

POST-HARVEST TREATMENT AND THE ACCUMULATION OF NITRITE AND *N'*-NITROSONORNICOTINE IN BURLEY TOBACCO

R.A. ANDERSEN & M.J. KASPERBAUER

*United States Department of Agriculture, ARS, Department of Agronomy, University of
Kentucky Lexington, Kentucky 40546, USA*

SUMMARY

Concentrations (dry-weight basis) of nitrate, nitrite and *N'*-nitrosonornicotine (NNN) in Burley tobacco were determined during successive processing stages of experimental homogenized-leaf-cured (HLC) material, after conventional air curing and during prolonged storage ('ageing') of HLC and air-cured tobaccos. During homogenized leaf curing, < 6 µg/g nitrite-N and < 10 µg/g NNN were found in tobacco frozen immediately after aerobic incubation of homogenates at 40 °C for 0, 4, 8, 20 and 25 h. Up to 550 µg/g nitrite-N and 850 µg/g NNN occurred in tobacco incubated similarly for 20 h, then allowed to stand 1 h without aeration. Samples of two genetic Burley lines of high and low alkaloid content were similarly incubated, allowed to stand 1 h, dried and 'aged' for up to one year in partially anaerobic environments. NNN contents were positively correlated with 'at-harvest' alkaloid content, and NNN increased at each subsequent stage of processing, reaching a maximum of 1 800 µg/g in the high-alkaloid line after one year of 'ageing'. Small increases of NNN that reached a final concentration of 50 µg/g occurred in tobaccos that were air-cured, then 'aged'.

INTRODUCTION

Nitrogenous constituents in Burley tobacco are influenced by post-harvest curing and ageing processes (Enzell & Wahlberg, 1980; Long & Weybrew, 1981). Investigations concerning carcinogenic nitrosamine alkaloids and 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone in tobacco and tobacco smoke have been reported (Hecht *et al.*, 1979; Hoffmann & Adams, 1981).

An investigation of the effects of controlled-environmental storage ('ageing') on components in air-cured and experimental homogenized-leafcured (HLC) Burley tobacco indicated that *N'*-nitrosonornicotine (NNN) content increased during storage (Andersen *et al.*, 1982). The HLC process involves successive homogenization of 'ripe' leaves, incubation and dehydration (Tso *et al.*, 1975). After 52 weeks of post-cure storage, NNN concentrations were 200 to 300 times higher in HLC than in air-cured tobacco. Prior to 'ageing', nitrite and NNN levels were much higher in HLC tobacco, whereas nitrate concentrations were similar for both cures. Nitrate and nitrite are precursors of *N'*-nitroso compounds derived from endogenous tobacco alkaloids, such as nicotine and nornicotine. The origin of high levels of nitrite in HLC tobacco

remains unknown. However, we suspect that activation of nitrate reductase from bacteria (Hamilton *et al.*, 1982) or disrupted leaf cells may occur during processing.

The purpose of this investigation was to study post-harvest practices that affect nitrite and NNN accumulations in tobaccos with genetically-varied alkaloid content.

MATERIALS AND METHODS

Plant materials, curing and storage

Burley tobacco genotypes with different alkaloid contents were field-grown to maturity at Lexington, Kentucky. Three studies were carried out, each in a different year. In Study I, a high-alkaloid line (Collins *et al.*, 1974), a nicotine to nornicotine-converter line and the cultivar Ky 14 were grown and homogenized-leaf-cured by the following successive steps: 'ripe' leaves homogenization slurry stage incubation, 40°, aerobic, post-incubated stage standing period (partially anaerobic), post-incubated-after standing stage air dry at <55°, air-dried stage prolonged storage (partially anaerobic), 'aged' tobacco stage. The HLC procedure was used as before (Andersen *et al.*, 1982), except that the maximum incubation was 25 h. In Study II, the three tobaccos in Study I and a low-alkaloid line (Collins *et al.*, 1974) were grown and homogenized-leaf-cured (Andersen *et al.*, 1982). In Study III, the low-alkaloid and the high-alkaloid lines were air-cured and homogenized-leaf-cured. One portion of air-cured tobacco was dried (in the same dryer used to dry HLC tobacco) to 3–4% moisture (re-dried) and another was not re-dried. Air-cured tobaccos were cut into strips and 1-kg lots of both air-cured and HLC tobaccos were used for controlled-environmental storage ('ageing') at 12% moisture (wet weight), at either 20° or 30°, for up to 1 year in the manner previously reported (Andersen *et al.*, 1982).

