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Formation of Tobacco-Specific Nitrosamines (TSNA) During Air-Curing: Conditions and Control*

by

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SUMMARY

The present review deals with studies performed during several consecutive years on the effect of air-curing on tobacco-specific nitrosamine (TSNA) formation and quality of tobacco. Temperature, relative humidity, water content and water activity data were collected during curing of dark tobacco in traditional air-curing barns and bulk-curing barns of different sizes, and chemical analysis of the cured tobacco were performed.
The results from the screening study of the traditional curing conditions support the idea that a high continuous relative humidity during the entire curing period results in high TSNA levels in the tobacco, while a drier climate produces tobacco with lower amount of TSNA and nitrite. The study also demonstrates that a high continuous relative humidity at the beginning of the yellowing phase followed by a pronounced decline of relative humidity at the critical period for TSNA formation, i.e., when the plant cell membrane breaks down due to moisture loss and the cell contents become available to nitrite forming micro-organisms, results in tobacco with relatively low TSNA levels.
Control of air-flow is important during the curing process and a uniform air-flow will increase the rate and amount of moisture loss from the tobacco, and also reduce possible gas-phase reactions between alkaloids and gaseous nitric oxides, derived from microbially generated nitrite, during air-curing of dark tobacco.
Curing experiments, in mini- and bulk-barns with controlled conditions, aiming to mimic a reduction in humidity at the end of yellowing confirm that a decline in humidity at the critical period reduces the accumulation of TSNA in the cured tobacco, while still producing a satisfactory tobacco quality in a shorter time than traditional air-curing.
[Beitr. Tabakforsch. Int. 21 (2005) 321–330]

ZUSAMMENFASSUNG

In dieser Übersicht werden Studien präsentiert, die während mehrerer aufeinander folgender Jahre durchgeführt wurden und in denen die Auswirkung der Lufttrocknung auf die Bildung tabakspezifischer Nitrosamine (TSNA) und die Qualität des Tabaks untersucht wurde. Die Temperatur, die relative Luftfeuchtigkeit, der Wassergehalt sowie Daten über die Wasseraktivität wurden während des Trockenvorgangs dunklen Tabaks in konventionellen Trockenschuppen und in Bulktrocknung unterschiedlicher Größe gesammelt, und der getrocknete Tabak wurde chemisch analysiert.
Die Ergebnisse der Untersuchung traditioneller Trocknungsbedingungen weisen darauf hin, dass eine hohe, andauernde relative Luftfeuchtigkeit während der gesamten Trocknungsperiode zu hohen TSNA-Konzentrationen im Tabak führt, während ein trockeneres Klima Tabake mit niedrigeren TSNA- und Nitritkonzentrationen hervorbringt. Die Untersuchung zeigt ebenfalls, dass eine hohe, andauernde relative Luftfeuchtigkeit zu Beginn der Trocknungsperiode, gefolgt von einem deutlichen Abfall der relativen Luftfeuchtigkeit zum kritischen Zeitpunkt der Entstehung der TSNA, und zwar wenn die Zellmembran aufgrund des Feuchtigkeitsverlusts aufbricht und der Zellinhalt für nitritbildende Mikroorganismen erreichbar wird, zu Tabaken mit relativ geringen TSNA-Konzentrationen führt.
Eine Kontrolle der Ventilation während des Trocknungsvorgangs ist wichtig, und ein gleichbleibender Luftstrom erhöht die Geschwindigkeit und den Feuchtigkeitsverlust des Tabaks und verringert mögliche Gasphasen-Reaktionen von Alkaloiden und gasförmigen Stickoxiden, die während der Lufttrocknung dunklen Tabaks aus mikrobiell erzeugtem Nitrit entstehen. Trocknungsexperimente in Kleinstschuppen und Trocknungsschuppen zur Bulktrocknung, in denen kontrollierte Bedingungen herrschen und

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eine Verringerung der Feuchtigkeit am Ende der Trocknungsperiode imitiert wird, bestätigen, dass eine Verringerung der Luftfeuchtigkeit während des kritischen Zeitpunkts die Ansammlung von TSNA im getrockneten Tabak reduziert. Hierbei wird eine zufriedenstellende Tabakqualität in kürzerer Zeit als während des traditionellen Trocknungsvorgangs erreicht [Beitr. Tabakforsch. Int. 21 (2005) 321–330]

RESUME

Dans cet article sont présentées des études menées au cours de plusieurs années consécutives, dans lesquelles l'effet du séchage à l'air naturel sur la formation des nitrosamines spécifiques du tabac (TSNA) et la qualité du tabac ont été examinés. Des données sur la température, l'humidité relative, la teneur en eau et l'activité de l'eau ont été recueillies lors du séchage à l'air de tabacs bruns dans des séchoirs conventionnels et des séchoirs en vrac de diverses dimensions, et l'analyse chimique du tabac séché a été effectuée.
Les résultats obtenus de l'étude sur les conditions conventionnelles de séchage suggèrent qu'une humidité élevée continue pendant toute la période du séchage mène à des teneurs élevées en TSNA dans le tabac, alors qu'un climat plus sec produit des tabacs ayant des teneurs en TSNA et nitrite plus basses. L'étude montre également qu'une humidité élevée continue au début du jaunissement suivi d'un déclin prononcé de l'humidité relative au moment critique de la formation des TSNA, c'est à dire au moment où la membrane cellulaire se décompose à cause de la perte d'humidité et le contenu cellulaire devient disponible aux microorganismes générateurs de nitrite, résultent en un tabac ayant des teneurs relativement réduites en TSNA.
Un contrôle des flux d'air est important au cours du séchage et un flux d'air uniforme augmente la vitesse et la quantité de la perte d'humidité du tabac, et réduit des réactions potentielles en phase gazeuse entre les alcaloïdes et les oxydes nitriques, issus du nitrite généré par l'activité microbienne lors du séchage à l'air du tabac brun. Des essais de séchage exécutés dans des petits séchoirs et des séchoirs en vrac sous des conditions contrôlées, permettant de reproduire une réduction de l'humidité à la fin du jaunissement, confirment qu'un déclin de l'humidité au moment critique réduit l'accumulation des TSNA dans le tabac séché, alors que la qualité du tabac est toujours satisfaisante et ceci en moins de temps qu'avec séchage à l'air conventionnel. [Beitr. Tabakforsch. Int. 21 (2005) 321–330]

