treatment was 5.9 and 4.4 times higher than that in low temperature treatment for burley and sun-cured tobacco, respectively.

Burley and sun-cured tobacco had substantially higher levels of NAB than flue-cured tobacco (Figure 2d), and the high-temperature treatment greatly increased NAB formation in burley and sun-cured tobaccos. Compared with low temperature treatment, NAB content in high-temperature-treated burley and sun-cured tobaccos were 16.1 and 11.0 times higher, but these values were still low.

Total TSNA content was calculated by adding the four individual TSNAs. As was the case for each individual TSNA, total TSNA in flue-cured tobacco was the lowest among the tobacco types and there was no significant increase when the tobacco was stored at high temperature over tobacco stored at low temperature. Contrarily, burley tobacco and sun-cured tobacco had a significant increase in TSNAs to high-temperature treatment. In samples stored at 45 °C, the total TSNA content was 5.8 and 5, a value 9 times higher than that stored in the 10 °C environment. Among the three tobacco types, total TSNA content was the highest in burley tobacco.

The temperature-controlled experiment with different types of tobacco indicated different results in terms of the response of TSNA formation to high temperature. TSNA formation in flue-cured tobacco did not increase after high-temperature treatment for 36 days, while burley and sun-cured tobacco had dramatic increases of TSNA content. This difference coincides with the fact that burley and sun-cured tobaccos had much higher levels of nitrate contents than flue-cured tobacco. Nitrate probably is the main source of nitrite or other nitrosating agents of alkaloids to give rise to TSNA, so it would also be one of the contributors of TSNA formation in stored tobacco under high temperatures.

Alkaloid, Nitrate, and Nitrite Content among Tobacco Types and the Response to Temperature. The samples stored at two different temperatures were also tested for alkaloid, nitrate, and nitrite contents. Alkaloid levels were different among flue-cured, burley, and sun-cured tobaccos. Burley tobacco had significantly higher nicotine content than flue-cured tobacco, although the nicotine level was lower than that in sun-cured tobacco. Nornicotine content and the percent nicotine conversion to nornicotine in burley tobacco were the highest among the three tobacco types (Table 1). The changes in individual alkaloids were not significant between low and high temperature conditions, although the nornicotine level and

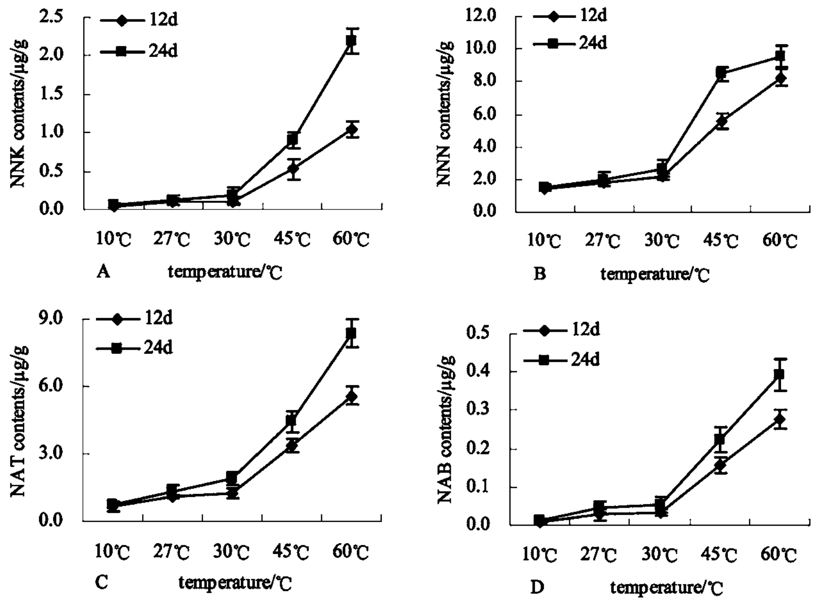

Figure 3. TSNA content of air-cured burley tobacco stored at different temperatures.

the percent nicotine conversion to nornicotine showed an increase at high temperatures.

