EFFECT OF ORDERING METHOD ON TOBACCO-SPECIFIC NITROSAMINES (TSNAs) CONTENT IN DARK AIR-CURED AND BURLEY TOBACCO

Mitchell D. Richmond[1], Colin R. Fisher[4], Anne M. Fisher[2], Robert C. Pearce[3], and William A. Bailey[4]*

Tobacco-specific nitrosamines (TSNAs) are known carcinogens in cured tobacco. They are produced primarily during the curing process, but agronomic practices occurring in the field as well as handling practices after curing may also influence TSNA levels, particularly if cured leaf is stored at high moisture. After curing and during market preparation, the cured leaf must be supple to avoid breakage. Ideally, this is after a period of wet weather during which the leaf absorbs moisture and comes into order or case. Often the weather remains dry for long periods after curing, and growers resort to artificial ordering to take down a sufficient amount of their crop to work on for several days, during which time the tobacco is bulked. The effect of this artificial ordering on TSNAs during short-term storage is not known. Field experiments were conducted in each of 3 years at two locations in Kentucky to evaluate TSNA accumulation following several ordering methods in dark air-cured and burley tobacco types. Treatments included natural ordering and variants of steaming and misting, which are both commonly used artificial ordering methods. At the Princeton location, samples were taken within 24 hr after the ordering treatments were done. In Lexington, samples were taken sequentially at takedown, after ordering, and after 14 d in the bulk. There were limited and inconsistent differences in total TSNAs between methods of ordering, and the TSNA levels were not affected by the moisture content of the leaf during bulking. There was a significant increase in TSNAs in the 24-hr period between takedown and bulking, which cannot be explained. We conclude that, in Kentucky, growers should use ordering methods that are best suited for their production system, but this may not be the case in warmer climates.

Additional key words: Curing, Market Preparation, Baling

INTRODUCTION

Kentucky leads the United States in burley and dark air-cured tobacco production, with an estimated 25,000 and 2,000 hectares harvested in 2016, respectively (19). The majority of burley tobacco is used as a component in blended cigarettes, because of its ability to accept flavoring compounds (16). Dark air-cured tobacco is used in smokeless products and specialty-type cigars (13).

Preparation of burley and dark tobacco types for market involves taking the tobacco down from the barn at the end of curing (takedown), removing the stalks from the stick, removing the leaves from the stalk (stripping), and baling for market. These processes can only be done when the leaves are sufficiently pliable to avoid breakage. This characteristic is referred to as order or case. Cured leaf is hygroscopic and will adsorb or desorb moisture as the relative humidity fluctuates and will equilibrate to ambient humidity after 8–24 hr (21). O'Bannon (15) concluded that medium to medium-high order, or a moisture content (MC) of at least 15%, should be the minimum leaf moisture target for stripping. Relative humidity >75% is necessary to achieve adequate order (18). Takedown and stripping of air-cured tobacco in Kentucky is done between November and February when the ambient humidity is often very low for extended periods and growers are compelled to use artificial ordering to enable them to continue preparing their crop for market. Steam, which requires a heat source, and misting are two common artificial ordering methods used in Kentucky (1).

During long-term storage, TSNAs accumulate if the MC of the packed tobacco is too high (20) but the effects of these different ordering methods on TSNA accumulation during on-farm short-term storage during market preparation has not been documented. As a result, growers in the United States are cautioned to allow tobacco to come into order naturally if possible, without the use of artificial ordering methods (11).

Four TSNAs are the components of total TSNAs: N'-nitrosonornicotine (NNN), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N'-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB). Only NNN and NNK are considered to be biologically active (2,6,9,10). Considerable progress has been made in reducing TSNAs in cured leaf since the 1970s by modifying agronomic, curing, processing, and manufacturing practices (4), with the most significant reductions occurring after seed screening was introduced (12).

The U.S. Food and Drug Administration (FDA) was granted authority to regulate tobacco products in 2009 (7) and recently proposed a limit of 1 $\mu g\ g^{-1}$ for NNN in finished smokeless tobacco products (8). Any practice that reduces TSNAs in raw tobacco leaf will have a direct impact on reduced TSNAs in tobacco products. The objective of this research was to determine if artificial ordering after takedown and during short-term storage affects TSNA accumulation.

