Nitrosated, Acylated, and Oxidized Pyridine Alkaloids during Storage of Smokeless Tobaccos: Effects of Moisture, Temperature, and Their Interactions†

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Each of three smokeless tobaccos was adjusted to two moisture levels before storage at two temperatures. Nitrosated, acylated, and oxidized alkaloids and nitrite in moist snuff and dry snuff generally accumulated at higher rates during storage up to 1 year at high moisture (ca. 50%) and high temperature (32 °C) compared to the rates at low moisture (<22%) and low temperature (24 °C). Higher moisture compared to higher temperature resulted in larger increases of nitrosated and acylated alkaloid levels and nitrite. Putative parent alkaloids and nitrate in moist and dry snuffs generally decreased during storage intervals, especially under the high-moisture–high-temperature environments. Frequent interactions occurred between effects of moisture and temperature. Concentrations of parent, nitrosated and oxidized alkaloids, nitrite, and nitrate in chewing tobacco did not differ during storage among treatments. The high sugar content of chewing tobacco may have inhibited change.

INTRODUCTION

Alkaloids in tobacco (*Nicotiana tabacum* L.) and tobacco smoke have been widely recognized for their contributions to tobacco quality and usability (Palmer, 1963). The nature and underlying neurobiology associated with nicotine, the principal tobacco pyridine alkaloid, and its role in pleasurable sensations and continuation of tobacco use have been reviewed (Ashton et al., 1979; Martin, 1987). Also, alkaloids and other chemical components associated with tobacco usage and attendant health risks were summarized (Davis, 1987). Unpublished results from our laboratory indicate that sums of the putative parent alkaloids of lesser occurrence than nicotine, i.e., nornicotine, anatabine, and anabasine plus nitrosated, acylated, and oxidized alkaloid derivatives, may range up to 20% of total alkaloids in tobacco after postharvest processing. Much less information is available on the agronomic, postharvest processing, human physiological, and health-related effects of the parent alkaloids of lower occurrence and their derivatives in tobacco, except in the cases of nitrosated alkaloids which may act as carcinogenic agents (Hecht et al., 1986; Hoffmann et al., 1987) and the oxidized derivative cotinine which may affect neurotransmitter release (Rowell, 1987). The present study is designed to determine effects of some postharvest processing conditions on concentrations of these alkaloids.

Chamberlain et al. (1988) reported on the composition of parent and nitrosated alkaloids in commercial smokeless tobaccos. Analyses of alkaloids and their nitrosated, acylated, and oxidized derivatives in leaves were reported for tobacco lines during growth and after postharvest treatments (Burton et al., 1988, 1989a,b; Andersen et al., 1989b, 1990) and in three reference smokeless tobaccos (moist snuff, dry snuff, and chewing tobacco) (Andersen et al., 1989a). Although moisture and temperature effects on levels of chemical components were measured during prolonged storage, the influences of moisture were not entirely separable from those of temperature in these former studies. Higher relative humidity was associated with significant increases of nitrite and nitrosated alkaloids in burley tobacco during a 28-day storage period (Burton et al., 1989a,b). Andersen et al. (1989a) showed that large increases of nitrosated alkaloids (14–33-fold) occurred in moist snuff (53% moisture) during prolonged storage, whereas decreases in all putative parent and most acylated alkaloids were observed in the same material. In contrast, smaller, but generally similar, directions of concentration changes in pyridine alkaloids occurred in dry snuff (8.9% moisture) and chewing tobacco (19% moisture) during storage.

In the present study, effects of moisture, temperature, and their interactions on levels of pyridine alkaloids and their derivatives were determined at intervals during prolonged storage in the three reference smokeless tobaccos of different tobacco matrix composition previously reported on (Andersen et al., 1989a), except that each was adjusted to two moisture levels prior to storage. Comparisons of putative parent, nitrosated, acylated, and oxidized alkaloids, as well as nitrate and nitrite, were made among four parameters consisting of two temperature environments and two moisture levels for each reference tobacco.

