ANALYSIS OF VARIABILITY IN CURING CONDITIONS AND TOBACCO-SPECIFIC NITROSAMINES WITHIN BARNS OF DARK AIR-CURED TOBACCO

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Significant variability in cured-leaf tobacco-specific nitrosamine (TSNA) content is commonly observed when sampling within dark air-curing barns. This variability may be due to inconsistency in the curing environment within different areas of the barn. A study was initiated in 2012, through support from a CORESTA Study Grant, to evaluate if cured-leaf TSNA content is related to microenvironmental conditions in the barn. Low-converter (TRsc) and high-converter (TRHC) selections of TR Madole dark tobacco were air cured in barns near Princeton and Lexington, KY. Temperature and relative humidity were measured with data loggers placed at 27 different locations within each barn for the duration of curing. There were no significant effects of individual data logger placement in either variety selection on hours above 24°C temperature, hours above 80% relative humidity, or TSNA; therefore, we investigated these data within the 3-dimensional aspects of tier, room, and bent within each barn. There were various effects of tier, room, and bent on temperature, relative humidity, and TSNA. Temperature data followed an understandable pattern across tiers in the barn within each year and location; however, relative humidity and TSNA were more difficult to characterize adequately. There was a significant relationship between hours above 24°C and TSNA, but not hours above 80% relative humidity. This study has shown that the effect of within-barn position on TSNA cannot be easily predicted.

Additional key words: tobacco-specific nitrosamines, TSNA, dark air-cured tobacco, curing environment

INTRODUCTION

Kentucky leads the United States in dark tobacco production with a combined total for both dark fire-cured and dark air-cured types of almost 39 million pounds, 70% of this being dark fire-cured tobacco. The average yield of dark air-cured tobacco is 3,024 kg/ha, and the average yield of dark fire-cured tobacco is 3,472 kg/ha. Dark fire-cured and dark air-cured tobaccos are currently valued at an average of $5.74 kg/ha and $5.17 kg/ha, respectively (36). These tobacco types are primarily used in smokeless products and specialty-type cigars (25). Smokeless product sales increased in the United States by 65.5% between 2005 and 2011, whereas cigarette consumption has continually decreased (13).

Tobacco-Specific Nitrosamines. The major carcinogens found in tobacco are tobacco-specific nitrosamines (TSNAs), which are produced primarily during curing. TSNAs are nitrogenous compounds that are formed from tobacco alkaloids and are detectable in the tobacco leaf and in the particulate phase of tobacco smoke. There are 4 major TSNAs: nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB) (4,14,19,20). The tobacco industry has demonstrated major interest in reducing TSNA content in tobacco products by funding research on this objective since a report was published showing that some TSNAs induce malignant tumors in mice, rats, and hamsters (21). Since the U.S. Food & Drug Administration (FDA) gained authority over tobacco products in 2009 (16), the tobacco industry has further emphasized reducing TSNA content to lower the health risk to consumers. TSNA reduction will likely become more important with pending tobacco regulation from the U.S. Food & Drug Administration (17). In fact, there has been a recent FDA proposal to limit NNN in smokeless tobacco products to 1 μg/g (18). A major focus of tobacco research for the past several years has been TSNA reduction in cured leaf by modifying agronomic, curing, processing, and manufacturing practices (15). The formation of TSNAs is influenced by many factors throughout the production process, and accumulation of TSNAs in cured leaf has been inherently variable, even within tobacco grown at one location and cured in the same facility (22).

Factors Influencing TSNA Accumulation. Alkaloids are an essential component of leaf quality in commercial tobacco and are important in providing a physiological stimulus that makes the consumption of tobacco products pleasurable (9). Bush (8) made the general conclusion that cultural practices and environmental conditions that improve plant growth will also increase alkaloid formation and accumulation. A commonly accepted mechanism of the formation of tobacco-specific nitrosamines is the nitrosation of naturally occurring alkaloids within the tobacco plant (29). Bush et al. (10) state that the most important of the reactions between alkaloids and nitrosating agents is the reaction between nitrite and the secondary amine alkaloids, which occurs during air curing. This reaction is most likely due to microbial activity, because nitrite does not accumulate in the plant. Burton et al. (5) concluded that nitrite was formed in significant quantities from the reduction of nitrate under aerobic conditions.

The amount of specific alkaloid precursor influences the amount of TSNA accumulation. The specific

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alkaloid precursor that is the most prevalent in burley and dark tobacco is nornicotine, which is converted from nicotine (22). Jack et al. (22) state that the relative amount of nornicotine depends on the degree of conversion and the concentration of nicotine originally present. The use of screened or low-converter (LC) seed has had a definite impact on reducing the amount of TSNA content in tobacco. Screened or LC seed reduces the amount of nornicotine, the precursor to nitrosonornicotine (NNN), and is one of the most effective steps in reducing TSNA accumulation (22).