[1]Department of Plant Sciences, University of Tennessee, Knoxville, TN 37996.
[2]Kentucky Tobacco Research & Development Center, University of Kentucky, Lexington, KY 40546.
[3]Department of Plant and Soil Sciences, University of Kentucky, Lexington, KY 40546.
[4]Department of Plant and Soil Sciences, Research and Education Center, University of Kentucky, Princeton, KY 42445.
*Corresponding author: William A. Bailey; email: abailey@uky.edu

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MATERIALS AND METHODS

Princeton. Field experiments were conducted in 2011, 2012, and 2013 at the University of Kentucky Research and Education Center near Princeton, KY to evaluate the effects of ordering method on TSNA content in dark air-cured and burley tobacco types. The cultivars used were low converter (LC; screened for low nicotine to nornicotine conversion) dark air-cured TR Madole (TRsc) and burley TN 90LC, and high converter (HC) selections of TR Madole (TRHC) and TN 90 (TN 90HC). All crop production practices, including transplant production, field preparation and fertilization followed University of Kentucky recommendations (17). Seedlings were transplanted in the field in late May. The four cultivars were grown in adjacent blocks within the same field at 12,100 plants ha^{-1}. Both tobacco types were topped (flower bud removed) at bud to early bloom (growth stages 51–61) (3) so that 16–18 usable leaves remained on the dark tobacco and 19–21 leaves remained on the burley. A manual stalk-rundown application of fatty alcohol and butralin was used to control suckers in all cultivars. All cultivars were stalk harvested 5 weeks after topping (mid-September) each year and allowed to field wilt adequately, and then six plants were placed evenly on each stick. Sixty sticks of each cultivar were harvested, housed, and cured in a standard air-curing barn. The sticks were spaced approximately 30 cm apart in the barn.

Takedown after curing was done when tobacco was just in sufficient natural order to allow takedown but on a dry day when weather forecasts predicted fog that night. The sticks were randomly loaded onto three scaffold wagons so that each of the three wagons was loaded with 20 sticks of each of the four cultivars and the 80 sticks on each wagon were evenly spaced. All three scaffold wagons were then parked outside the barn and allowed to dry and go out of order in the ambient, low humidity, outside daytime conditions, and then two of the three wagons were pulled back in the barn. One of these two wagons received misting from a garden hose and was covered with plastic for the night. The other scaffold wagon was covered with plastic and a steaming rod was placed under the wagon. This wagon was steamed for 3 min, allowed to equilibrate for an hour, and then steamed again for 6 min. Both wagons in the barn were left tightly covered with plastic for the night. The next morning, the wagon outside the barn was naturally ordered and was pulled in alongside the misted and steamed wagons and also covered with plastic to maintain order. Approximately 1 hr later, the covers were removed from each wagon as needed to allow moisture/order to reach the same level by feel. Samples were collected when all three wagons felt similar in moisture/order and at moisture levels typical for stripping dark air-cured and burley tobacco in western Kentucky.

Samples were collected as four replicates within each cultivar and within each ordering method (wagon). Each sample consisted of 20 leaves collected from the fourth leaf position from the top of the center four plants on each of five adjacent sticks (the first and sixth plants on each stick were not sampled). Whole-leaf samples were then immediately placed in a freezer (−18°C) until TSNA analysis. The TSNA analysis (14) was done at the University of Kentucky Tobacco Analytical Laboratory located at the Kentucky Tobacco Research & Development Center (KTRDC) using gas chromatography with a thermal energy analyzer (GC-TEA). A control sample (ground reference cigarette filler 2R1) provided by the Center for Tobacco Reference Products at the Kentucky Tobacco Research and Development Center was used as the check for the instrument and method before and after each sequence of eight test samples.