Sampling procedures

In Study I, samples were taken immediately after the chopping-homogenization (slurry) stage and after 4, 8, 20 and 25 h incubation. In Study II, samples were taken immediately after homogenization, then after incubation was completed and the homogenate was allowed to stand 1 h without aeration and, finally immediately after drying. Samples were freeze-dried and stored at -70° until analysed. Cured tobaccos in Study III were taken at the start of 'ageing' and after 3 days and 1, 3, 10, 20, 30, 40 and 52 weeks. Samples were stored at -70° until analysed.

Chemical analyses

Total alkaloids, nitrate-N and nitrite-N were determined as previously described (Andersen *et al.*, 1982). *N*-Nitrosornicotine (NNN) was determined either by high-performance liquid chromatography-thermal energy analysis, by modification of the Hecht *et al.* procedure described by Andersen *et al.* (1982), or by the following gas chromatographic-nitrogen-phosphorus detector (NPD) procedure. A 0.1–0.5-g sample was extracted at room temperature for 45 min with 3.0 mL saturated barium hydroxide, 0.15 g barium hydroxide and 10 mL ethyl acetate. A 92- μ L aliquot of the upper layer was mixed with 8- μ L of an ethyl acetate solution containing 4 μ g azobenzene as internal standard. A 0.5- μ L aliquot was injected into a Hewlett-Packard 5880A GC with a fused silica 30 m \times 0.25 mm i.d. SE-54 column of 0.25 μ m film-thickness. The GC was operated using a splitless/split injection technique with inlet at 220°C and the detector at 270°C, inlet purge flow at 1.2 mL/min, He carrier linear velocity at 31 cm/s, inlet septum purge inactivation time of 35 s and initial column temperature of 100°C. After

1 min, the oven was temperature-programmed at 4 °C/min to 220 °C, then maintained at 220 °C for 20 min. Quantification was carried out by means of internal standards, using authentic compounds for calibration. GC-mass spectrometer analyses were employed to verify peak identities in samples. A Hewlett-Packard Model 5985A system in the electron-impact mode at 70 eV was used with a 30 × 0.31 mm fused-silica SE-54 capillary column at 300 °C, He carrier gas (linear flow rate, 30 cm/s), inlet and flame-ionization detector temperatures of 300 °C and 2,4'-dipyridyl as internal standard.

RESULTS AND DISCUSSION

Slurry stage of HLC tobacco (Studies I and II)

Immediately after homogenization, nitrate-N contents were less than 6.0 g/kg and nitrite-N was less than 1.4 mg/kg for all the tobacco genotypes, with no appreciable differences in amounts among them. NNN was present at less than 8 mg/kg for all genotypes, except that none was detected in the low-alkaloid line.

Incubation of the HLC slurry (Study I)

Nitrate-N contents in Ky 14 or in the alkaloid lines did not vary significantly with tobacco type or incubation time. Nitrite-N levels doubled between 4 and 25 h of incubation. NNN ranged from 3–9 mg/kg and did not vary appreciably with genotype or incubation time.

The absence of significant increases of NNN in the HLC materials indicated that nitrosation did not occur during incubations, *per se*. Since concomitant increases in nitrite did not result in elevated NNN levels, there may have been insufficient time for nitrosation of nicotine or nornicotine by the scheme cited by Enzell and Wahlberg (1980). The slurry pH values were 4.7–5.3; slight decreases (0.1–0.6 pH units) occurred during incubations.

Post-incubated HLC tobacco after a standing period (Study II)

Nitrate concentrations in the three alkaloid lines remained unchanged during the post-incubation 1 h standing period. Nitrate-N levels after the standing period ranged from 0.4–2.0 g/kg.