EXPERIMENTAL PROCEDURES

Smokeless Tobacco Products and Storage Conditions. Three reference smokeless tobacco research products, loose-leaf chewing tobacco (1S1), dry snuff (1S2), and moist snuff (1S3) previously described in part (Andersen et al., 1989a), were obtained from the Tobacco and Health Research Institute, University of Kentucky, Lexington, KY. The principal materials besides moisture in the products as received were as follows: 1S1, sucrose (23%), Wisconsin air-cured leaf (17%), Pennsylvania air-cured leaf (15%), other corn syrup solids (6.2%), crushed burley stems (5.8%); 1S2, dark air-cured stem (33%), dark fire-cured leaf (23%), fire-cured Virginia leaf (20%), flue-cured stem

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(15%); 1S3, dark fire-cured leaf (26%), dark air-cured leaf (7.8%), NaCl (7.4%), burley stems (3.7%). The pH values of suspensions of the tobacco products as received were 6.4 for 1S1, 6.4 for 1S2, and 8.0 for 1S3. Moisture contents in the products as received were 22.3% for 1S1, 12.3% for 1S2, and 55.5% for 1S3. Portions of each product were adjusted to one additional moisture content to provide a high–low range of moisture for the three tobacco products. This was accomplished by spraying with a mist of distilled water or air-drying at room temperature and monitoring moisture contents with a C. W. Brabender moisture volatile testor. The resultant additional moisture levels were 49.3 for 1S1, 51.4% for 1S2, and 21.9% for 1S3.

Approximate 150-g portions of 1-kg lots of each reference product of given moisture content were transferred to 1-qt Mason jars with rubber-sealed screw tops for storage in the dark in temperature-controlled environment chambers. Three replications of 12 treatment combinations were used consisting of three tobacco products, two moisture levels per product, and two temperatures, 24 ± 1 and 32 ± 1 °C. Seals were broken at 2-week intervals throughout storage to equilibrate to atmospheric pressure, and samples were mixed during a 30-s period. Samples (15 g) were removed at the beginning of storage (0 time) and after storage durations of 6, 12, 24, and 48 weeks, placed in paper bags, and then frozen at −70 °C. The bag contents were then freeze-dried, ground to 100–200-mesh size, and equilibrated overnight to ambient moisture and temperature (at about 60% relative humidity and 25 °C) in darkness on a laboratory bench to decrease tendency for weight fluctuations to occur in subsequent weighings. All samples were then stored in sealed plastic containers at −70 °C until analyzed.

Reference Compounds. Nornicotine and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were obtained from Chemsyn Science Laboratories, Lenexa, KS. Anabasine, cotinine, and nicotine were obtained from Aldrich Chemical Co., Milwaukee, WI. 2,3′-Dipyridyl was obtained from K and K Laboratories, Plainview, NY. Anatabine was synthesized according to the procedures of Quan et al. (1965). *N*′-Nitrosonornicotine (NNN) and *N*′-nitrosoanatabine (NAT) were synthesized by nitrosation of nornicotine and anatabine, respectively, in a manner described for the nitrosation of morpholine (Lijinsky and Taylor, 1975). Syntheses of *N*′-formylnornicotine (FNN), *N*′-formylanatabine (FAT), *N*′-acetylnornicotine (ANN), *N*′-*n*-butanoylnornicotine (BNN), *N*′-*n*-hexanoylnornicotine (HNN), and *N*′-*n*-octanoylnornicotine (ONN) were described by Burton et al. (1988). *N*′-Acetylanatabine (AAT) and ANN (alternately) were synthesized as previously described (Andersen et al., 1989b).

Chemical Analyses. Putative parent alkaloids, i.e., nicotine, nornicotine, anatabine, and anabasine, were determined by a capillary GC procedure (Andersen et al., 1990). FNN, FAT, ANN, AAT, BNN, HNN, ONN, NNN, NNK, NAT, cotinine, and 2,3′-dipyridyl were extracted, partitioned, and quantitatively analyzed by capillary GC as described (Burton et al., 1988, 1989a; Andersen et al., 1989b) except with modification as also described (Andersen et al., 1990). Nitrite (Crutchfield and Burton, 1989) and nitrate [Armstrong et al., 1967; see also Technicon method (Technicon Industrial Systems, 1978)] were determined by spectrophotometric procedures. Calcium was determined as previously described (Andersen et al., 1989b). Quantitative results for all alkaloid derivatives and parent alkaloids in tobacco were expressed as micrograms per gram of dry weight normalized during storage on the basis of Ca concentration in the appropriate tobacco and moisture level at the start of storage; this corrected for dry-weight changes during storage. ANOVA was performed on results, and Fisher's least significant difference (LSD) test was used only where the *F* test was significant (Einot and Gabriel, 1975).