It has been observed that nitrogen fertility of the soil can influence the accumulation of TSNA in tobacco. The amount of alkaloids and nitrate accumulated in the plant is influenced by the rate of nitrogen fertilizer applied (10). An experiment using differing rates of nitrogen fertilizers concluded that TSNA accumulation did not increase in green leaf samples (fresh tobacco), but cured-leaf samples had significant increases in TSNA accumulation at higher rates of applied nitrogen fertilizer (3). Bailey (2) reported that excessive nitrogen applications of 560–1,120 kg N/ha resulted in increased TSNAs in 2 of 6 dark fire-cured experiments and 2 of 3 dark air-cured experiments. Caldwell et al. (11) conducted a study using differing rates of nitrogen (112 kg/ha,168 kg/ha, and 224 kg/ha), and found that lower application rates of nitrogen resulted in reduced TSNA content in cured leaf but also had a negative impact on yield and quality. The effect of increased N fertilizer on TSNA accumulation is believed to be largely due to the increase in alkaloid precursors.

Previous literature discussing the relationship between TSNA accumulation and tobacco plant maturity is limited because of the innate complexity of this relationship. Nicotine accumulation reaches its maximum content when the tobacco plant reaches maturity (9). Burton et al. (6) conducted a study to determine how senescence influenced the accumulation of TSNA and nitrite using burley tobacco cured at 2 temperature/relative humidity conditions in curing chambers. This study showed that under normal curing conditions (24°C/70% relative humidity [RH]), a rapid increase of TSNA accumulation took place during the first 14 days of air curing, but no significant conclusions were drawn that linked maturity to TSNA content.

Although negligible amounts of TSNA can be found in green leaf, nearly all TSNA formation occurs during curing (10,29,32), and specifically during the yellowing to early browning stage (2–3 weeks after harvest) in air-cured tobacco (37). Temperature and relative humidity during these critical curing stages are thought to significantly impact the level of TSNA formed in the cured leaf.

Under conditions where there are higher concentrations of nitrite, there were also correspondingly higher concentrations of TSNAs under an environment considered ideal for curing burley tobacco (5). A study conducted by Burton et al. (7) using dark air-cured tobacco found that only a small amount of the total nitrate in the leaf was converted to nitrite under normal air-curing conditions and that factors other than nitrate concentration influenced nitrite accumulation. Rapid drying or desiccation of the leaf limits the formation of nitrite, which also reduces the formation of TSNA (30).

The air curing of dark tobacco generally occurs in a period of 6–8 weeks, which is comparable to burley tobacco (1). Measured levels of TSNAs in the cured leaf have been inherently variable even within the same tobacco and same curing barn (22). Temperature, relative humidity, and air flow are the environmental conditions that are believed to be the most important factors that influence the variability of TSNA within curing facilities.

Massey and Smiley (24) concluded that the most favorable curing conditions for air-cured burley tobacco depended on keeping average daily RH between 65 and 70%. However, this humidity range tended to be associated with lighter "buff"-colored burley tobacco that the market demanded in the 1960s and 1970s. Today's market generally demands darker cured leaf, which requires average daily humidity of 72–75% (34). This optimum relative humidity range would also apply to dark air-cured tobacco.

Relative humidity also determines the rate of moisture loss by the tobacco plant (35). Relative humidity and temperature are factors of the curing environment that may affect the variability of TSNA content even within the same curing barn. Staaf et al. (33) suggested that traditional air-curing conditions support the idea that high relative humidity during curing results in higher TSNA levels, whereas drier curing conditions result in tobacco with lower TSNA and nitrite, but drier conditions also generally result in lower cured leaf quality. It was also suggested that the critical period of TSNA formation during air curing can be defined as when the plant cell membranes break down because of the loss of moisture, therefore causing cell contents to become available to microorganisms (that is, microbial reduction of nitrate to nitrite) that exist on or in the leaf of tobacco. It was concluded that this critical period of cell membrane breakdown can be shortened if this moisture loss from the tobacco leaf is rapid, or lengthened if the environmental conditions favor microbial growth (33).