INTRODUCTION

After harvest, all tobaccos are cured in order to produce a leaf that has appropriate physical and sensory properties and desirable chemical composition. The curing method selected is dependent upon the outcome desired and on the tobacco type. Dark tobacco that is used for production of smokeless products is traditionally air- or fire-cured. In traditional air-curing, the tobacco is normally stalk-cut and hung in a wooden barn. It is cured at ambient temperature and relative humidity (RH) for four to eight weeks depending on the weather conditions. The desired chemical changes that occur include moisture loss, degradation of chlorophyll, starch and sugar, and formation of brown pigments and aroma compounds. An unwanted consequence of the air-curing is formation of tobacco-specific nitrosamines (TSNA) via reaction of tobacco alkaloids with microbially generated nitrite. The latter reaction is highly dependent on ambient conditions during the curing season, particularly on RH, temperature and air-flow. The objective of the present study was to:

a) evaluate the curing conditions during traditional air-curing to achieve a deeper insight into the importance of each of the factors for TSNA formation
b) use that knowledge to design controlled curing regimes that will result in a tobacco having consistently low levels of TSNA and a quality that is acceptable for production of smokeless tobacco.

MATERIAL AND METHODS

Traditional curing

Curing barns: In Princeton and Owensboro, KY, air-curing was performed in traditional wooden barns with one to six tiers. In the Philippines, temporary curing structures (bamboo poles with thatch roof and removable vinyl sidings) were used for the curing. The barns in Italy were traditional outside structures and in France a steel-frame structure covered with transparent plastic was used for the curing.

Data collection: Temperature and RH data were logged by self-contained sensing units, Tinytag Ultra TGU-1500. The units were centrally located inside the curing barns/structures within the tobacco mass and recorded data continuously. In Princeton, units were located at several different tiers and sections of the barn. One Tinytag was located outside each structure to measure ambient conditions.

Controlled curing

Curing facilities: In Owensboro, KY, the curing facility consisted of three mini-curing barns (model Brl 120, inside dimension 1.64 × 2.58 m) manufactured by Vencon-Varsos S.A, Greece. A curing computer (VK 981) monitored and controlled the dry-bulb (DB) and wet-bulb (WB) temperatures of the individual barns, and was set manually according to a pre-determined curing schedule. Introduction of misting water to provide humidity, when WB readings were below set points, was controlled automatically. The fan speed controlling the forced-air ventilation was set manually. All barns were equipped with heat-exchangers.
The curing facility in Reidsville, NC, consisted of twelve micro- and four bulk-curing barns located on the Upper Piedmont Research Station. The micro-curing barns were 0.5 (w) × 0.6 (d) × 2.3 (h) m, and each had the capacity to hold two racks of stalk-cut dark tobacco (32 plants). The bulk-curing barns were standard Taylor barns with the capacity to hold eight boxes. All barns were automated to permit a centralized computer to monitor and control DB and WB temperatures on a programmable schedule. Forced-air ventilation and introduction of misting water to provide humidity at WB set points were controlled by the computer program.

In year 2000, the single bulk-barn was equipped with a 7.5 HP motor and a 22-degree pitch fan blade, which provided 31,800 m^3/h airflow at 0.0254 m static pressure. In 2001, the facilities were extended to contain four bulk-barns. An airflow of 38,200 m^3/h was provided by a 10 HP motor and a 17-degree pitch fan blade in each barn. All barns were equipped with heat-exchangers.

Standard flue-curing boxes were modified to handle stalk-cut tobacco by removing the doors and center divider screen from each box. A length of 5.1 × 5.1 cm angle iron was welded across the top front of each box, spanning from end to end, to strengthen the box and to provide support for the wire mesh. A lateral support bar was welded midway between the two box ends to span from the top rear of the box to the newly added angle iron at the top front. A sheet of wire mesh (0.65 cm diameter rod spaced in a 10.2 × 10.2 cm grid) was welded across the top of each box.

Harvesting and loading of barns: At Owensboro, the tobacco was stalk cut and left to wilt on sticks that were hung on a trailer for two days. Each barn was loaded with 200 plants with a spacing of approximately 0.02 m^2/plant, and produced about 36 kg of cured leaf tobacco.

In Reidsville, 2000, all tobacco was stalk cut and laid on the ground for one day to wilt. At the research station, wire hooks were driven into the tobacco stalks about 5 cm from the lower (cut) end, and 278 plants were hung in each box (approximately 0.01 m^2/plant). The boxes were weighed after filling and upon removal from the bulk barns after the curing was completed. Each barn contained approximately 4100 kg (stalk incl.) of green tobacco and produced about 385 kg of cured leaf tobacco.

In Reidsville, 2001, two bulk barns were used for stalk-cut tobacco, and two bulk barns were used for primed leaf tobacco. For the curing of primed leaves, regular flue-curing boxes were placed on trailers and filled with hand-primed leaves (from the upper two-thirds of the plant) to 160 kg/m^3 (900 kg/box) in the field. After the boxes were weighed, pins were inserted to hold the leaves in place after the boxes were rotated to the upright position. The cured weights were about 1000 kg/barn of cured tobacco.