After a 1-h standing period (without forced aeration) which followed incubation, large increases in nitrite occurred compared with non-incubated tobaccos (Fig. 1). Nitrite-N increases in the genotypes were observed in the following order: Ky 14 (× 41) < low-alkaloid line (× 82) < high-alkaloid line (× 87) < nornicotine-converter line (× 322). The greatest elevations in nitrite levels apparently occurred during the post-incubation standing period. Nitrite is formed by reduction of endogenous nitrate, mediated by nitrate reductase (Fig. 2). Fresh tissues of plants contain nitrate reductase, and this may be responsible for nitrite formation in homogenates. Induced activities of dissimilatory nitrate reductase in tobacco bacterial flora under anaerobic conditions during processing may also contribute to nitrate reduction in leaves (Hamilton *et al.*, 1982).

The genotype-dependent increases in NNN concentrations, observed after the start of incubation, were greatest in the nornicotine-converter line (Fig. 1). It can be inferred from Study I that elevations in NNN did not occur during the aerobic incubation. The brief standing period (without forced aeration) following incubation apparently provided conditions for nitrite formation and nitrosation of nicotine and nornicotine. Our quantitative results seem consistent with the knowledge that conversion of nornicotine to NNN requires fewer metabolic steps than does that of nicotine to NNN (Hecht *et al.*, 1979).

Fig. 1. *N'*-Nitrosornicotine and nitrite-N concentrations at post-slurry stages of homogenized-leaf-cured (HLC) tobacco processing. ○, low alkaloid; □, high alkaloid; ●, nornicotine converter; ■, Ky 14

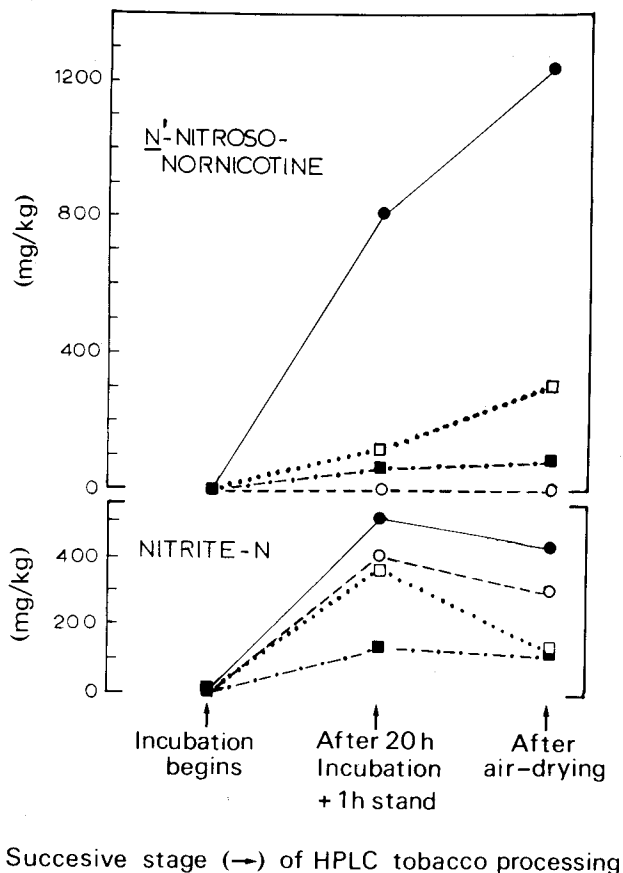
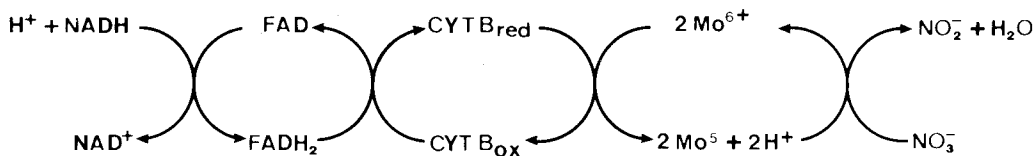