Previous literature suggests that during the yellowing stage of dark air-cured tobacco, the barn should be held at about 80% relative humidity and only ventilated enough to prevent house burn (35). Curing of mature tobacco at higher temperature and humidity (32°C/83% RH) led to a 400-fold increase in TSNA level (37). Burton et al. (5) found a positive correlation between nitrite and TSNA when tobacco is air cured in a normal environment (24°C/70% RH). Curing tobacco at higher temperature and humidity (32°C/83% RH) dramatically increased the accumulation of individual TSNAs and nitrite (6). Roton et al. (30) concluded that microbial populations responsible for the formation of nitrite may grow in cured tobacco, and TSNA concentrations may continue to increase after curing if the leaves are kept hanging in the barn under humid conditions after the end of curing. It is likely that the level of residual nitrite in cured tobacco and temperature play a major role in the reaction (30).

It is well known that TSNA levels can vary tremendously between samples collected within the same barn (22), which leads to questions about the spatial variability of curing conditions within air-cured barns and how

well this variation in conditions correlates to variations in TSNA accumulation in the cured leaf. The objectives of this study were to evaluate curing conditions and attempt to correlate changes in TSNA levels of cured leaf with environmental conditions within dark air-cured barns.

MATERIALS AND METHODS

Research was conducted in 2012 and 2013 at the University of Kentucky Research and Education Center near Princeton, KY and at the Kentucky Agricultural Experiment Station Spindletop Farm near Lexington, KY to evaluate variability in curing conditions within dark air-cured barns. The curing barn used at each location was a 3-tiered design with tiers parallel to the length of the barn. Soil types at each location were Crider silt loam (fine–silty, mixed, active Typic Paleudalfs) at Princeton and Bluegrass-Maury silt loam (fine–silty, mixed, active, mesic Typic Paleudalfs) at Lexington. Drip irrigation was used 3 times at the Princeton location throughout the growing season in 2012, totaling 33.8 cm/ha.

TR Madole (TRsc) screened for low nicotine to nornicotine conversion and TR Madole high converter (TRHC), with greater propensity for high conversion of nicotine to nornicotine, were used in this experiment. Approximately 4,500 plants (750 sticks of tobacco) were grown at each location, with 2,250 plants (375 sticks of tobacco) of each variety. Transplants were grown using current University of Kentucky recommendations (28). Tobacco plants were transplanted in the field in Princeton on May 31, 2012 and June 4, 2013 and in Lexington on June 5, 2012 and May 29, 2013. Field management at each location followed current University of Kentucky recommendations. Nitrogen was applied at Princeton at 336 kg N/ha with 224 kg N/ha broadcast prior to transplanting and 112 kg N/ha side dressed 4 weeks after transplanting. Urea (46–0–0) was used as the nitrogen source for broadcast application and urea–ammonium nitrate (32% N liquid) was the nitrogen source used for side dressing at Princeton. Nitrogen was applied at Lexington at 308 kg N/ha with 168 kg N/ha broadcast prior to transplanting and 140 kg N/ha side dressed 4 weeks after transplanting. Urea (46–0–0) was used as the nitrogen source for broadcast and ammonium nitrate (34–0–0) was the nitrogen source used for side dressing at Lexington. Urea was incorporated immediately after broadcast applications at both locations. Phosphorus and potassium were applied broadcast prior to transplanting, following soil test recommendations at each location. Tobacco was topped at bud-early bloom stage to 16–18 usable leaves. Manual stalk-rundown applications of fatty alcohol (4% v/v) followed by fatty alcohol (5% v/v) plus butralin (1.5% v/v) were used to control suckers. Tobacco was harvested on September 28, 2012 and September 5, 2013 in Princeton and on August 20, 2012 and August 21, 2013 in Lexington. Both varieties were stalk harvested and allowed to field wilt adequately, and then 6 plants were placed evenly on each stick.

HOBO® data loggers (27) were placed in 27 locations throughout each curing barn as tobacco was housed. The Princeton barn was only 5 tiers wide, and this study occupied the entire barn, whereas in the Lexington barn only the northeast corner was used for this study, and other tobacco was housed to fill the remainder of the barn. Each barn was a 3-tiered design with 5 rooms used in the experiment, as demonstrated in Figure 1.

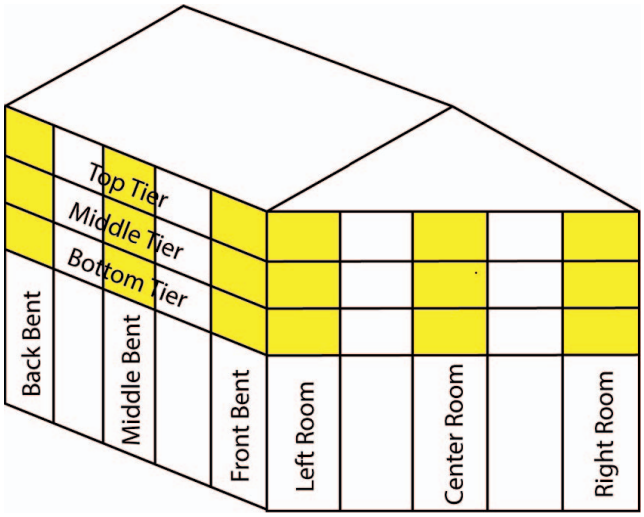

Figure 1. Diagram of long-tier–orientation barn demonstrating the 3-dimensional areas that were studied.

