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Tobacco-Specific Nitrosamine Accumulation and Distribution in Flue-Cured Tobacco Alkaloid Isolines

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Changes in three tobacco-specific nitrosamines (TSNA) and their alkaloid precursors during growth and the curing process were quantified in seven NC 95 flue-cured isolines with different alkaloid levels. *N'*-Nitrosonornicotine (NNN), *N'*-nitrosoanatabine (NAT), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were separated by gas chromatography and measured with a thermionic N-P detector. Mature green leaves contained small amounts (0.6-1.5 $\mu g\ g^{-1}$) of TSNA that increased (0.9-17.8 $\mu g\ g^{-1}$) during curing. Leaves from higher stalk positions and leaves with increased time in the curing process had greater amounts of TSNA. Among the alkaloid isolines, highest significant correlation coefficients between a TSNA and the related alkaloid precursor were 0.95 and 0.76 for the correlations between nornicotine with NNN and anatabine with NAT, respectively. The correlation between nicotine and NNN (r = 0.40) was not significant.

Tobacco-specific nitrosamines (TSNA) in flue-cured tobaccos have not been as extensively studied as they have in other tobaccos and tobacco products. Hecht et al. (1977) quantified (1.31 $\mu g\ g^{-1}$ average) *N'*-nitrosonornicotine (NNN) in Coker 139, a flue-cured genotype, and reported that there was no definitive trend between TSNA and leaf stalk position. Chamberlain and Arrendale (1983) analyzed NNN content in five flue-cured genotypes plus TI-1112 and concluded that there was no significant correlation between NNN levels (70-1000 $\mu g\ g^{-1}$) and the levels of the related alkaloid precursors nornicotine (600-31 400 $\mu g\ g^{-1}$) or nicotine (4000-43 800 $\mu g\ g^{-1}$). Chamberlain et al. (1984) concluded on the basis of effects of nitrogen fertilization on NNN accumulation that there was no significant correlation between nicotine/nornicotine ratios and the levels of NNN in different genotypes of flue-cured tobacco. However, they suggested that some positive correlation existed between the total amount of alkaloids and NNN in cured tobacco. NNN was not detected in green immature tobacco. Chamberlain and Chortyk (1986) and Chamberlain et al. (1987) studied the effect of curing regime on NNN formation in both lamina and midrib tissue of the flue-cured cultivar Speight G-28. It was found that midrib tissue contained a higher concentration of NNN than lamina of air-cured samples of this flue-cured cultivar. Very low levels of NNN were found in midribs of the

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Table I. Genotypes Used in This Study[a]

genotype identifier	typical alkaloid content,[b] mg/g	genotype identifier	typical alkaloid content,[b] mg/g
LAFC 53	3	TA 3.5	35
TA 1.0	10	TA 3.9	36
TA 2.0	20	NC 95	33
TA 3.1	31		

[a] Isoline background NC 95. Reported by Chaplin and Burk (1984).

flue-cured samples. Flue-cured lamina contained a higher concentration of NNN than air-cured lamina of this flue-cured genotype.

Besides NNN, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a nitrosated degradation product of nicotine, *N'*-nitrosoanatabine (NAT) and *N'*-nitrosoanabasine (NAB) are found also as tobacco-specific nitrosamines in tobacco and tobacco products (U.S. Surgeon General, 1982). Adams et al. (1983b) detected 0.64 μg/cigarette of NNK in an experimental cigarette (62 mm) made from flue-cured tobacco. Presence of NAT in tobacco was first reported by Hoffmann et al. (1979) and often was found in equal or greater amounts than NNN (Adams et al., 1983a; Andersen et al., 1987a,b; Brunnemann et al., 1985; Djordjevic et al., 1986, 1987). NAB has been found in smaller amounts in tobacco than the other tobacco-specific nitrosamines (Brunnemann et al., 1982, 1983a, 1985; Adams et al., 1983a). Recently, Brunnemann et al. (1987) reported the presence (0.07–2.5 μg g^{-1}) of another tobacco-specific nitrosamine, 4-(*N*-methyl-*N*-nitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) in tobacco snuff, but evidence of this nitrosamine in other tobacco types or commercial products is lacking.

The objectives of this study were (a) to measure accumulation of NNN, NAT, and NNK tobacco-specific nitrosamines in flue-cured tobacco genotypes near isogenic for alkaloid levels at different stages of tobacco production; (b) to determine TSNA distribution in leaves from different stalk position; and (c) to calculate the correlation between each tobacco-specific nitrosamine and the related alkaloid precursor.