Fig. 2. Postulated scheme of electron transport in a nitrate reductase system during post-harvest processing of tobacco (adapted from Hageman & Reed, 1980)



Air-dried stage of HLC tobacco (Study II)

No appreciable change in nitrate content occurred during drying, but nitrite decreased in all genotypes (Fig. 1). The following nitrite changes occurred: low-alkaloid line, -25%; high-alkaloid line, -61%; nornicotine-converter line, -15%; and Ky 14, -16%. Drying may

inhibit bacterial growth and nitrite formation; pre-formed nitrite might decrease after chemical reaction.

Further NNN increases occurred during drying. Genotype influences paralleled those of the preceding stage, and the greatest NNN content was reached in the nornicotine converter (1 240 mg/kg), with lesser amounts in Ky 14 and the high-alkaloid line and none in the low-alkaloid line.

Prolonged storage ('ageing') of HLC and air-cured tobacco (Study III)

Nitrate-N contents in HLC tobaccos before storage were 5.5–8.5 g/kg, and those in air-cured tobaccos of the same genotype were 8.0–9.5 g/kg. All tobaccos contained less nitrate after 52 weeks of storage, and net changes were: HLC low-alkaloid, –33%; HLC high-alkaloid, –50%; air-cured re-dried low-alkaloid, –29%; and air-cured re-dried high-alkaloid, –20%. Smaller decreases occurred in non-re-dried air-cured tobaccos. Losses in nitrate during 'ageing' contrast with apparent increases of nitrate that occur during conventional curing (Long & Weybrew, 1981).

Nitrite in the high-alkaloid HLC tobacco gradually decreased during storage and fell at a faster rate for tobacco stored at 30°C than for that at 20°C (Fig. 3); nitrite-N in the low-alkaloid line increased slightly to about 10 mg/kg. Air-cured and air-cured re-dried tobaccos showed 6- to 15-fold elevations of nitrite after 52 weeks and, in 30°C environments,

Fig. 3. Comparison of nitrite-N contents in HLC alkaloid lines during 'ageing' at different temperatures at 12% moisture

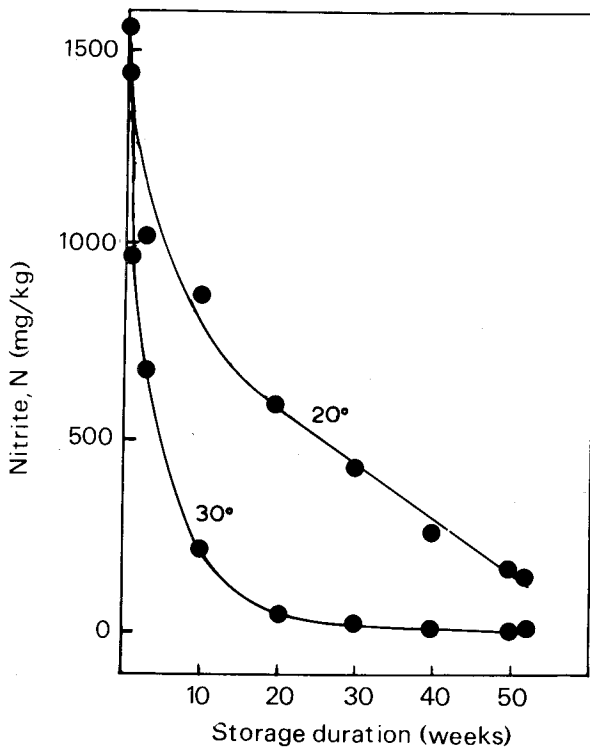
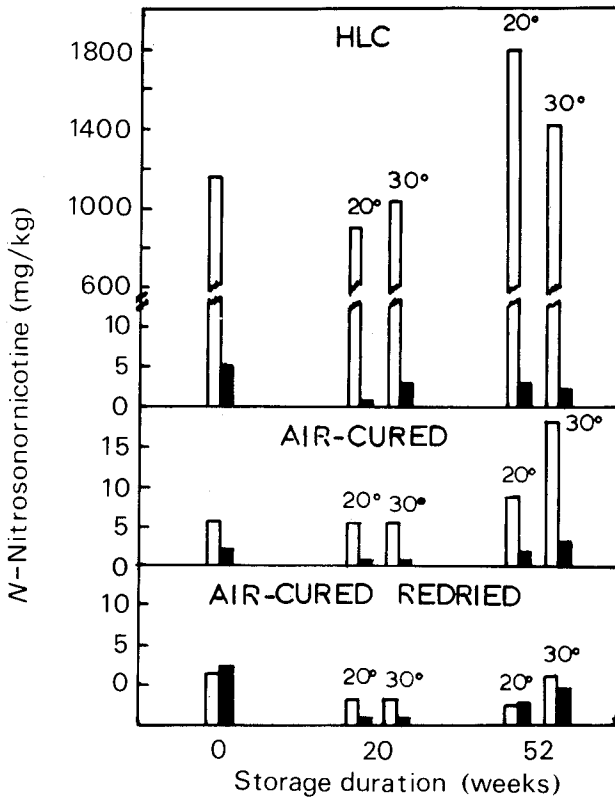