All data loggers were positioned vertically on each tier at 3 locations across the width of each barn (left side room 1, center room 3, and right side room 5), and 3 locations down the length of the barn (front bent, middle bent, and back bent), as represented in Figure 1 (3 locations × 9 loggers at each location = 27 data loggers). Rooms 2 and 4 were also filled with tobacco at the same density as in rooms 1, 3, and 5. Figure 2 illustrates the tobacco housing and meter placement scheme within each room. Each data logger was launched to collect temperature and RH data every hour for the entire cure at the time of tobacco housing. Ambient temperature and RH data were collected with the use of a single data logger outside each barn backed up by data from a permanent field weather station nearby.

Stick spacing used at housing of each barn was approximately 30 cm between sticks. Tobacco was housed in each barn by alternating 5 sticks TRsc followed by 5 sticks TRHC, so that 10 sticks will be allocated as a set for each of the 27 monitoring and sampling locations. Tobacco between each monitoring and sampling location within the barns was also placed with 5-stick alternations of TRsc and TRHC. Sticks for sampling were tagged and housed in the designated monitoring locations. Each data logger was placed between the 5 sticks of TRsc and the 5 sticks TRHC at each location. Loggers were placed at approximately the same level as the fourth leaf on plants. All data loggers were taken down with the tobacco and downloaded after curing. Leaf samples were collected from the TRsc and TRHC tobacco on each side of each data logger, totaling 54 leaf samples collected for nitrite and TSNA analysis from each barn (27 TRsc samples and 27 TRHC samples). Each sample consisted of 20 leaves, which were taken from the fourth leaf from the top of

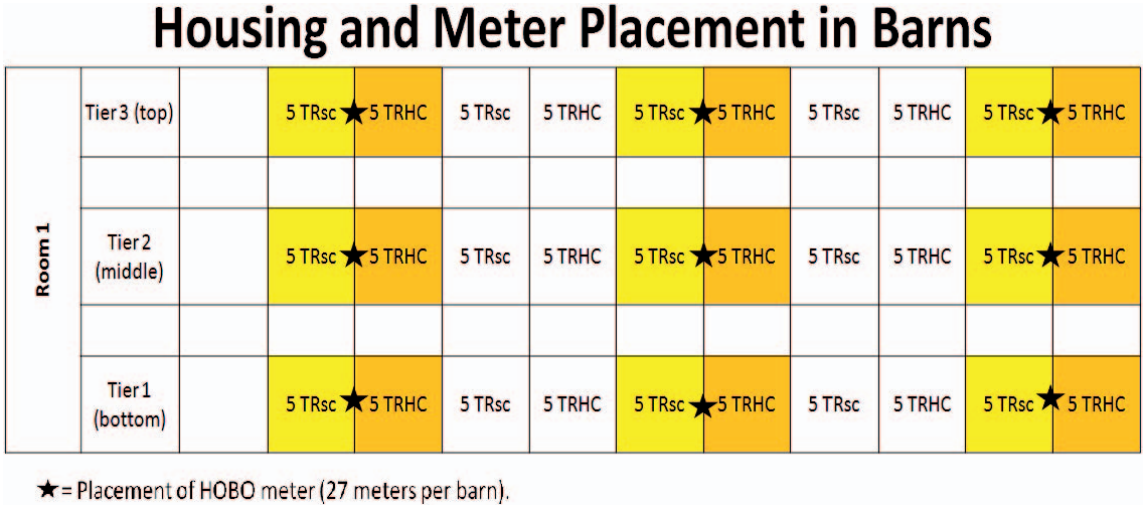

Figure 2. Tobacco housing and logger placement scheme showing the placement of data loggers and tobacco varieties in each room of the 3 sampled rooms within the barns.

20 different plants in each 5-stick segment. If the fourth leaf was absent, that plant was not included in the sample. Leaves were only collected from the center 4 plants on each stick and were not collected from the outside plants on each stick. Samples were then freeze dried, ground to 1 mm, and sent to be analyzed for nitrite and TSNA content.