RESULTS AND DISCUSSION

Results for effects of moisture and temperature on amounts of nitrosated, acylated, and oxidized pyridine alkaloids in smokeless tobaccos during prolonged storage up to 48 weeks are given in Table I. Effects on parent pyridine alkaloids, nitrite, and nitrate are given in Table II. We postulate that parent alkaloids are precursors of alkaloid derivatives and that nitrosation, acylation, and oxidation reactions may compete for the same available parent alkaloids during the postharvest processing of tobacco. Differences in concentrations of some analyzed compounds in the same reference tobaccos at 0-time storage reported in this investigation and in the earlier paper (Andersen et al., 1989a) can be explained by our uses of an improved method (in this paper only) to determine GC detector response factors as recently described (Andersen et al., 1990) and different batches of the tobaccos.

Moist Snuff. Concentrations of each nitrosated alkaloid were significantly higher in the higher water content moist snuff at a given temperature than in the lower water content counterpart at each storage duration (Table IA). Also, concentrations of each nitrosated alkaloid were significantly higher in the 32 °C stored snuff at the higher water content (55.5%) than in 24 °C stored counterpart materials at each storage duration (except for NAT at 48 weeks). Lower water content snuff (21.9%) stored at the two temperatures did not differ significantly in NNN, NNK, and NAT levels. This moisture-dependent temperature effect on nitrosated alkaloid contents was indicative of a moisture–temperature interaction. Sums of nitrosated pyridine alkaloids (Table IA) in snuff with 55.5% moisture content were similar in concentration levels, relative amounts of NNN, NNK, and NAT, and trends of concentration changes to those determined in the previous study (Andersen et al., 1989a), although maximal levels were lower in the present study. Moisture clearly had a greater effect than temperature on the accumulations of NNN, NNK, and NAT as evidenced by the maximal sums of these components stored at 55.5% moisture compared to 21.9% (approximately 750 vs 50 μg/g, respectively). Epidemiological investigations indicated that the use of snuff, particularly the practice of snuff dipping, was associated with increased risk of oral cancer (Winn et al., 1981). Therefore, reductions of carcinogenic nitrosamine formations in smokeless tobaccos are desirable, and it would appear that this might be feasible by control of moisture during all phases of postharvest processing.

There was a lesser trend noted for effects of moisture on levels of acylated compounds in moist snuff than was the case for nitrosated compounds (Table IA). Individual acylated compounds were significantly elevated in 11 of 56 cases (not including 0-time storage tobacco) in the higher water content tobacco stored at a given temperature and duration.

Acylated compounds in moist snuff (Table IA) were significantly elevated in 12 of 56 cases when stored at 32 compared to 24 °C at the same moisture content and storage duration. In all cases, these storage temperature mediated concentration increases were observed for the alkaloid derivatives having shorter acyl hydrocarbon chain lengths ($C < 4$). There were five cases of negative correlations of acylated compounds with increased storage temperatures which only occurred for the longer chain compounds. There were several moisture–temperature interactions on acylated alkaloid contents in snuff compared at the same storage duration.

Oxidized alkaloids in moist snuff were significantly increased in 9 of 16 cases in the higher compared to the lower water content tobacco stored at the same temperature and duration (Table IA). This class of alkaloid derivatives when similarly compared was elevated in 8 of 16 cases when stored at 32 compared to 24 °C. Moisture–temperature interactions for oxidized compounds occurred

Table I. Effect of Moisture, Temperature, and Their Interactions on Concentrations of Nitrosated, Acylated, and Oxidized Pyridine Alkaloids in Smokeless Tobaccos during Prolonged Storage[a,b]