MATERIALS AND METHODS

Materials. Seven genotypes of flue-cured tobacco type with different total alkaloid levels were chosen for this study. The genotypes, NC 95 and six isolines derived from NC 95, with typical alkaloid content ranging from 3 to 36 mg g^{-1} were used (Table I). These were described by Chaplin and Burk (1984). Tobacco was grown and cured under recommended conditions for flue-cured tobacco at Oxford, NC, in 1983, except an additional 69 kg of N fertilizer ha^{-1} was applied. The experimental design was a randomized complete block with three replications. Leaves from three plants within a plot were combined at each sampling time to represent an observation.

Samples were taken at harvest (mature leaves), during flue curing at end of yellowing, and after curing was complete. Leaves were harvested at three primings (first = lower stalk position; second = middle stalk position; third = upper stalk position) with the interval between each priming being about 2 weeks. A 10-cm strip was cut from the widest part of each leaf, midrib removed, and lamina frozen (−20 °C). Lamina tissue remained frozen until freeze-dried and then ground to pass a 40-mesh screen prior to chemical analysis.

Methods. Individual alkaloids, nicotine, nornicotine, anatabine, and anabasine were determined by using the method developed by Severson et al. (1981) and modified by Madsen et al. (1985).

Tobacco-specific nitrosamines (TSNA) were determined with a modification of the extraction procedures described by Hecht et al. (1975) and Andersen and Kemp (1985). A 1-g sample of ground lamina was extracted with 12 mL of citrate–phosphate buffer, pH 4.5, containing 20 mM of ascorbic acid by shaking in a stoppered centrifuge tube on a wrist action shaker overnight at room temperature. The suspension was centrifuged at 4000*g*, and the supernatant was decanted and saved. The pellet was washed twice with 10-mL portions of citrate buffer extracting solution by suspending the pellet, centrifuging, and combining each wash with the saved extract prior to adjusting to pH 5.0 with 1 N NaOH. The aqueous extract was partitioned with ethyl acetate (3 × 30 mL). Combined ethyl acetate extract was partitioned with 1 N HCl (3 × 5 mL). The combined aqueous acid phases were adjusted to pH 5.0 with 10 N NaOH at ice bath temperature and partitioned with chloroform (3 × 5 mL). Combined chloroform phases were diluted with chloroform to 25 mL, and anhydrous sodium sulfate was added as a drying agent. A known portion (5–25 mL) was evaporated to near-dryness under vacuum and then dried thoroughly under nitrogen. The residue was dissolved in 50–300 μL of acetone solution containing azobenzene as an internal standard. A 2.0-μL portion was injected into a Varian 3700 GC equipped with thermionic N–P specific detector under the same conditions as for alkaloid analysis except that injector and detector temperatures were maintained at 280 °C and column temperature was programmed from 140 °C, with an initial 3-min hold, to 245 °C at 2 °C min^{-1} and then held at 245 °C for 5 min.

Quantification was carried out by internal standardization with azobenzene after calibration of retention times and response factors with authentic tobacco-specific nitrosamines. Detection limit for individual tobacco-specific nitrosamines was 10 ppb.

Peak identification in some samples was verified by use of a gas chromatography–mass spectrometry computer system (a Varian 3700 GC equipped with an on-column injector and a Finnigan Model 705 ion trap detector).

Table II. Alkaloid Content (Milligrams per Gram Dry Weight) in Flue-Cured Lamina from the Third Priming (Leaves from Upper Stalk Position) of Alkaloid Isolines of NC 95[a]

genotype	NIC	NNIC	ANAT	total
LAFC 53	6.52	0.14	0.14	6.85
TA 1.0	30.64	0.39	1.10	32.56
TA 2.0	43.69	0.43	1.06	45.72
TA 3.1	39.64	6.47	1.73	49.28
TA 3.5	49.64	0.86	1.82	52.99
TA 3.9	54.19	0.66	0.77	56.20
NC 95	60.40	1.20	2.17	64.70
LSD[b]	12.82	3.32	0.50	12.86

[a] Alkaloid abbreviations: NIC, nicotine; NNIC, nornicotine; ANAT, anatabine; total, total alkaloids. [b] LSD, least significant difference ($P = 0.05$).