All leaf samples were analyzed at the University of Kentucky Tobacco Analytical Laboratory located at the Kentucky Tobacco Research and Development Center. The TSNA analysis method followed the method used by Morgan et al. (26) with use of a gas chromatography–thermal energy analyzer (GC-TEA). Nitrate and nitrite contents were analyzed with the use of the method developed by Crutchfield and Grove (12) at the University of Kentucky.

Data were analyzed with the use of PROC GLIMMIX (31). Data were analyzed as a factorial combination of room, bent, and tier that corresponds to the placement of the data loggers. Because of the nature of the barns, each site location (Princeton and Lexington) was analyzed separately. Year served as the replicate for the experiment. As expected, TSNA concentration was significantly different between TRsc and TRHC; thus, each variety was analyzed separately. Means were separated with the use of least-squares (LS) means, and significance was determined at alpha of 0.10. PROC REG was used to analyze regressions between continuous variables.

RESULTS AND DISCUSSION

Recording curing conditions every hour for the entire duration of curing at each site location resulted in nearly 1,500 individual data points for each measured variable. For the purposes of this analysis, critical thresholds for temperature and relative humidity were established and used to develop a cumulative index to best represent conditions during the yellowing stage (first 14 days of curing) and the entire curing season. The analysis process compared temperature and relative humidity from each data logger and corresponding TSNA content for each position in the curing barn to all other positions. Within-barn temperature data are presented as the cumulative number of hours where temperature exceeded 24°C for the yellowing phase of the cure. This was selected based on previous research suggesting that temperature above 24°C during curing tended to result in higher levels of TSNA in the cured leaves (6,37). Within-barn RH data are presented as the number of hours with RH above 80% for the yellowing phase of the cure. This RH threshold was chosen because previous research has shown elevated TSNA levels for tobacco cured under conditions of high RH (6). TSNAs are presented as total TSNA in micrograms per gram, which is the sum of all individual TSNAs (NNN, NAT, NAB, NNK). TRHC data were analyzed and are presented separately from TRsc data.

Logger and tobacco placement could be visualized as 27 individual points within each barn. Each logger placement was housed with 5 sticks of each variety on either side of each data logger (Figure 2). There was no significant effect of individual data logger placement on temperature, RH, TRHC total TSNA, or TRsc total TSNA at either barn location because of the overall high variability in TSNA content throughout each barn. This result was not unexpected, because the barns at the 2 locations differ with respect to size, orientation, and construction. This reflects the real-world conditions in which many different types of barns and curing structures are often used and each may have its own unique pattern of air movement. It may be inferred from this research that the relative curing conditions or accumulation of TSNAs cannot be simply predicted based on the relative position of the curing leaf within the barn.

In an attempt to detect additional patterns in these data, the 3-dimensional aspects of tier (bottom, middle, top), room (left, center, right), and bent (front, middle, back) within each barn (Figure 1) were investigated. Tier, room, and bent were included as classification variables,

Table 1. Average number of hours above 24°C in tier, room, and bent during the yellowing phase for Princeton and Lexington.[a]

Temperature					
Tier		Room		Bent	
Hours above 24°C					
Princeton					
Bottom	60.83 b	Left	61.61 b	Front	61.72 b
Middle	58.72 c	Middle	61.00 b	Middle	63.05 ab
Top	69.27 a	Right	66.22 a	Back	64.06 a
P value	*<0.0001*		*<0.0001*		*0.0739*
Lexington					
Bottom	137.17 c	Left	149.33 a	Front	153.61 a
Middle	145.56 b	Middle	143.94 b	Middle	144.28 b
Top	158.67 a	Right	148.11 a	Back	143.50 b
P value	*<0.0001*		*0.0053*		*<0.0001*

[a] Means followed by the same letter within location and within tier, room, or bent are not significantly different according to Fisher's Protected LSD at $\alpha = 0.10$.

thus allowing some of the variability to be apportioned to these positional factors. Simple linear regression was used in order to determine relationships between temperature, RH, and TSNA accumulation.

Influence of Barn Position on Temperature, RH, and Total TSNA. *Tier.* There was a significant effect of tier level on cumulative temperature, as shown in Table 1. In general, the number of hours above 24°C tended to increase with tier height, but the response varied between locations. However, there appeared to be differences in the pattern of increase between barns. An increase in the cumulative hours above 24°C was observed with each increase in tier height at the Lexington location. However, the bottom tier had significantly higher cumulative temperature >24°C than the middle tier at the Princeton location, which is likely not biologically relevant, as this difference was only 2 hr. These results generally follow expectations, as heat tends to rise and accumulate at the top of the barn. The cumulative hours of high relative humidity were also different among tiers (Table 2). The number of hours >80% RH increased in higher tiers at Princeton, but generally decreased with higher tiers in Lexington. Total TSNA for TRHC increased with tier height in Princeton, but decreased with tier height in Lexington (Table 3). The opposing relationship between TSNA and tier increment at each location is an interesting result, yet difficult to explain with hours >24°C or >80% RH. Total TRsc TSNA levels among tiers were not statistically different at either location (Table 4).