Lexington. Field experiments were conducted in 2010, 2011, and 2012 at the University of Kentucky Agricultural Experiment Station Spindletop Farm near Lexington, KY, using only burley. In 2010, only a high converter line of TN 90 (TN 90HC) was used, whereas both high and low converter lines (TN 90HC and TN 90LC) were used in 2011 and 2012. All crop production practices, including transplant production, field preparation, and fertilization followed University of Kentucky recommendations (17). Seedlings were transplanted into the field in late May at 17,300 plants ha^{-1}. Tobacco was topped at bud to early bloom (growth stages 51–61) (3) to 19–21 usable leaves. Four replications of the ordering treatments were randomly allocated to plots in the field before stalk cutting 10 sticks per plot 4 weeks after topping (mid-September) each year. Six plants were placed evenly on each stick. Sticks were housed in a standard air-curing barn at a stick spacing of 20 cm. When the tobacco was naturally in order after a period of high humidity at the end of the cure, all the tobacco was taken down and hung on scaffold wagons.

Samples were collected immediately after takedown ("takedown"), after ordering and packing into the bulk ("after ordering"), and after 14 d in the bulk ("after 14 days"). Bulking was done by removing the tobacco from the sticks and laying the separate bundles of eight stalks of unstripped tobacco from each plot in 2010, or 16 stalks of each plot in 2011 and 2012, on a layer of border tobacco in prefabricated six-micron-thick black polyethylene plastic bags that were 1.8 m long, 0.9 m wide, and 0.45 m deep. Bundles of extra tobacco were then placed on either side and on top so that none of the sampled tobacco was in contact with the plastic bag. A data logger that recorded temperature and humidity was placed in each bundle of sampled tobacco and the bags were then heat-sealed so that they were airtight.

Ordering treatments included natural ordering and variations of mist and steam applications. Samples consisted of the fourth leaf from the top of the plant from the central four plants on each stick. Samples were collected from two sticks per plot at takedown, and from four sticks before bulking and after 14 d in the bulk.

The "natural" treatment was bulked as soon as possible after taking down all the tobacco, so the "takedown" and "after ordering" samples for that treatment were collected within 2 hr of each other. The cured tobacco for all the other treatments was then allowed to dry out in a low-humidity environment overnight and then the artificial ordering treatments imposed. Steaming (steam) was

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Table 1. Total TSNA content as affected by ordering method for dark air-cured and burley cultivars at Princeton.

Cultivar and Ordering	Total TSNAs[a] ($\mu g\ g^{-1}$) 2011	2012	2013
Dark air-cured			
TRHC			
Mist	8.9	2.8	9.8
Natural	10.5	3.2	10.1
Steam	6.4	1.9	12.8
P value	0.1343	0.3386	0.5695
TRsc			
Mist	0.9	0.6 ab	1.9
Natural	1.0	0.8 a	1.6
Steam	0.8	0.5 b	2.4
P value	0.3967	0.0417	0.2668
Burley			
TN 90HC			
Mist	22.8	3.1 b	6.3
Natural	23.8	6.3 a	4.4
Steam	24.2	5.8 a	4.6
P value	0.9826	0.0305	0.4897
TN 90LC			
Mist	7.1 a	1.2	1.2 ab
Natural	6.9 a	0.9	0.9 b
Steam	5.0 b	1.4	1.5 a
P value	0.0186	0.1107	0.0374

[a] Means within a cultivar and year followed by the same letter are not significantly different according to Fisher's protected least significant difference at P = 0.10.

done by hanging the sticks with the stalks in a chamber and introducing steam underneath until the leaf was in sufficient order. This process was repeated after about 30 min for the steamed-twice treatment (steam 2×). The misting (mist), misted twice (mist 2×), and coarse spray (spray) treatments were done using the mist or shower settings on a garden water hose attachment. Each side of the stalks was sprayed as evenly as possible without runoff and then left hanging on the scaffold wagon. This tobacco was not bulked until there was no free moisture on the leaf surface. In the third year (2012), two additional ordering treatments were added by placing tobacco in controlled environment chambers for 24 hr at 70 or 80% relative humidity for ordering.

All samples were weighed immediately after sampling, the lamina was separated from the midrib, and both components were freeze-dried and weighed again. These weights were used to calculate MC. Only the lamina was ground and analyzed for TSNA content. The tobacco was ground in a Wiley mill, to pass through a 1-mm screen. TSNA analysis was done at the University of Kentucky Tobacco Analytical Laboratory with the GC-TEA and the 2R1 reference tobacco as a control standard.