Curing schedules: The experimental curing schedules were designed for four phases, i.e., yellowing, critical, browning and drying. The yellowing phase was continued for 8 days, at which time most of the leaves had turned brown. The following critical period was designed to reduce the moisture content of the lamina below a certain level within a short time frame so as to severely restrict microbial activity. The subsequent browning and drying phase were devised to allow the development of body and aroma, and complete the drying of the stem.

In Owensboro, 2000, the yellowing phase was continued for eight days, with set points of 29 ˚C and 81% RH. The following 2.5 day critical period was designed to maintain or reduce (at a linear rate) the RH to 81%, 61% and 44% in the three barns, respectively. In 2001, the yellowing phase temperature was set to 33 ˚C and the RH to 82% between day 0–2 and 75% between days 2– 8. The subsequent two-day critical phase was designed to decrease the RH to a set point of 60% in all barns. During the subsequent browning phase (10 days) the temperature was set to 35 ˚C and 44% RH in the daytime. At night the barn doors were opened and the tobacco was exposed to ambient temperature and RH. In 2001, the tobacco was moved to a trailer after 23 days to complete the drying of the stem, and the curing was finished after about 35 days.

At Reidsville, the RH set points were set to cycle during the eight-day yellowing phase from 89% to 70% (2000) or 61% (2001) and back to 89% (in a linear manner) during each 24-hour period to resemble ambient conditions. The temperature set points were 30 ˚C in 2000 and 35 ˚C in 2001. Once the leaves had turned brown, they were dried over a two-day critical period by a distinct decrease (at a linear rate) to a final RH of 54%. During the subsequent browning phase the RH set points were cycled in a range between 45% and 88% during each 24-hour period, for 10 days. The temperature was set to 30 ˚C. The final drying phase was continued at 34% and 30 ˚C for five days, and the total curing time was approximately 26 days.

Data collection: In addition to the DB and WB temperature data collected by the computer during monitoring and control of the curing barns, temperature and RH data were logged by self-contained sensing units, Tinytag Ultra TGU-1500. The Tinytags were centrally located in each barn or curing box, and were inserted within the tobacco mass and measured data on a continual basis. The temperature and RH data in Table 2 represent the average of all the sensors in the boxes/barn.

At Reidsville, 2001, one additional sensor (Vaisala), capable of both monitoring and controlling RH, was placed in each bulk-curing barn near the lower WB temperature thermocouple used to control the RH. This provided a comparison of the WB temperature with the RH sensor itself, and indicated that the sensor could be used to control the RH rather than by the DB and WB temperatures.

Water activity and water content measurements

A Rotronic hygromer® AwV C was used for the water activity analysis. The water content was determined by drying pieces of approximately 2 g tobacco at 110 ˚C for 2 h.

Samples for water activity and water content were taken every day the first 9–10 days of the controlled curing, and then every second or third day until days 25–29. The leaves were separated into lamina and midrib. Samples were taken from the centre of each leaf-half, one for water activity and one for water content. The midrib was cut into pieces (about 2 cm) and every other piece was combined into a sample for water activity measurement. The remaining pieces were combined into a sample for water content measurement. The measurements were performed during controlled curing in micro-and bulk-barns at Reidsville, in 2000 and 2001.

Tobacco varieties

The tobacco cured at Princeton consisted of various dark tobacco types, including the most common commercial dark varieties. In the Philippines, KY 160 was used. In Owensboro, VA 309 was used in both the traditional and controlled curing barns. Dark MT and Malawi Western were cured in the barns in Italy and France, respectively. In

Table 1. Data from Tinytag measurements and concentrations of TSNA and nitrite in tobacco from traditional curings

Location	Tier/barn	RH average (%)	Temp. average (°C)	TSNA [a] (µg/g)	Nitrite [a] (µg/g)
Princeton, 2000	Tier 2	85	24	6.0	16
	Tier 4	78	25	5.0	11
Princeton, 2001	Tier 1	85	22	9.6	78
	Tier 2	83	22	7.0	60
	Tier 3	80	23	6.2	17
	Tier 4	79	23	11.0	67
Princeton, 1998	Tier 2	~73	~25	1.3	1
	Tier 4	~69	~25	1.4	1
	Tier 6	~65	~25	1.1	1
Princeton, 1999	Tier 2	71	24	2.6	3
	Tier 4	67	25	2.8	3
Philippines, 2001	Barn 1	69	28	0.2	nd [b]
	Barn 2	72	30	0.8	nd [b]
Owensboro, 2001	Barn 1	87 / 72 / 70 [c]	23 / 19 / 18 [c]	1.2	2
	Barn 2	84 / 75 / 84 [c]	24 / 25 / 22 [c]	1.7	3
Italy, 2001	Barn 1	76 / 72 / 78 [d]	18 / 19 / 19 [d]	1.1	3
	Barn 2	77 / 59 / 77 [d]	21 / 20 / 16	0.1	3
France, 2001	Barn 1	77 / 79 / 84 [d]	16 / 13 / 16 [d]	0.1	5

[a] Average values reported on dry weight basis.
[b] Not determined.
[c] Days 1–12 / 12–18 / 18–45.
[d] Days 1–16 / 16–20 / 20–45.

Reidsville, NC, the tobacco varieties KY 171 and McNair 944 were used in year 2000 and VA 359 in year 2001. Cultural practices commonly used for dark tobacco production were used at all locations.
The controlled cured tobaccos were evaluated for the usability for production of smokeless products.