Room. There were significant room effects for the number of hours above 24°C (Table 1), number of hours above 80% RH (Table 2), total TRHC TSNA (Table 3), and total TRsc TSNA at Princeton (Table 4). The right room within the Princeton barn had significantly more hours above 24°C, more hours above 80% relative humidity, and higher total TRHC TSNA when compared to the

Table 2. Average number of hours above 80% RH in tier, room, and bent during the yellowing phase for Princeton and Lexington.[a]

RH					
Tier		Room		Bent	
Hours above 80%					
Princeton					
Bottom	91.33 c	Left	79.45 b	Front	94.39 b
Middle	103.67 b	Middle	114.11 a	Middle	99.73 b
Top	112.34 a	Right	113.78 a	Back	113.22 a
P value	*<0.0001*		*<0.0001*		*0.0002*
Lexington					
Bottom	157.28 a	Left	120.17 c	Front	124.44 c
Middle	149.24 a	Middle	171.00 a	Middle	165.52 a
Top	140.28 b	Right	155.63 b	Back	156.83 b
P value	*0.0024*		*<0.0001*		*<0.0001*

[a] Means followed by the same letter within location and within tier, room, or bent are not significantly different according to Fisher's protected LSD at $\alpha = 0.10$.

Table 3. TR Madole High Converter (TRHC) total TSNA accumulation in tier, room, and bent during the yellowing phase for Princeton and Lexington.[a]

Total TRHC TSNA					
Tier		Room		Bent	
μg/g					
Princeton					
Bottom	5.44 b	Left	5.28 b	Front	5.63 ab
Middle	5.21 b	Middle	5.52 b	Middle	5.17 b
Top	6.91 a	Right	6.76 a	Back	6.75 a
P value	*0.0345*		*0.0859*		*0.0708*
Lexington					
Bottom	6.15 a	Left	5.29 b	Front	4.82 b
Middle	5.42 b	Middle	6.04 a	Middle	6.12 a
Top	4.87 b	Right	5.12 b	Back	5.50 ab
P value	*0.0136*		*0.0698*		*0.0146*

[a] Means followed by the same letter within location and within tier, room, or bent are not significantly different according to Fisher's Protected LSD at $\alpha = 0.10$.

Table 4. TR Madole Low Converter (TRsc) total TSNA accumulation in tier, room, and bent during the yellowing phase for Princeton and Lexington.[a]

Total TRsc TSNA					
Tier		Room		Bent	
μg/g					
Princeton					
Bottom	1.25	Left	1.15 b	Front	1.73
Middle	1.50	Middle	1.91 a	Middle	1.39
Top	1.78	Right	1.47 b	Back	1.42
P value	*0.1288*		*0.0175*		*0.3375*
Lexington					
Bottom	1.02	Left	1.02	Front	0.89
Middle	0.95	Middle	1.00	Middle	0.95
Top	0.97	Right	0.92	Back	1.09
P value	*0.7309*		*0.5222*		*0.1063*

[a] Means followed by the same letter within location and within tier, room, or bent are not significantly different according to Fisher's Protected LSD at $\alpha = 0.10$.

left room. Both the left and right rooms at Princeton were along exterior walls as the barn was only 5 rooms wide, and the room designated as "right" faced the west. The higher temperature in the right room could be explained by the warming of that side of the barn by the afternoon sun. The TRsc TSNA levels were higher in the middle room (Table 3), which was not similar to TRHC TSNA (Table 4). The left room of the Lexington barn had more hours above 24°C and fewer hours above 80% RH compared to the middle room, but temperature was not significantly different than the right room. The left room at Lexington was adjacent to an east-facing exterior wall, whereas the right room was near the center of the barn. Because only the left room was along an exterior wall in the Lexington barn, this could explain the higher temperatures and lower relative humidity in the left room compared to the middle room. The right and center rooms of the Lexington barn were more likely influenced by air that may have been cooler and more humid as a result of passing through the large mass of adjacent tobacco. However, this does not explain a lack of significance in temperature between the left and right rooms at Lexington. Despite significant differences in curing conditions, there were no significant differences in total TRsc TSNA between rooms at Lexington. Total TRHC TSNA seemed to be inversely related to temperature at Lexington, as rooms with increased hours above 24°C also had significantly lower total TRHC TSNA.