A major difference between the treatments at the two locations is that at Princeton, samples were taken within 24 hr of treatment, with no storage; whereas at Lexington, final samples were taken after 14 days of storage in the bulk.

RESULTS AND DISCUSSION

Princeton. The TSNA data are presented by cultivar because of known differences in TSNA accumulation between high converter and low converter lines. Total TSNA content for each ordering method in each year for the dark and burley cultivars at UKREC are shown in Table 1.

The only differences in total TSNAs between the ordering treatments for dark tobacco was in TRsc in 2012 (P = 0.0417). The natural ordering had significantly higher total TSNAs than steam (0.8 and 0.5 $\mu g\ g^{-1}$, respectively), but was not different from the mist treatment (0.6 $\mu g\ g^{-1}$). These results were unexpected, as we would have anticipated the natural ordering to have the lowest TSNAs. However, the results were inconsistent and occurred in only 1 year out of 3.

In the burley, the only significant effect of ordering method within TN 90HC was in 2012 (P = 0.0305). Mist ordering (3.1 $\mu g\ g^{-1}$) had significantly lower total TSNAs than natural and steam (6.3 and 5.8 $\mu g\ g^{-1}$, respectively). For TN 90LC, ordering method did not affect total TSNAs in 2012, but the 2011 and 2013 results were inconsistent. In 2011, the steam treatment had significantly lower TSNAs than natural ordering or misting (P = 0.0186); but in 2013, steam (1.5 $\mu g\ g^{-1}$) was significantly higher than natural ordering (0.9 $\mu g\ g^{-1}$), with mist not significantly different from either (1.2 $\mu g\ g^{-1}$). The inconsistency of the data means that we cannot draw any valid conclusions about the effect of the treatments. Overall, TSNAs were much higher in 2011 than in 2012 and 2013 in both TN 90LC and TN 90HC, which is likely explained by a more humid curing season in 2011.

Lexington. There were no significant differences in total TSNAs between ordering methods in either TN 90LC or TN 90HC in any of the 3 years at Lexington (Table 2). Total TSNAs ranged from 3.8 to 6.2 $\mu g\ g^{-1}$ in TN 90HC across all years and ordering methods and from 0.9 to 2.5 $\mu g\ g^{-1}$ in TN 90LC.

In TN 90LC in 2011 and 2012, there was a significant increase in TSNAs between takedown and bulking (after ordering), but not between bulking and after 14 d in the bulk (Table 3). The same was true of TN 90HC in 2011, but in 2012, TSNAs after 14 d in the bulk were not significantly different from either takedown or before bulking (after ordering). There is no explanation for the consistent increase of up to 80% in TSNAs between takedown and bulking, a period of no more than 30 hr. This increase in TSNA is unlikely to be an error associated with the chemical analysis process because the 2R1 reference product (Table 3) that is routinely included before and after every eight samples demonstrates that the difference between the takedown and before bulking samples is a true difference or could be even greater.

There was about a 10% range of MC each year (Table 4; 19.8–25.9% in 2010, 20.2–32.0% in 2011, and 14.8–25.3% in 2012), but most were within the normal 20–25% range at which tobacco would be handled during market preparation. Within any year, there were significant differences in MC between the ordering treatments. The naturally ordered treatment in each year varied from

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Table 2. Total TSNA content after 14 days in bulk for burley cultivars at Lexington.

	Total TSNAs[a] ($\mu g\ g^{-1}$)				
	2010	2011		2012	
Ordering	TN 90HC	TN 90HC	TN 90LC	TN 90HC	TN 90LC
Natural	4.9	4.6	1.4	5.7	1.6
Mist 1	5.1	4.8	1.2	5.0	1.8
Spray	6.2	3.8	1.3	–	–
Steam	5.0	3.8	0.9	–	–
Double steam	–	4.4	1.1	–	–
Mist 2	–	–	–	5.1	2.5
70% relative humidity	–	–	–	5.4	1.5
80% relative humidity	–	–	–	5.6	1.3
P value	0.5680	0.6000	0.5200	0.8760	0.5670

[a] Treatments were significantly different according to Fisher's protected least significant difference at P = 0.10.
– = treatments not included in some years.