Sampling

All samples for chemical analysis were taken at the end of curing. Representative whole leaf samples were pulled from each section in the traditional barns or from each box/barn in the controlled curing barns and combined into one sample before grinding.

TSNA analysis

The tobacco-specific *N*-nitrosamines, *N*-nitrosonornicotine (NNN), *N*-nitrosoanatabine (NAT), *N*-nitrosoanabasine (NAB), and 4-methyl-*N*-nitrosoamino-1-(3-pyridyl)-1-butanone (NNK), were analysed according to SPIEGELHALDER *et al.* (1). The results were reported as the sum of the four *N*-nitrosamines as total TSNA; detection limit: 0.1 µg/g.

Nitrite analysis

The nitrite concentrations were analysed according to the ISO Standard 13395 (July 1996) and reported as nitrite-ion; detection limit: 1 µg/g.

RESULTS AND DISCUSSION

Previous studies on the mechanisms of TSNA formation have shown that green tobacco contains virtually no TSNA. These compounds are formed during curing and are derived from the reaction of tobacco alkaloids and nitrite-derived species (2,3). According to the current hypothesis, which is based on several experimental studies on air-curing, nitrite is formed after about two to three weeks of traditional air-curing, i.e. at the end of the yellowing stage when the leaf turns brown (3–5). This is the time when the plant cell membranes break down due to moisture loss and the cell contents become available to micro-organisms that naturally exist on or in the tobacco leaf, and for chemical reactions that lead to TSNA formation (5,6). These reactions include microbial reduction of nitrate to nitrite and chemical nitrosation of the alkaloids by nitrite and other NO_x compounds. We define this time period as the critical period during air-curing. If the moisture loss from the tobacco is rapid and complete soon after end of yellowing, i.e., the critical period is shortened, accumulation of TSNA should be minimized. On the other hand, if the moisture loss period is lengthened, the conditions become favourable for microbial growth and the production of nitrite may be enhanced. As a result, higher levels of TSNA may be formed. Results of a study of environmental parameters such as rainfall, temperature and humidity on the accumulation of TSNA during air-curing of tobacco in Kentucky, USA, for three consecutive years support this view (7). These studies show that high humidity during a curing season, especially at the end of the yellowing period, which is a critical time for the formation of nitrite, resulted in tobacco with significantly higher levels of TSNA than tobacco that was cured in a drier year. Thus, implementation of curing procedures in which the time for microbial growth is reduced would hence be a means to prevent or at least reduce the formation of TSNA. Support for this view was provided by a study that showed that quick drying of tobacco at the end of yellowing at 70 °C resulted in tobacco with significantly lower levels of TSNA than conventionally cured tobacco, which confirm the current hypothesis of

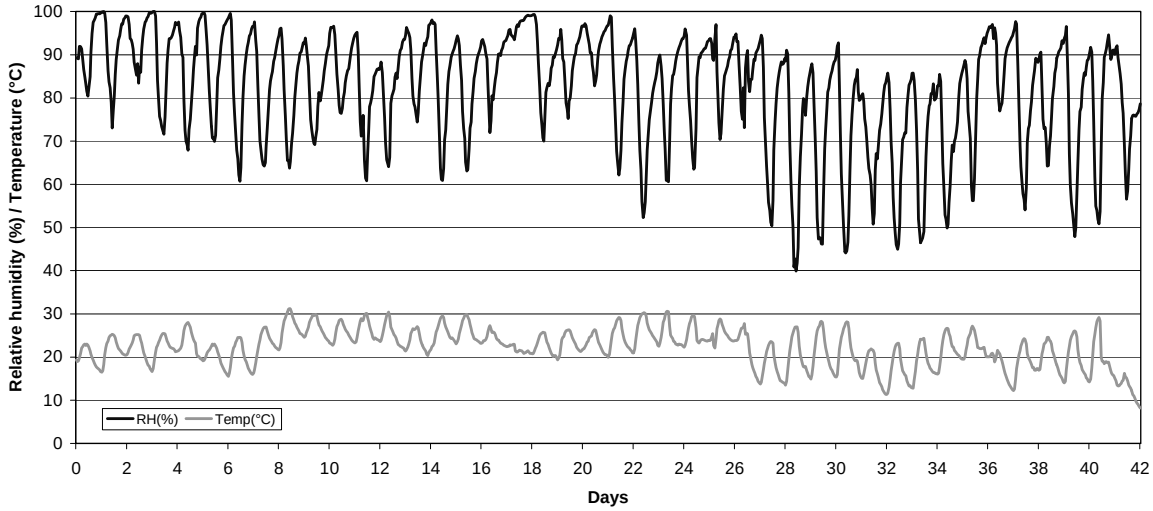


Figure 1. Traditional curing in Princeton, KY, 2001 (average of all tiers)