Burley tobacco had extremely high nitrate content relative to the other tobacco types, 10 times higher than that in flue-cured tobacco. This was expected as it is a significant characteristic in burley tobacco chemistry. Compared with low temperature treatment, the high temperature did not cause many changes in nitrate content for each tobacco type. There was no significant difference in nitrite content for flue-cured tobacco, while the nitrite contents in burley and sun-cured tobacco were increased substantially in 45 °C treated tobacco over the 10 °C treated tobacco.

Effects of Temperature on TSNA Formation of Air-Cured Burley Tobacco. To accurately determine the effect of temperature on TSNA formation in burley tobacco, five shredded tobacco samples were stored in controlled temperature chambers at 10, 27, 30, 45, and 60 °C. The results presented in Figure 3 illustrate that high temperatures significantly promoted TSNA formation. Especially when temperatures exceeded 30 °C, TSNA accumulation increased dramatically. There was a significant, but small, TSNA increase from 10 to 30 °C for the tobacco stored at 30 °C for 24 days. The contents of NNK, NNN, NAT, NAB, and total TSNA increased by 257, 71.9, 156, 370, and 104%, respectively, compared with the values at 10 °C. For tobacco stored at 60 °C for 24 days, the increased percentages were 4030, 519, 1026, 3220, and 772%, respectively.

From the above temperature-controlled experiments, the relationship between temperature and TSNA accumulation was reasonably established. Storage temperature as high as 27 °C was enough to induce increased TSNA formation, while the most significant effect was shown when the temperature was above 30 °C. The rate of increase became greater as the temperature increased, indicating that the high temperature was very favorable to TSNA formation and that the high temperature was one of the main contributors for the TSNA formation during the storage of air-cured burley tobacco.

Effect of Nitrate Level in Cured Leaves on TSNA Formation at High Temperature. In order to investigate the effect of nitrate level on TSNA formation during leaf storage at high temperature, different applied nitrogen amounts were used in the field experiment to obtain burley tobacco leaves with varied levels of nitrate nitrogen. Freshly air-cured leaves were sampled before being stored at 45C for 15 days; then, the samples before and after storage were tested for nitrate and TSNA contents. The results in Table 2 show that as the applied nitrogen amount increased, nitrate nitrogen level increased significantly. TSNA content in freshly cured tobacco leaves also showed a trend of increase with increased nitrate nitrogen, but with a poor correlation. Nevertheless, in leaves being stored at 45 °C for 15 days, TSNA contents increased significantly with the increased nitrate nitrogen, and the net increase of TSNAs, which was the difference of TSNA content before and after storage, became much greater as the nitrate nitrogen level increased and showed a significant positive correlation with the R^2 of 0.96. This result indicated that the nitrate nitrogen level in tobacco leaves played a more important role in TSNA

Table 2. Effect of Nitrate Level on TSNA Formation during Leaf Storage at High Temperature

applied N amount (kg/hm^2)	nitrate-N (mg/g)	NNN (ng/g)	NAT (ng/g)	NAB (ng/g)	NNK (ng/g)	total TSNA (ng/g)
		in freshly cured leaves				
120	1331.2	199.1	491. 7	9.7	68.9	769.4
180	2000.8	545. 6	692. 2	13. 1	79.3	1330.2
240	2277. 2	551. 6	613. 7	13. 6	73.9	1252.8
300	3184.4	629.4	737. 2	14.2	84.3	1465.1
		in leaves stored at 45 °C for 15 days				
120	1328.6	247.5	602. 2	19.7	82. 6	952
180	1984.5	1649.5	1347.6	23. 6	114.7	3135.4
240	2253. 2	2235. 8	1525.6	29. 6	156.8	3947.8
300	3098.6	2730.2	2325. 5	44.3	204.1	5304.1
		increase after 15 days of storage				
120	−2.6	48.4	110. 5	10.0	13.7	182.6
180	−16. 3	1103.9	655. 4	10. 5	35.4	1805.2
240	−24. 0	1684.2	911. 9	16. 0	82.9	2695.0
300	−85.8	2100.8	1588.3	30.1	119.8	3839.0

Table 3. Effect of Adding Nitrate and Nitrite to Flue-Cured Tobacco on TSNA Formation under High-Temperature Treatment ($\mu g\ g^{-1}$)

	NNN /$\mu g\ g^{-1}$	NAT /$\mu g\ g^{-1}$	NAB /$\mu g\ g^{-1}$	NNK /$\mu g\ g^{-1}$	TSNAs /$\mu g\ g^{-1}$
water only	0.016	0.115	0.003	0.008	0.131
NH_4NO_3	0.317	1.160	0.080	0.140	1.698
KNO_3	0.749	2.404	0.155	0.727	4.036
$NaNO_3$	1.020	4.301	0.421	0.574	6.315
$NaNO_2$	82.76	184.76	17.19	116.09	400.81

[a]Temperature in the controlled chamber was 60 °C, the humidity was 60%, and samples were stored for 19 days.

formation during leaf storage at high temperature than TSNA formation during air-curing.