Fig. 4. Effects of storage duration at 12% moisture, temperature and tobacco alkaloid genotype on *N*-nitrosornnicotine (NNN) contents in post-cured tobacco; □, high alkaloid; ■, low alkaloid



these were twice as high as in 20 °C environments; however, maximum levels reached only 5–15 mg/kg. These elevations contrasted with the small nitrite decreases we reported for Ky 14 air-cured tobacco during ‘ageing’ (Andersen *et al.*, 1982).

At the start of storage, NNN in the low- and high-alkaloid lines was less than 8 mg/kg for each method of curing, except that it was much higher in the HLC high-alkaloid line (Fig. 4). After 20 weeks, NNN contents in the variously cured batches of low-alkaloid line were < 3 mg/kg at either temperature; the contents in the air-cured and air-cured re-dried high-alkaloid line tobaccos were < 6 mg/kg. In contrast, the concentrations remained much higher in the HLC high-alkaloid line, *viz.*, about 1 g/kg. The longest ‘ageing’ period did not result in significant changes of NNN contents among the cured low-alkaloid isolate tobaccos, which remained at or below 5 mg/kg. NNN contents in the HLC and air-cured high-alkaloid line were higher after ‘ageing’ for 52 weeks than after 20 weeks. No corresponding increase occurred in air-cured re-dried tobacco. Increases in NNN (compared to 0-time values) of 36 and 18% were seen for HLC high-alkaloid tobacco stored at 20 °C and 30 °C, respectively, and 36 and 71% for air-cured high-alkaloid tobacco at 20 °C and 30 °C. The maximum NNN content (1 800 mg/kg) in the HLC high-alkaloid line stored for 52 weeks at 20 °C contrasted with the highest content of the air-cured counterpart of this line, *viz.*, 18 mg/kg after 52 weeks at 30 °C. NNN

contents in cured and 'aged' high-alkaloid tobaccos were generally higher at corresponding stages of post-harvest processing than those reported earlier for Ky 14 tobacco (Andersen *et al.*, 1982).

CONCLUSION

NNN accumulation depended on alkaloid genotype and occurred mainly during the following processing steps: standing period following incubation, forced-air drying and prolonged storage ('ageing') for HLC tobaccos and 'ageing' for air-cured lines. The greatest increases of nitrite contents in HLC tobaccos occurred during the standing period following homogenate incubation. Presumably, reduction of nitrate during this same period was fostered by partially anaerobic conditions in the post-incubated homogenates. Nitrite formation *via* nitrate-reducing bacteria or tobacco nitrate reductase seems probable. Nitrate and nitrite decreases during the forced-air drying of HLC, and 'ageing' of HLC and air-cured tobaccos can be partially accounted for by increases in NNN. The results of this investigation may provide information needed to lessen nitrosamine contents in tobacco.

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