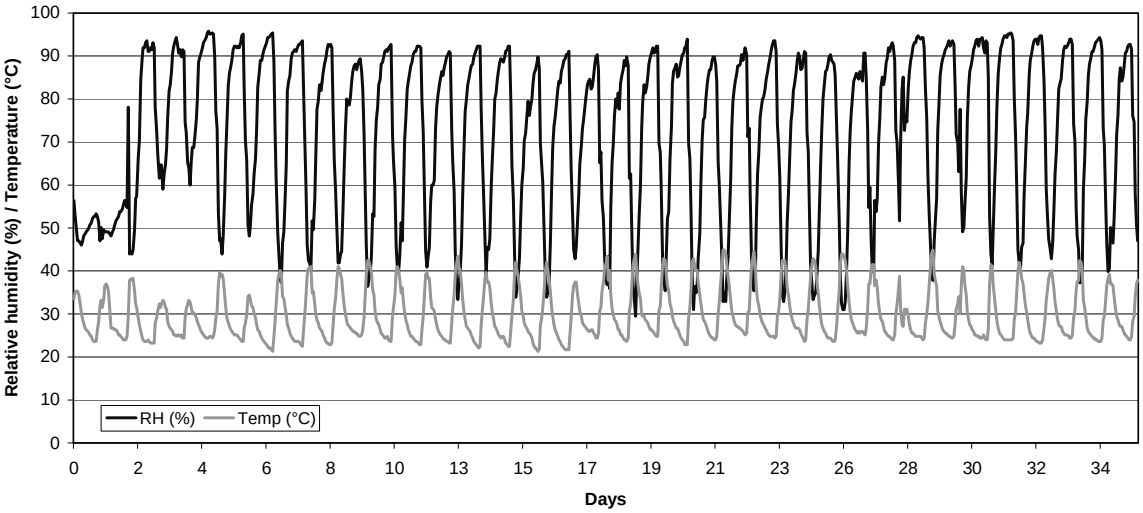


Figure 2. Traditional air-curing in the Philippines, 2001 (Barn 2)

TSNA formation (3). However, the quick-dried tobacco was not evaluated for usability in a commercial product. Also, attempts to improve curing conditions by modifying the plant or by introducing different versions of "field structures" designed to speed up the moisture loss in tobacco during curing have been made (8). Use of these techniques has reduced TSNA levels in tobacco to some extent but the curing process was still found to be dependent on ambient conditions.

Traditional curing

Results from the present study of temperature and RH during curing in traditional air-curing barns, and chemical analysis of the cured tobacco are presented in Table 1 and also exemplified in Figures 1 to 4. Relative humidity and temperature data collected from a traditional air-curing barn in Princeton, KY, during seasons 2000 and 2001, showed average continuous RH between 78–85% and average temperatures between 22–25 ˚C for the whole curing period resulting in TSNA and nitrite levels between 5–11 µg/g and 11–78 µg/g, respectively, in the cured tobacco. Temperature and RH inside the barn are illustrated in Figure 1.

A drier climate with average continuous RH between 65–73% and average temperatures around 25 ˚C was observed during the 1998 and 1999 curing seasons in the same curing barn in Princeton. The TSNA and nitrite levels ranged between 1.1–2.8 µg/g and between 1–3 µg/g, respectively (see Table 1). In the Philippines, 2001, where temporary curing structures (bamboo poles with thatch roof and removable vinyl sidings) were used, the RH values were similar to those in Princeton, i.e, 69–72%, but temperature slightly higher, 28–30 ˚C. The resultant TSNA level was between 0.2–0.8 µg/g (nitrite was not determined) (see Figure 2 and Table 1).

In two traditional air-curing barns in the Owensboro area, a high continuous RH in the beginning of the yellowing phase followed by a pronounced decline when the leaves turned brown, i.e, at the critical period, and a subsequent aroma developing browning phase (Figure 3), resulted in good quality tobacco with TSNA levels of 1.2 and 1.7 µg/g

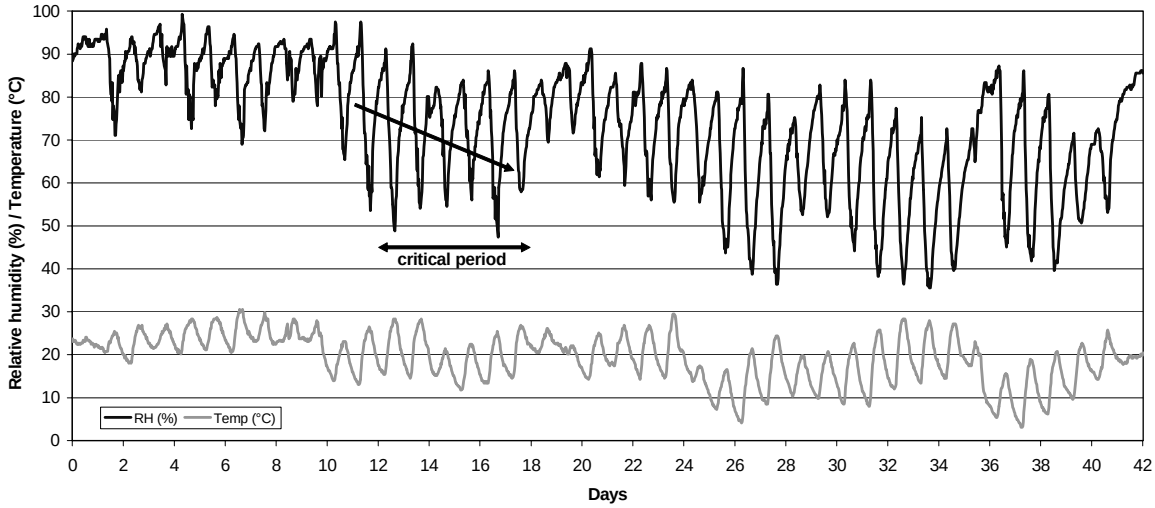


Figure 3. Traditional air-curing in Owensboro, KY, 2001 (barn 1)