Effect of Addition of Nitrate and Nitrite to Flue-Cured Tobacco on TSNA Formation at High Temperature. Very low levels of nitrate content are characteristic of cured leaves of flue-cured tobacco, and they had minimal response of increased TSNA to high-temperature treatments. To determine the effect of nitrate content on TSNA formation, we added nitrate to flue-cured tobacco to levels equivalent to the nitrate content of air-cured burley tobacco to determine if increased nitrate content can trigger the response of TSNA formation to high temperature. Nitrates from three different nitrate salts (NH_4NO_3, KNO_3, $NaNO_3$) were added separately to cut flue-cured tobacco before high-temperature treatment. High nitrate content in flue-cured tobacco equivalent to burley tobacco resulted in a strong increase of TSNA with high-temperature treatment (Table 3). However, the degree of increase of TSNAs varied with different nitrate source. Sodium nitrate had the most significant effect on TSNA formation under high temperature, which was 47 times higher than that in no-nitrate-addition group of the flue-cured tobacco. Potassium nitrate was also very effective. Ammonia nitrate had the smallest effect, but it still gave rise to TSNA level 12 times higher than the control. When the same concentration of sodium nitrite (NO_2) was added to cut flue-cured tobacco, the TSNA content was dramatically increased with the total TSNA content in flue-cured tobacco stored at 60 °C for 10 days, increasing by 3063 times over no-treatment control. These results indicated that the nitrogen oxide content played a pivotal role in TSNA formation under high storage temperatures. The fact that addition of nitrate to flue-cured tobacco at levels equivalent to burley tobacco followed by high-temperature treatment increased TSNA concentration comparable to burley tobacco strongly suggests that high levels of nitrate in burley and sun-cured tobacco coupled with high temperature were responsible for the increase of TSNA formation during storage.

From the above analysis, we could make the reasonable hypothesis that TSNA formation during leaf storage was the consequence of the interaction between high temperature and abundant nitrate content in cured tobacco. Nitrate is a chemically unstable compound, and high temperature could cause it to give rise to gaseous nitrite or other volatile nitrogen oxides, which may easily react with alkaloids to form TSNAs. Therefore, the controlling of storage environment, reduction of leaf nitrate content, and the scavenging of gaseous nitrosating agents would be effective pathways to reduce or inhibit TSNA formation during the storage of cured tobacco. Current studies being conducted in the laboratory include optimization of storage conditions and controlling approaches and the elucidation of the mechanism of higher level nitrate accumulation in burley tobacco than in flue-cured tobacco so as to chemically and agronomically regulate nitrogen metabolism in burley tobacco to reduce nitrate content in harvested and cured leaves. Removal of gaseous NO*x* from storage tobacco by absorbents and antioxidants is also in progress.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Bush L. P.; Cui M. W.; Shi H. Z.; et al. Formation of tobacco-specific nitrosamines in air-cured tobacco. *55th Tobacco Science Research Conference* . Greensboro, NC. September 9−12, 2001.

(2) Fischer, S.; Spiegelhalder, B.; Eisenbarth, J.; et al. Investigation on the origin of tobacco-specific nitrosamines in mainstream smoke of cigarettes. *Carcinogenesis* **1990**, *11*, 723−730.

(3) Hecht, S. S. Biochemistry, biology, and carcinogenicity of tobacco-specific nitrosamines. *Chem. Res. Toxicol.* **1998**, *11* (6), 559−603.

(4) Hoffmann, D.; Brunnemann, K. D.; Prokopczyk, B. D.; Djordjevic, M. V. Tobacco-specific *N*-nitrosamines and areca-derived *N*-nitrosamines—Chemistry, biochemistry, carcinogenicity, and relevance to humans. *J. Toxicol. Environ. Health* **1994**, *41* (1), 1−52.