moisture–temp; H = high, L = low	storage conditions: moisture, %	storage conditions: temp, °C	storage conditions: duration, weeks	pyridine alkaloid derivatives, μg/g: nitrosated: NNN	nitrosated: NNK	nitrosated: NAT	acylated: FNN	acylated: ANN	acylated: BNN	acylated: HNN	acylated: ONN	acylated: FAT	acylated: AAT	oxidized: cotinine	oxidized: 2,3′-dipyr
							(A) Moist Snuff								
L	21.9		0	10.1	2.3	25.0	110.8	32.0	1.1	5.7	10.1	16.4	2.8	181.5	347.3
H	55.5		0	9.9	2.3	25.8	111.1	30.1	1.1	5.5	10.4	16.1	2.8	169.7	383.0
		LSD 0.05		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LL	21.9	24	6	8.7 C	2.0 C	23.2 C	75.2 B	21.8 B	1.0	4.7	7.5	13.4 B	2.1	133.3 B	449.6
HL	55.5	24	6	22.2 B	5.2 B	114.6 B	77.3 B	21.9 B	0.9	4.9	9.0	13.7 B	1.7	146.6 B	581.2
LH	21.9	32	6	10.7 C	2.5 C	29.0 C	119.8 A	34.7 A	1.1	5.4	8.8	16.4 A	1.9	225.3 A	457.0
HH	55.5	32	6	43.2 A	6.8 A	216.2 A	118.5 A	33.7 A	1.0	4.8	7.8	15.8 AB	2.3	204.9 A	479.4
		LSD 0.05		6.2	1.2	31.7	19.3	5.5	NS	NS	NS	2.4	NS	28.1	NS
LL	21.9	24	12	9.4 C	2.0 C	26.7 C	101.2 B	28.9	1.1	5.0	9.9	14.2	2.4	169.5 AB	533.0 B
HL	55.5	24	12	37.7 B	5.7 B	211.5 B	108.3 AB	30.8	1.1	5.7	12.4	16.4	2.7	181.6 AB	766.8 A
LH	21.9	32	12	9.7 C	2.2 C	28.8 C	75.5 C	26.7	1.0	5.1	11.8	14.4	2.0	128.0 B	520.9 B
HH	55.5	32	12	71.2 A	9.7 A	332.2 A	118.2 A	35.3	1.0	5.5	11.5	17.7	2.7	220.0 A	767.4 A
		LSD 0.05		13.5	1.6	50.9	14.8	NS	NS	NS	NS	NS	NS	71.4	174.6
LL	21.9	24	24	12.9 C	2.5 C	39.9 C	84.7 C	22.9 B	1.3 B	8.0 A	17.1 A	19.6	3.3	103.2 C	307.2 C
HL	55.5	24	24	100.3 B	26.9 B	409.1 B	92.6 BC	29.6 B	1.5 A	7.6 A	14.1 AB	20.1	3.3	125.6 B	338.0 BC
LH	21.9	32	24	10.9 C	1.7 C	34.2 C	102.0 B	30.4 B	1.1 B	6.3 B	14.3 AB	18.4	2.8	139.1 B	377.2 B
HH	55.5	32	24	198.2 A	44.0 A	526.8 A	122.0 A	164.5 A	1.3 B	5.1 C	11.3 B	20.8	3.2	222.4 A	603.1 A
		LSD 0.05		4.7	3.9	52.2	11.7	15.2	0.2	1.0	3.8	NS	NS	14.7	55.1
LL	21.9	24	48	10.4 C	1.0 C	31.6 C	99.1 AB	31.7 C	1.0 C	5.1 A	10.7 A	15.0 B	1.7 B	147.7 C	385.9 C
HL	55.5	24	48	172.0 B	24.0 B	543.0 A	114.3 A	95.4 B	1.6 A	4.4 B	8.4 B	16.2 B	1.5 B	214.2 AB	515.5 BC
LH	21.9	32	48	10.0 C	1.1 C	27.5 C	92.6 B	36.6 C	1.0 C	4.7 AB	9.0 B	14.9 B	2.1 A	183.0 BC	674.1 B
HH	55.5	32	48	274.0 A	32.4 A	461.1 B	104.8 AB	182.6 A	1.4 B	4.3 B	9.1 B	22.4 A	1.5 B	250.5 A	1310 A
		LSD 0.05		36.8	5.9	66.6	18.1	22.0	0.2	0.5	1.4	1.9	0.3	59.3	189.5
							(B) Dry Snuff								
L	12.3		0	66.6	64.0	137.7	87.5	23.4	1.3	3.6	6.9	12.3	2.1	144.7	293.0
H	51.4		0	66.8	66.9	135.5	87.7	24.0	1.4	3.9	7.1	12.4	1.8	140.5	273.9
		LSD 0.05		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
LL	12.3	24	6	28.5 B	59.0	119.4 B	69.5 AB	20.2 AB	1.0	3.7	7.4 A	10.2 AB	1.6 A	131.6 AB	289.2
HL	51.4	24	6	57.8 B	54.3	126.9 B	62.7 B	17.9 B	1.0	3.4	6.7 AB	9.4 B	1.3 AB	116.9 B	296.7
LH	12.3	32	6	64.0 B	59.4	125.3 B	79.9 A	22.7 A	1.2	3.5	6.4 AB	10.9 AB	1.1 B	149.5 AB	325.1
HH	51.4	32	6	81.7 A	57.5	189.6 A	79.6 A	22.5 A	1.1	3.2	5.9 B	11.5 A	1.3 AB	153.7 A	323.8
		LSD 0.05		7.5	NS	26.3	11.7	3.7	NS	NS	1.5	1.6	0.5	34.2	NS