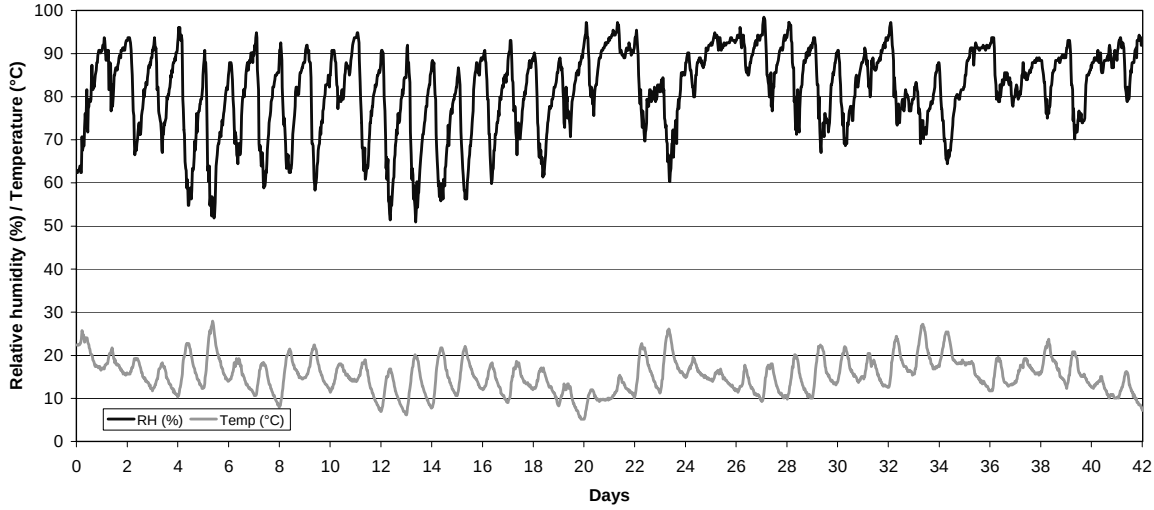


Figure 4. Traditional air-curing in France, 2001

and nitrite concentrations of 2 and 3 μg/g, respectively. This is also exemplified in Table 1, by two curing barns in Italy (2001), where barn 2, with a distinct decrease in RH after 16 days in the yellowing phase, i.e, at the critical period, exhibited lower levels of TSNA in the cured tobacco than barn 1 with the lesser decline. The importance of temperature is clearly demonstrated in Figure 4 for a curing structure in France (2001). An average continuous RH of approximately 80% with no decline during the critical period and an average temperature during curing around 16 ˚C still resulted in extremely low levels of TSNA (0.1 μg/g).

The present screening study of the traditional curing conditions during several consecutive years supports the idea that high continuous RH during the entire curing period results in high TSNA levels in the tobacco, while a drier climate produces tobacco with lower amounts of TSNA and nitrite. Additionally, the current study shows that a high continuous RH at the beginning of the yellowing phase followed by a pronounced decline of RH at the critical period of time for TSNA formation results in tobacco with relatively low TSNA levels. Also, tobacco cured at low temperatures (around 16 ˚C) but at relatively high RH accumulates very low levels of TSNA. This is most probably due to a reduced bacterial activity at that low temperature. Low curing temperatures, however, are not common in tobacco producing countries. On the contrary, the average temperature during the curing period is usually above 20 ˚C, and therefore our further curing studies with controlled curing were focused on the decline of the RH at the critical period.

Controlled curing

Curing experiments with the objective to introduce a pronounced decline of humidity at the end of yellowing have been performed in mini- and bulk-curing barns with controlled conditions during curing seasons 2000 and 2001. To reduce the accumulation of TSNA, curing schedules were developed containing a distinct decline in RH during the critical period. In a Japanese study of burley tobacco in plastic curing chambers, the RH was alternated between

Table 2. Data from Tinytag measurements and concentrations of TSNA and nitrite in tobacco from controlled curings [a]

Location [b]	Barn	RH average (%)			Temp. average (°C)	TSNA (µg/g) [c]	Nitrite (µg/g) [c]
		Yellowing	Decline [d]	Browning [e]			
			Mini-barns				
Owensboro, 2000 (sc)	Barn 1	90	90⇒65	68	29	2.3	4
	Barn 2	87	85⇒61	68	29	2.7	5
	Barn 3	87	85⇒55	68	29	2.0	4
Owensboro, 2001 (sc)	Barn 1-3	75	60	62	31	0.7	2
			Bulk-barns				
Reidsville, 2000 (sc)	Barn 1	69 [e]	70⇒35	47	30	1.0	8
Reidsville, 2001 (sc)	Barn 1 (cure 1)	65 [e]	80⇒50	57	33	1.3	2
	Barn 2 (cure 1)	63 [e]	78⇒50	58	32	1.4	2
	Barn 2 (cure 2)	77 [e]	82⇒51	60	32	4.9	2
	Barn 3 (cure 2)	76 [e]	83⇒48	58	33	4.6	2
Reidsville, 2001 (pr)	Barn 1 (cure 2)	81 [e]	83⇒48	59	33	6.4	4
	Barn 4 (cure 2)	73 [e]	72⇒32	45	33	3.5	2

[a] For details see Materials and Methods.
[b] sc = stalk cut, pr = primed.
[c] Average values reported on dry weight basis.
[d] Absolute values.
[e] Cycling average.

60% and 80% at two-hour intervals during the yellowing and browning phase to resemble natural curing conditions. The study showed that alternate and repeated dehydration and moistening (cyclic curing) produced cured leaves with a higher apparent quality compared to leaves cured in a conventional plastic curing barn without RH cycling. Also, the levels of solanone and norsolanadione, which are markers for generation of aroma compounds, were higher during cycling than during constant RH (9).

The present study, conducted both in Owensboro and Reidsville, had the objective to mimic the normal day and night cycles and allow the natural development of body and aroma of the tobacco. The RH was then cycled in a linear manner on a 24-h basis during the browning phase. In the large bulk-curing barns in Reidsville, cycling was also performed during the yellowing phase. The results from RH and temperature measurements, and the results from chemical analyses of tobacco cured in bulk-curing structures of different sizes are summarized in Table 2.