(5) El-Bayouny, K.; Latropoulos, M.; Amin, S.; et al. Increased expression of cyclooxygenase in rat lung tumors induced by the tobacco specific nitrosamine 4-(methylnitrosamino)- 4-(3-pyridyl)-1-butanone: The impact of a high-fat diet. *Cancer Res.* **1999**, *59*, 1400−1403.

(6) Bush, L. P.; Hempfling, W. P.; Burton, H. R. Biosynthesis of nicotine and related compounds. In *Analytical Determination of Nicotine and Related Compounds and their Metabolites*; Gorrod, J. W., Jacob, P., III, Eds.; Elsevier: Amsterdam, 2000, pp 13−44.

(7) Shi, H.; Zhang, J. Tobacco Alkaloids. China Agriculture Press: Beijing, China, 2004.

(8) Cui, M. The source and the regulation of nitrogen oxide production for tobacco-specific nitrosamine formation during air-curing tobacco. Ph.D. Dissertation. University of Kentucky, Lexington, KY, 1998

(9) Burton, H. R.; Childs, G. H., Jr.; Anderson, R. A.; Fleming., P. D. Changes in chemical composition of burley tobacco during senescence and curing. 3. Tobacco-specific nitrosamines. *J. Agric. Food Chem.* **1989**, *37*, 426.

(10) Djordjevic, M V; Gay, S L; Bush, L P; Chaplin, J F. Tobacco-specific nitrosamine accumulation and distribution in flue-cured tobacco alkaloid isolines[J]. *J. Agric. Food Chem.* **1989**, *37*, 752−756.

(11) Burton, H. R.; Dye, N. K.; Bush, L. P. Relationship between TSNA and nitrite from different air-cured tobacco varieties. *J. Agric. Food Chem.* **1994**, *42*, 2007.

(12) De Roton C.; Girard C. Potential changes of TSNA composition in stored tobacco powder: Consequences for sample preparation and ground storage. *2004 CORESTA Congress*. Kyoto, Japan, October 3−7, 2004.

(13) Jackisch, R. M.; Rovedder, J. H. Burley tobacco post-curing management and its effect in the nitrosamine amount. *2007 CORESTA Joint Study Group Meeting*. Krakow, Poland, October 8–12, 2007.

(14) Verrier J. L.; Wiernik A.; Staaf M.; Cadilhac J. L.; Onillon, M.; Vidal, B. The influence of post-curing of burley tobacco and dark air-cured tobacco on TSNA and nitrite levels. *2008 CORESTA Congress*. Shanghai, China, November 2–7, 2008

(15) Saito, H.; Miyazaki, M.; Miki, J. Role of nitrogen oxides in tobacco-specific nitrosamine formation in burley tobacco. *2006 CORESTA Congress*. Paris, France, October, 15–20, 2006

(16) Shi, H.; Wang, R.; Bush, L. P.; Yang, H.; Fannin, F. F. The relationships between TSNAs and their precursors in burley tobacco from different regions and varieties. *J. Food, Agric. Environ.* **2012**, *10* (3&4), 1048–1052.

(17) Morgan, W. T.; Reece, J. B.; Risner, C. H.; Bennett, C. B.; Midgett, C. H.; Johnson, K. S.; Burton, H. R. A collaborative study for the determination of tobacco specific nitrosamines in tobacco. *Beitrage Tabakforschung Int.* **2004**, *21*, 192–203.

(18) Jack A.; Bush L. (2007) The "LC" protocol. http://www.uky.edu/Ag/Tobacco/Pdf/LC-Protocol.pdf

(19) Crutchfield, J. D.; Grove., J. H. A new cadmium reduction device for the microplate determination of nitrate in water, soil, plant tissue and physiological fluids. *J. AOAC Int.* **2011**, *94*, 1896–1905.

(20) Hongzhi Shi, N.; Kalengamaliro, M.; Krauss, W. P.; Hempfling, F. Gadani Stimulation of nicotine demethylation by $NaHCO_3$ treatment using greenhouse-grown burley tobacco. *J. Agric. Food Chem.* **2003**, *51*, 7679–7683.