Bent. There was a significant effect of bent on hours above 24°C (Table 1), number of hours above 80% RH (Table 2), and total TRHC TSNA (Table 3). Princeton had significantly higher hours above 24°C in the back bent, whereas Lexington had significantly higher hours above 24°C in the front bent. Each barn location had more hours of RH greater than 80% in the back bent when compared to the front bent. The back bent at the Princeton barn was at the east end of the barn and the back bent at Lexington was near the middle of the barn. This could explain the opposing trends for temperature and RH in bents at each location. The front bent was on the north end of the Lexington barn and was the only bent that was exposed to an external wall on 2 sides. The back bent of the barn at Princeton had significantly higher total TRHC TSNA content when compared to the middle bent, but was similar to TSNA in the front bent (Table 3). Lexington had significantly higher total TRHC TSNA content in the middle bent compared to the front, but was similar to the back bent. The curing environment data does not explain this variation well, although relative humidity was significantly higher in the middle bent at Lexington and temperature was the highest in the back bent at Princeton.

There were many tier, room, and bent effects on temperature, RH, total TRHC TSNA, and total TRsc TSNA shown in this paper. It is probable that all barns will not behave similarly when attempting to characterize barn behavior. Temperature data followed a pattern that was understandable within each year and location. However, relative humidity and TSNA accumulation did not follow similar trends within each barn. These differences in relative humidity within the microenvironments of each

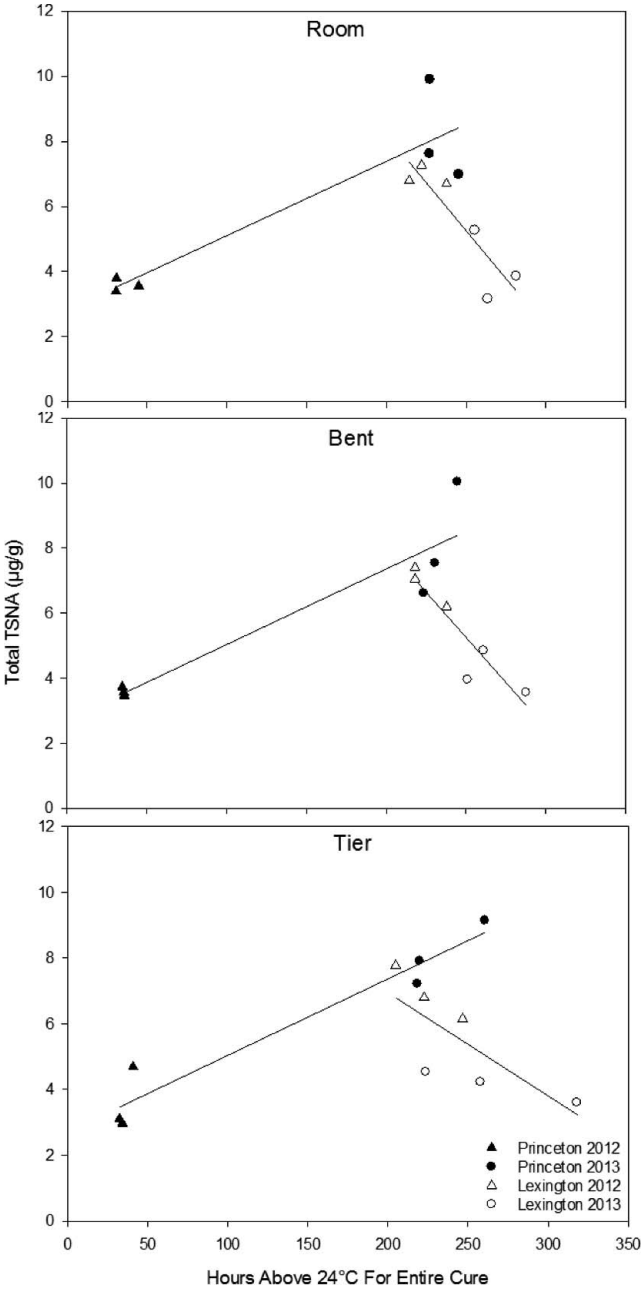

Figure 3. Temperature above 24°C during the entire cure of air-curing affects TRHC total TSNA accumulation for room, bent, and tier.

barn could be due to differences in barn structure, directional orientation, dimensions, and/or ventilation structures. These differences in TSNA content could be affected by many other factors in both the field and curing seasons.