In Owensboro, experiments in mini-sized bulk barns (approximately 1/6 the size of a regular flue-curing barn) were performed in year 2000. Following the yellowing period of eight days, a decline in RH from ~85% to ~60% during a 2.5 day critical period before the browning phase resulted in cured tobacco with TSNA levels between 2–3 µg/g and nitrite concentrations around 4 µg/g. In the experiment performed in 2001, the yellowing phase RH had an average of ~80% for the first four days with a slow decline to 60% during the next four days. The average RH for the eight-day period was approximately 75%. The RH was maintained at 60% during the two-day critical period before the browning phase. This curing scheme produced tobacco containing only 0.7 µg/g TSNA and 2 µg/g of nitrite.

In Reidsville, NC, experiments were conducted in bulk-curing barns adapted for air-curing of stalk-cut tobacco. The RH was cycled during both the yellowing and browning phases. In 2000, after a yellowing period of eight days with an average RH of 69%, a two-day decline from ~70% to 35% followed by a ten-day browning phase gave cured tobacco containing around 1 µg/g TSNA and 8 µg/g nitrite. In 2001, the curing experiments were performed in two barns and during two consecutive curing periods. In cure 1, the average RH during the 8.5 days of yellowing was approximately 65% in both barns. This was followed by a decline in RH from ~80% to 50% in both barns over a two-day period, and then diurnal cycling between 72% and 45% RH during the browning phase (Figure 5). The cured tobacco from barns 1 and 2 contained 1.3 and 1.4 µg/g TSNA, respectively. The nitrite concentrations in the tobacco in both barns were 2 µg/g. In the second cure, the average RH during yellowing was about 77% in both barns. A two-day decline from ~82% to ~50% followed by a browning phase, produced tobacco with a TSNA level of 4.9 and 4.6 µg/g in barns 2 and 3, respectively. The nitrite levels in the tobacco from both barns were 2 µg/g. The relatively higher levels of TSNA in the second cure was unexpected, but may be explained by presence of nicotine to nornicotine converter plants (unpublished data).

An air-curing experiment using primed dark tobacco packed in boxes commonly used during flue-curing was also conducted in Reidsville in 2001. In barn 1, a yellowing period with an average of 81% RH was followed by a decline from 83% to 48% during the critical phase, and an average of 59% during the browning phase. This produced cured tobacco with 6.4 µg/g TSNA and 4 µg/g nitrite. In barn 2, the RH during yellowing averaged 73% and declined from 72% to 32% during the critical phase followed by an average of 45% during the browning phase. This produced tobacco with 3.5 µg/g TSNA and 2 µg/g nitrite. A limited distribution of air through the tightly packed primed leaves during the air-curing process in the bulk barn may have contributed to the unexpectedly high TSNA levels in the cured tobacco. Support for this view is provided by the fact that a uniform airflow increases the rate and uniformity of moisture loss from the tobacco, and proper space and ventilation generally produces tobacco with lower amounts of TSNA than tobacco confined in close proximity (10). Also, it cannot be excluded that gas-phase reactions via gaseous oxides released from nitrite in the tobacco leaves may affect the amount of TSNA in the air-cured tobacco, if the ventilation is insufficient. Support

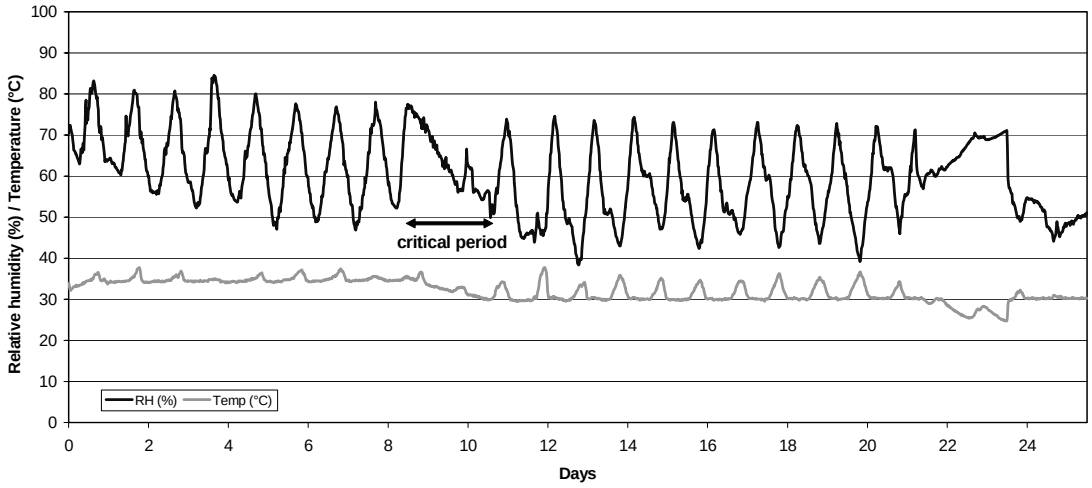


Figure 5. Controlled curing in Reidsville, NC, 2001 (barn 1; cure 1)

for this view has been provided by SAITO *et al.* (11), who showed that TSNA formation in burley tobacco by heat treatment in a sealed container was caused by reaction of alkaloids with NO_x gas that was formed from nitrite already existing in the lamina.

It should be noted that the air-curing process in a bulk-curing barn differs significantly from flue-curing in a bulk-curing barn, with respect to temperature parameters. This has the consequence that a properly conducted flue-curing occurs with virtually no bacterial involvement. In fact, NESTOR *et al.* have shown that the formation of TSNA in flue-cured tobacco derives mainly from reaction between combustion by-products (i.e., oxides of nitrogen) and tobacco alkaloids (12). All bulk barns used in this study were equipped with heat exchanger on the burner, which eliminate gas-phase nitrosation due to combustion by-products.