RESULTS AND DISCUSSION

Alkaloids. The range of nicotine among the seven genotypes in the third priming was from 6.52 mg g^{-1} in LAFC 53 to 60.4 mg g^{-1} in NC 95 (Table II). The levels of nicotine in the isolines and NC 95 were higher than expected based on previous history (Chaplin and Burk, 1984) and probably reflected the environmental conditions of the 1983 growing season. Excluding genotype TA 3.1, nornicotine content generally increased among the isolines from LAFC 53 to NC 95. TA 3.1 contained 5–50 times more nornicotine than the other genotypes. Anatabine concentrations were higher than nornicotine except in TA 3.1. Anabasine levels were 2–7 times lower compared to nornicotine and anatabine with the lowest amount measured in LAFC 53 and the greatest in TA 3.1 and NC 95 genotypes. Secondary amine alkaloids, nornicotine, anabasine, and anatabine, were 3.1–6.1% of the total alkaloids except in line TA 3.1 where they were just over 18% of the total alkaloids.

Nicotine accumulation increased in NC 95 from harvest through end of yellowing to cured tobacco (Table III). Nornicotine, anatabine, and anabasine accumulation showed trends for increased amounts from harvest through curing, although the increased nornicotine accumulation was not as pronounced as that of nicotine, anatabine, or

Table III. Alkaloid Content (Milligrams per Gram Dry Weight) in Flue-Cured Lamina of NC 95 from Three Primings at Harvest, at End of Yellowing during the Curing Process, and in the Cured Lamina[a]

sample	priming	NIC	NNIC	ANAT	total
harvest	1	22.63	0.56	0.60	24.50
	2	32.82	0.48	0.88	34.62
	3	47.33	0.87	1.65	50.45
	LSD[b]	13.68	ns[c]	0.57	13.60
end of yellowing	1	36.81	0.46	1.12	38.72
	2	46.34	0.45	1.21	48.38
	3	59.23	1.03	2.22	63.19
	LSD	11.60	0.21	0.24	11.86
cured	1	37.37	0.57	1.23	39.58
	2	48.98	0.55	1.43	51.46
	3	60.40	1.20	2.17	64.70
	LSD	9.33	0.62	0.45	9.42

sample means	NIC	NNIC	ANAT	total
harvest	34.26	0.64	1.05	36.16
end of yellowing	47.46	0.65	1.52	49.93
cured	48.92	0.77	1.61	51.65
LSD	5.24	ns	0.20	5.27

[a] Alkaloid abbreviations: see footnote, Table II. [b] LSD, least significant difference ($P = 0.05$). [c] Not significant.

Table IV. TSNA Content (Micrograms per Gram) in Flue-Cured Lamina from the Third Priming of Alkaloid Isolines of NC 95[a]

genotype	NNN	NAT	NNK	total
LAFC 53	0.35	0.88	0.76	1.98
TA 1.0	1.23	4.48	2.33	8.08
TA 2.0	2.23	7.03	2.96	12.22
TA 3.1	10.90	14.42	2.70	28.11
TA 3.5	2.54	12.19	3.24	18.04
TA 3.9	3.35	7.96	1.30	12.69
NC 95	2.80	11.76	3.11	17.77
LSD[b]	0.76	2.58	1.39	5.17

[a] TSNA abbreviations: NNN, *N*-nitrosonornicotine; NAT, *N'*-nitrosoanatabine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. [b] LSD, least significant difference ($P = 0.05$).

anabasine. While nornicotine concentrations in lamina of air-cured burley tobaccos are either similar or higher than anatabine (Djordjevic et al., 1986, 1987) these flue-cured tobaccos excluding TA 3.1 contained approximately 2 times more anatabine than nornicotine.

At all sampling dates, nicotine content of NC 95 lamina from the upper (priming 3) stalk positions exceeded that of the lower positions (Table III), a trend well documented in the literature (Tso, 1972). Nornicotine content in the leaves from lower and midstalk positions was approximately the same, and it was about half that in the upper stalk position leaves from the third priming. Anatabine level increased also with each priming, with the top leaves containing about twice as much of this alkaloid as leaves from the earlier primings (Table III).

Tobacco-Specific Nitrosamines. NNN content increased in the flue-cured NC 95 isolines as nornicotine content increased (Tables II and IV). Excluding TA 3.1, NNN content generally increased in these lines with increased total alkaloid content. The largest amount of NNN was found in TA 3.1, which contained the largest amount of nornicotine. Relatively large amounts of NAT were measured in lamina of TA 3.1, TA 3.5, and NC 95, the genotypes containing the largest amounts of anatabine. NNK contents in lamina of these flue-cured tobacco genotypes were not highly correlated with nicotine contents, contrary to what was expected from the published evidence that nicotine is the principal precursor of NNK (Adams et al., 1983b).