LL	12.3	24	12	66.2 B	59.8	140.8 B	92.3	28.0	1.3 AB	4.0	9.5	11.4	1.1 B	177.6 B	432.1
HL	51.4	24	12	75.0 B	57.1	168.2 B	97.6	29.7	1.2 AB	4.0	9.1	12.5	2.2 A	193.7 B	563.3
LH	12.3	32	12	69.4 B	60.6	143.6 B	96.0	28.3	1.4 A	3.8	8.5	11.7	1.8 AB	152.4 B	390.7
HH	51.4	32	12	129.6 A	55.7	359.8 A	112.2	34.9	1.0 B	3.3	6.9	12.9	1.9 AB	259.2 A	387.1
		LSD 0.05		12.3	NS	30.7	NS	NS	0.3	NS	NS	NS	0.8	61.9	NS
LL	12.3	24	24	70.7 C	68.7 A	160.2 C	81.8 C	26.0 D	3.0 A	5.5 A	10.9 B	14.1 AB	2.4 AB	101.7 D	256.5 C
HL	51.4	24	24	87.5 B	58.7 A	258.0 B	93.5 BC	29.8 C	2.3 B	5.2 AB	10.1 B	16.1 A	2.7 A	126.3 C	284.8 BC
LH	12.3	32	24	72.5 C	69.6 A	157.2 C	105.8 B	36.2 B	2.1 C	4.9 B	12.5 A	13.7 B	1.7 B	158.5 B	292.9 B
HH	51.4	32	24	137.9 A	19.5 B	377.2 A	122.0 A	42.9 A	1.5 D	4.3 C	9.5 B	13.3 B	2.5 AB	207.4 A	364.0 A
		LSD 0.05		13.1	24.6	39.9	12.4	3.4	0.3	0.5	1.5	2.1	0.9	22.5	33.1
LL	12.3	24	48	71.6 C	64.2 A	143.2 C	104.9	37.8 B	1.8	3.7 AB	8.4 AB	11.9 AB	1.8	175.0 B	266.4 B
HL	51.4	24	48	253.3 A	49.3 B	596.5 A	110.1	136.7 A	2.0	3.3 B	6.7 C	12.9 A	1.6	211.3 B	386.3 B
LH	12.3	32	48	71.2 C	63.8 A	144.4 C	97.3	34.7 B	2.1	3.8 A	9.2 A	11.5 AB	1.8	204.9 B	438.7 B
HH	51.4	32	48	126.6 B	23.8 C	274.0 B	102.4	34.0 B	1.6	3.4 AB	7.6 BC	10.6 B	1.4	303.7 A	1086 A
		LSD 0.05		32.8	13.9	60.0	NS	16.4	NS	0.5	1.5	2.2	NS	85.0	579.5
							(C) Chewing Tobacco								
L	22.3		0	2.8	0.5	5.4	63.6 A	10.9 A	0.4	2.3	6.0	6.7	1.6	60.4	134.3
H	49.3		0	2.7	0.5	4.3	52.3 B	8.7 B	0.4	2.0	3.9	5.9	1.5	52.6	123.0
		LSD 0.05		NS	NS	NS	7.4	1.2	NS	NS	NS	NS	NS	NS	NS
LL	22.3	24	6	3.2	0.7	6.6 A	65.5 A	12.9 A	0.5	2.2	5.2	7.1 A	1.6 A	71.1 A	146.9 AB
HL	49.3	24	6	3.2	0.6	6.2 A	63.1 A	12.4 A	0.4	2.2	4.9	6.2 AB	1.5 AB	74.1 A	153.6 A
LH	22.3	32	6	2.9	0.8	4.9 B	51.5 B	9.4 B	0.4	2.1	4.1	6.4 AB	1.5 AB	59.4 B	142.9 AB
HH	49.3	32	6	3.0	0.7	5.2 B	49.8 B	8.8 B	0.4	2.0	3.9	5.9 B	1.4 B	59.9 B	131.8 B
		LSD 0.05		NS	NS	0.9	8.1	1.8	NS	NS	NS	1.1	0.2	9.2	17.1
LL	22.3	24	12	2.8	0.3	4.6	61.6	11.6	0.4	2.0	4.4	6.2	1.3 A	66.1 AB	158.8 AB
HL	49.3	24	12	2.5	0.3	4.2	48.8	9.3	0.3	1.8	3.9	5.0	1.0 A	55.3 B	136.9 B
LH	22.3	32	12	2.6	0.5	5.0	59.1	12.1	0.4	1.8	4.2	6.0	1.2 AB	69.3 AB	154.8 AB
HH	49.3	32	12	3.1	0.4	5.0	64.4	11.7	0.4	2.0	4.5	6.0	1.2 AB	81.1 A	168.5 A
		LSD 0.05		NS	NS	NS	NS	NS	NS	NS	NS	NS	0.3	25.1	29.1
LL	22.3	24	24	3.2	0.3	7.5	46.7 A	7.0	0.4	2.3	4.5 B	7.0	1.3 A	39.3	127.2 B
HL	49.3	24	24	3.2	0.4	7.6	39.6 B	7.4	0.4	2.6	5.2 AB	6.5	1.2 B	37.2	131.2 AB
LH	22.3	32	24	3.0	0.3	6.5	46.7 A	8.2	0.3	2.7	6.3 A	7.1	1.4 A	42.4	140.0 AB
HH	49.3	32	24	3.3	0.5	6.3	43.7 AB	7.1	0.4	2.7	6.9 A	6.6	1.4 A	45.5	141.5 A
		LSD 0.05		NS	NS	NS	6.8	NS	NS	NS	1.7	NS	0.1	NS	13.0
LL	22.3	24	48	3.2	0.4 B	8.2 A	64.1 A	11.9 A	0.5 AB	1.7	3.4	6.1	0.5	72.9	121.9 AB
HL	49.3	24	48	2.6	0.4 B	6.5 B	52.9 AB	11.4 A	0.4 B	1.7	3.7	5.2	0.8	61.0	113.2 B
LH	22.3	32	48	2.8	0.5 AB	6.9 AB	53.4 AB	11.9 A	0.5 AB	2.1	4.2	6.1	0.6	58.1	142.5 A
HH	49.3	32	48	2.9	0.6 A	6.0 B	49.6 B	8.1 B	0.5 A	1.8	3.9	5.4	0.4	64.3	130.3 AB
		LSD 0.05		NS	0.2	1.5	13.2	3.3	0.1	NS	NS	NS	NS	NS	21.5