19.8% in 2010 to 21.4% in 2012. The MC of the single misting treatment (Mist 1) was nearly 3% greater than the naturally ordered tobacco in 2010, but was the same as the naturally ordered tobacco in 2011 and 2012. The double misting treatment (Mist 2) increased the MC relative to the natural ordering, but was no different from the single misting treatment. The steamed in 2010 and 2011, and the double steamed tobacco in 2011, was the same MC as the naturally ordered leaf. In both years that it was tested, the sprayed tobacco had higher MC than all the other treatments, most likely because it was difficult to distribute the water evenly across both sides of the stalks on the stick and from the top to the bottom of the plant with the high volume emitted from a coarse spray. The MC of the tobacco ordered at a controlled humidity of 80% was no different from the naturally ordered tobacco, but at 70% controlled humidity, it had lower MC than naturally ordered tobacco.

The data logger recordings of the relative humidity in the bulks showed that the humidity stabilized in the bulk after 7 d. The MC of the leaf at the end of the 14 d in the bulk corresponded well with the median humidity in the bulk over the last 7 days, and is consistent with published sorption rates of cured leaf (5).

CONCLUSIONS

The differences between the ordering treatments at Princeton were inconclusive and in Lexington, there were no differences at all despite the wide range of moisture levels. Therefore, these data suggest that ordering method does not consistently affect TSNA accumulation. However, takedown and market preparation in Kentucky is done during the winter months when the ambient temperatures range from −4 to 13°C. The median temperatures in the bulks during each of the 3 years of the Lexington test were 15, 12, and 10°C, respectively. There could well be an effect of both the ordering method and MC

Table 3. Total TSNA content, time after ordering, and year for burley cultivars at Lexington.

	Total TSNAs[b] ($\mu g\ g^{-1}$)		
Cultivar and Sample Time[a]	2010	2011	2012
TN 90HC			
At takedown	5.7	2.9 b	4.6 b
After ordering	5.8	4.5 a	5.8 a
After 14 days	5.3	4.3 a	5.3 ab
P value	0.6750	0.0170	0.0550
TN 90LC			
At takedown	–	0.6 b	1.3 b
After ordering	–	1.1 a	1.6 a
After 14 d	–	1.2 a	1.6 a
P value	–	<0.0001	0.0320
2R1 reference[c]			
At takedown	–	2.9 a	3.2 a
After ordering	–	2.7 b	3.2 a
After 14 days	–	3.1 a	3.1 b
P value	–	0.0010	0.0080

[a] Data pooled over ordering method.
[b] Means within a cultivar and year followed by the same letter are not significantly different according to Fisher's protected least significant difference as $P = 0.10$.
[c] Control samples ran with each time sampling.
– = treatments not included in some years.

Table 4. Moisture content after 14 days in bulk at Lexington.

	Moisture Content (% w/w)		
Ordering	2010	2011	2012
Natural	19.8 c	21.1 b	21.4 b
Mist 1	22.7 b	20.2 b	22.6 ab
Spray	25.9 a	32.0 a	–
Steam	18.7 c	20.5 b	–
Double steam	–	24.0 b	–
Mist 2	–	–	25.3 a
70% relative humidity	–	–	14.8 c
80% relative humidity	–	–	22.4 ab
P value	<0.0001	<0.0001	<0.0001

[a] Means within a cultivar and year followed by the same letter are not significantly different according to Fisher's protected least significant difference as $P = 0.10$.
– = treatments not included in some years.

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in warmer climates. It is important to note that the ordering methods used at the Lexington and Princeton locations were different and that dark air-cured and burley tobacco growers may use different ordering methods in their production systems. There was no strong evidence to suggest that the method used to bring burley or dark air-cured tobacco into order has any consistent impact on TSNA accumulation under Kentucky climate conditions during the late fall and winter months. This may not be the case in warmer climates. The reason for the increase in TSNAs in the very short time between takedown and ordering should be investigated further, especially if regulation will limit TSNA levels.

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