Temperature Is Related to TRHC TSNA. Data were subjected to linear regression across both sites to understand better the behavior within 3-dimensional aspects of the curing barns and the relationship between hours above 24°C, hours above 80% RH, and TSNA. Total TRsc TSNA was not significantly related to hours above 24°C or 80% RH when regressed for the yellowing phase

or the entire cure, which was also observed for hours above 80% RH and total TRHC TSNA. However, there was a significant linear relationship between hours above 24°C and total TRHC TSNA for the entire cure as shown by location in Figure 3, but this relationship was not observed for only the yellowing phase of the cure. There was a positive linear relationship between total TRHC TSNA and temperature in Princeton, whereas this relationship is negative at Lexington. The opposing difference across years and between locations may be explained by the different curing environments. The Lexington curing environment for the entire cure in both years of this experiment observed at least 200 hr above 24°C, whereas Princeton was closer to 60 hr in 2012. This can be attributed to the time of year that the tobacco was harvested at the 2 locations. Regardless, more hours above 24°C resulted in decreased total TRHC TSNA at Lexington but increased total TRHC TSNA at Princeton. It is also interesting to note the point at which the directions of the regressions change, as it suggests that increased hours about 24°C results in increased TSNA up to approximately 200–230 hours >24°C (Figure 3). Beyond approximately 230 hr >24°C, drying rate of tobacco may increase to the point where TSNA production actually decreases. Similar to Roton et al. (30), this may be a function of rapid drying or desiccation of the leaf, limiting nitrite formation with more hours above 24°C during the yellowing phase of curing at Lexington. Another possibility involves studies that have suggested relationships between microbial communities and TSNAs (23,33). Staaf et al. (33) suggested that a critical period exists for cell contents to become available to microorganisms and chemical reactions as plant cell membranes are broken down because of moisture loss. These reactions include microbial reduction of nitrate to nitrite and chemical nitrosation of alkaloids by nitrite. Law et al. (23) found a corresponding shift in microbial communities in samples with higher levels of TSNAs, nitrate, and nitrite under curing conditions with increased relative humidity. It may be possible that frequent temperatures above a certain threshold promote rapid drying and can detrimentally impact microbial communities that correlate with TSNA content.

CONCLUSION

The formation and accumulation of TSNAs in curing tobacco is a complex process influenced by many factors. Studies of TSNA formation under controlled environmental conditions have shown that temperature and relative humidity can impact TSNA levels in cured leaves. However, the environmental conditions in air-curing barns are constantly changing, and one of the major challenges is trying to characterize the conditions the tobacco is exposed to adequately. Instrumentation used to measure barn environments must be durable as well as accurate. The HOBO data loggers used in this experiment were found to be reasonably reliable, but variation in RH between individual loggers may be as high as 10% (C. Fisher, personal communication). Placement of loggers is important to get an accurate reflection of the conditions, and loggers in direct contact with leaf material will generally have erroneous readings. For this study, small shields were used to keep the loggers from being in direct contact with leaves; however, the impact the shields may have had on measurements is unknown. Data loggers placed near the external walls of the barn can experience more fluctuation and influence from ambient conditions outside the barn, particularly if placed near a vent opening.

One thing that is clear from this study is that the effect of within-barn position cannot be easily predicted. For example, it cannot be assumed that the highest TSNA level will always be at the top or bottom or even the middle of a barn. Factors such as barn construction, including the location and design of vents, vertical distance between tier rails, and siding materials will influence the flow of air through the crop. Additional research is needed to determine if there is spatial structure of conditions within curing barns and how to sample the conditions to be able to predict and ultimately manage the barn for optimal curing.

With any TSNA research, inherent variability in TSNA formation, air-curing barns, environmental conditions, and other complex variables are not easily measured. Correlation between curing environment and TSNA accumulation can be difficult to prove due to these intricate interactions and relationships.

This study was designed to correlate changes in curing conditions within dark air-cured barns to TSNA accumulation in specific areas throughout the barn. The differences between these 2 barns are tremendous. The distance between these 2 locations is around 200 miles (320 km). Lexington's elevation above sea level is about 978 ft (298 m) when compared to 482 ft (147 m) in Princeton. The barns at both locations have different directional orientation and significant dimensional differences. Therefore, differences were expected between these 2 barns. Future studies could utilize an increased barn size to allow more distance between sensors and sampling areas as a technique to detect variability in curing conditions and TSNA better.

Major progress has been made in understanding the formation of TSNA, but there is still much to learn. There are other complex processes that influence accumulation of TSNA. High variability in cured-leaf TSNAs is still observed, and this variability needs to be addressed if federal regulations specify a maximum TSNA limit. In this study, we found limited significant relationships between temperature and relative humidity on TSNA formation, which suggests that other factors may be involved. More precise methods of analyzing the within-barn environment could help clarify how temperature, relative humidity, and TSNA interact.

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