Table V. TSNA Content (Micrograms per Gram) in Flue-Cured Lamina of NC 95 from Three Primings at Harvest, at End of Yellowing, during the Curing Process, and in the Cured Lamina[a]

sample	priming	NNN	NAT	NNK	total
harvest	1	0.19	0.41	0.30	0.91
	2	0.19	0.25	0.11	0.55
	3	0.39	1.72	0.42	2.54
	LSD[b]	0.17	0.34	0.12	0.36
end of yellowing	1	0.23	0.65	0.12	1.00
	2	0.17	0.63	0.08	0.88
	3	0.44	1.89	0.21	2.54
	LSD	0.24	ns[c]	ns	1.14
cured	1	0.88	3.83	1.32	6.03
	2	1.02	4.43	1.01	6.45
	3	2.80	11.76	3.11	17.77
	LSD	1.18	3.42	0.88	5.40

sample means	NNN	NAT	NNK	total
harvest	0.26	0.79	0.28	1.34
end of yellowing	0.28	1.06	0.13	1.47
cured	1.56	6.67	1.81	10.08
LSD	0.28	0.85	0.26	1.29

[a] TSNA abbreviations: see footnote, Table IV. [b] LSD, least significant difference ($P = 0.05$). [c] Not significant.

Table VI. Correlation Coefficients (r) of Tobacco-Specific *N*-Nitrosamines (TSNA) with the Precursor Alkaloid in NC 95 Flue-Cured Tobacco during Curing from Three Primings and in Flue-Cured Leaf of Seven NC 95 Alkaloid Isolines from Third Priming

independent variable[a]	dependent variable	NC 95 sampling			NC 95 alkaloid isolines
		harvest	EOY[b]	cured	
NIC	NNN	0.82[c]	0.41	0.73[d]	0.40
NNIC	NNN	0.15	0.59	0.47	0.95[c]
ANAT	NAT	0.87[c]	0.52	0.83[c]	0.76[c]
NIC	NNK	0.42	0.32	0.62	0.51[d]
TA	TSNA	0.74[d]	0.37	0.73[d]	0.65[c]

[a] Abbreviations: see footnote *a* of Tables II and IV. [b] EOY = end of yellowing. [c] $P \leq 0.01$. [d] $P \leq 0.05$.

At each priming, mature green leaves contained NNN, NAT, and NNK (harvest samples: Table V). Occurrence of TSNA in these green leaf samples could indicate the presence of TSNA in normal mature tissue, in senescent tissue, in localized necrotic cells present in mature leaf tissue, or possibly on the leaf surface formed in situ from secretions from trichomes, glands, and/or hydathodes. Similar observations have been reported for green leaves of burley tobacco (Bush et al., 1979; Djordjevic et al., 1985, 1987). The levels of TSNA at harvest and at end of yellowing do not differ significantly. However, TSNA concentrations increased during the final curing stage and were greatest in the cured tobacco. Sample means for NAT concentration were greater than for NNN for harvest, end of yellowing, and cured leaf (Table V, statistics not shown). In the alkaloid isoline genotypes the mean NAT value (8.39 $\mu g\ g^{-1}$) was significantly higher than the mean NNN value (3.43 $\mu g\ g^{-1}$). The higher levels of NAT in comparison to NNN levels in these samples coincide with the higher levels of anatabine in comparison to nornicotine. Highest concentrations of TSNA in lamina of NC 95 flue-cured tobacco were measured in the leaves from third priming (top position). Usually the individual *N*-nitrosamines, NNN, NAT, and NNK, were also in significantly greater concentrations in leaves from the upper stalk position (third priming) than in leaves from the first or second primings, regardless of sample date. The upper leaves also had, as stated earlier, the greatest alkaloid content.

Correlation coefficients of the alkaloids with the TSNA were positive and generally significant (Table VI). Within the NC 95 maturation and curing samples, the correlation coefficients between the alkaloid precursors and the corresponding *N*-nitrosamine were nonsignificant for the end-of-yellowing samples. However, during curing the end of yellowing is a time at which TSNA precursors and perhaps TSNA concentrations are changing (Hamilton et al., 1982; Madsen et al., 1985), and consequently the correlations at this time may reflect this dynamic state and were nonsignificant. Correlations calculated from data obtained from cured samples should reflect static summation of associations in the leaf between alkaloids and TSNA formation during plant growth, maturation, and curing. Highest correlation was obtained for anatabine with NAT. The low correlation between nornicotine and NNN probably was the result of the small differences in nornicotine among samples within NC 95 genotypes. The positive and significant associations between NNN and nicotine at harvest and in cured tobacco do not necessarily indicate that this tertiary amine tobacco alkaloid should be precluded as an immediate precursor for NNN. Hecht et al. (1978) showed that detached tobacco leaves fed either [2′-^{14}C]nicotine or [2′-^{14}C]nornicotine prior to air-curing yielded similar amounts of NNN (0.009% and 0.007%, respectively) in the air-cured leaf.