The time window for the critical period was investigated in the bulk-curing barns and also in the micro-barns. The results from the water content and water activity measurements in the lamina and in the midrib during controlled curing in micro- and bulk-barns from experiments conducted at Reidsville in 2000 and 2001 are presented in Figures 6 and 7. The figures represent the average of four different cures. As can be seen from Figure 6, the water content decreased much more rapidly from the lamina than from the midrib. Assuming that the cell membrane breakage, and thus leakage of the cell content into the intercellular spaces, occurs when the water content in the tobacco is below 70% (13), the cell membrane breakage in the lamina occurred on average after three days of curing. The water activity limit for the growth of most bacteria, 0.9 (14), was reached after five days, leaving two days for microbial activity on average. In the midrib the cell membrane breakage occurred after 11 days and water activity declined to under 0.9 after 14 days. This shows that the curing schedules used in the experiments resulted in a rather short window of time for bacterial growth after the cell breakage, both in the lamina and the midrib, and thus for nitrite and TSNA formation. Accordingly, nitrite in the cured tobacco from different cures ranged between 1 and 39 µg/g and TSNA between 0.6 and 1.8 µg/g. These results also show that lamina and midrib are two different matrices that lose moisture and reach the "safe" water activity at different rates. Considering the fact that the concentration of alkaloids, one of the precursors of TSNA formation, is about five times higher in the lamina than in the midrib (15), it is important to dry out the lamina as quickly as possible at the critical time for nitrite formation in order to minimize the amounts of nitrite being formed. Analyses of traditionally air-cured commercial tobaccos during the years have shown that the contribution of lamina TSNA is greater in tobacco cured in a dry year than in tobacco cured in a wet curing season. In a wet curing season when drying of midrib is prolonged, substantial amounts of nitrite are formed and the levels of TSNA are usually significantly higher in the midrib than in the lamina.

The quality of the tobaccos cured under controlled conditions was in general somewhat lower than the quality of tobaccos cured in traditional barns. Nevertheless, the controlled cured tobaccos were deemed usable for the production of smokeless products.

CONCLUSIONS

The curing experiments in the bulk-barns confirm that a controlled decrease in relative humidity at the critical period reduces the accumulation of TSNA in the cured tobacco, while still producing a satisfactory tobacco quality in a shorter time than traditional air-curing. In year 2001, curing experiments utilising primed leaves showed promising results, although the TSNA level in the cured tobacco was somewhat higher than expected. One factor that could have influenced the TSNA level negatively is the poor distribution of air through the tightly packed primed leaves in the boxes. Due to insufficient ventilation, it cannot be excluded that gas-phase reactions via gaseous oxides formed from nitrite in the tobacco leaves affected the amount of TSNA in the cured tobacco.

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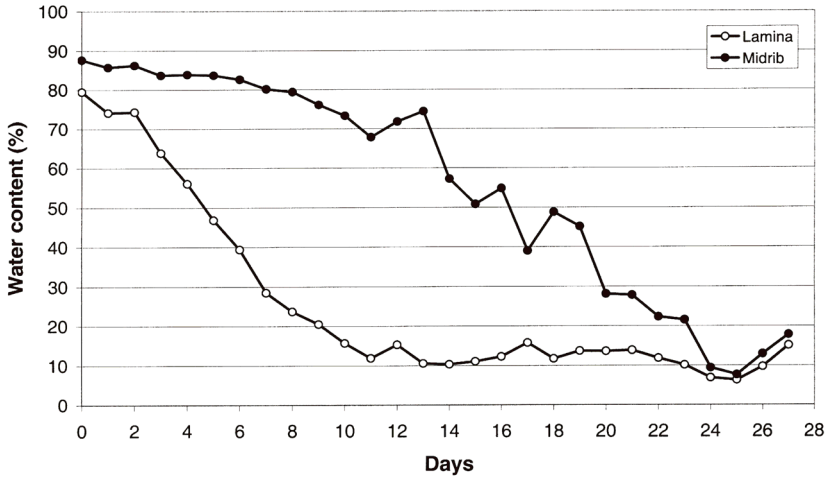


Figure 6. Water content in lamina and midrib during controlled curing in micro- and bulk-barns 2000 and 2001. The values represent the average from four cures.

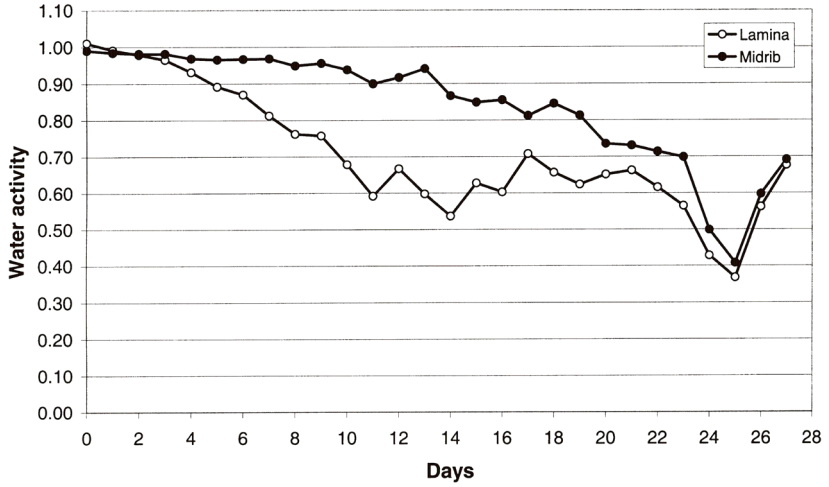


Figure 7. Water activity in lamina and midrib during controlled curing in micro-and bulk-barns 2000 and 2001. The values represent the average from four cures.

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