In the alkaloid isoline samples, the two highest correlation coefficients, 0.95 and 0.76, were for the correlation of nornicotine with NNN and anatabine with NAT, respectively. There was approximately a 10-fold difference between the lowest and highest concentrations of alkaloids in the alkaloid isoline series of tobacco. With this large range of alkaloids, the prediction equations should be more accurate than when determined over a very small range of alkaloid concentrations. Prediction equations for NNN based on nornicotine were NNN = 1.38 + 1.35[nornicotine] and NAT = 2.14 + 4.9[anatabine] with nitrosamines in micrograms per gram and the alkaloids in milligrams per gram.

MacKown et al. (1984) reported positive and significant correlation coefficients for nicotine and nornicotine with NNN for three burley tobacco alkaloid isolines. None of their correlation coefficients were as high as we obtained for nornicotine and NNN in the flue-cured alkaloid isoline samples, but they did report a significant correlation between nicotine and NNN that we did not observe. Within the NC 95 samples we did observe positive and significant correlations between nicotine and NNN. Brunnemann et al. (1983a) reported a significant r of 0.65 between nicotine and NNN in experimental blended cigarettes but a nonsignificant r of 0.49 between nicotine and NNN in commercial tobacco products (Brunnemann et al., 1983b). Individual alkaloid data were not reported, and correlations between the alkaloids nicotine, nornicotine, and anatabine with the corresponding *N*-nitrosamines NNK, NNN, and NAT were not calculated.

Our results demonstrate that a small amount of TSNA was present in mature green tobacco leaves and that the amount increased during curing. Data are not available to suggest whether the increase in TSNA during curing was a result of physiological and biochemical processes directly related to the leaf or an interaction between the tobacco leaf and microflora associated with the leaf. Results from these samples indicate that the most important alkaloid for formation of a particular TSNA was the individual alkaloid nitrosated to that TSNA.

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Effect of Salt Concentration and Duration of Boiling on Peanut Seed Composition

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Green peanuts (*Arachis hypogaea* L. Cv. Florunner) were boiled in water containing various amounts of salt (0–5%) for periods of 0–2 h to determine the effect of wet heat on seed composition. The results showed that boiling caused a reduction in soluble carbohydrates, soluble protein, and free amino acids, while total protein, insoluble carbohydrates, and oil remained unaffected. Gel filtration and gel electrophoresis data showed the disappearance of major protein and polypeptide entities, indicating that while total seed protein content is unaffected during boiling, the protein and polypeptide composition are greatly altered.

In the United States, about 70% of the peanuts produced are consumed locally as food and the remaining is used as peanut byproducts. The aroma, texture, and flavor of raw peanuts can be improved by several methods. All methods of peanut preparation for food involve heating, which in turn alter or destroy various seed components such as protein, carbohydrates, vitamins, and natural antioxidants (Woodroof, 1983).

Liardon and Hurrel (1983) reported that the proteins are the most reactive of the major food components and during food processing they react with sugars, fats, and their oxidation products, polyphenols and various food additives. Previous studies have shown conformational changes in seed proteins (Neucere, 1974; Jacks et al., 1975; Srikanta and Rao, 1974; Kumar et al., 1980; Yamada et al., 1983) due to heating. Recently, Basha and Young (1985) reported changes in protein and polypeptide composition following oil roasting of peanut seeds for periods greater than 4 min.

Most of the previous reports were concerned with roasted peanut products. Although large amounts of peanuts are consumed as roasted products, freshly dug, unshelled, immature peanuts boiled in a brine are consumed as a delicacy in certain areas of the United States and various peanut growing regions of the world. However, little information exists on the effects of boiling on seed composition and its nutritional value. The objective of this study was to determine the effects of boiling periods and the presence of different concentrations of salt during boiling on peanut seed composition.

MATERIALS AND METHODS

Peanut (*Arachis hypogaea* L. Cv. Florunner) seeds were grown under recommended cultural conditions at the University of Florida Agricultural Experiment Station, Marianna, FL. After harvesting, green peanuts were pulled from the plant, washed, and then divided into 150-g batches. In the salt concentration study, peanuts were boiled for 45 min in 700 mL of water containing 0, 1, 2, 3, 4, and 5% (w/v) salt. For the study of the effect

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