[a] Mean values are for three replicated samples and are corrected for Ca content departures from those at 0-time storage. [b] LSD values are least significant differences between mean values of the same tobacco product at an equivalent storage duration. LSD values are significant at the 0.05 level of probability; NS, not significant at $P = 0.05$. Mean values in a vertical column subset for a given tobacco product and storage duration followed by no corresponding letter are significantly different at $P = 0.05$.

Table II. Effect of Moisture, Temperature, and Their Interactions on Concentrations of Nitrite, Nitrate, and Parent Pyridine Alkaloids in Smokeless Tobaccos during Prolonged Storage[a,b]

moisture–temp; H = high, L = low	storage conditions: moisture, %	temp, °C	duration, weeks	NO_2 N, µg/g	NO_3 N, µg/g	parent pyridine alkaloid, µg/g: nicotine	nornicotine	anatabine	anabasine
				(A) Moist Snuff					
L	21.9		0	14.1	4414	16 875	147.1	264.0	70.6
H	55.5		0	12.8	3731	17 635	157.7	265.5	74.5
		LSD 0.05		NS	NS	NS	NS	NS	NS
LL	21.9	24	6	15.5 B	4897 A	17 934	162.1	277.4 A	80.6 A
HL	55.5	24	6	21.4 B	4797 AB	17 416	164.3	246.6 B	72.2 B
LH	21.9	32	6	9.3 B	4989 A	18 443	177.0	274.0 A	76.6 AB
HH	55.5	32	6	589.4 A	4287 B	18 181	156.9	215.0 C	71.8 B
		LSD 0.05		32.1	582.3	NS	NS	17.6	5.2
LL	21.9	24	12	13.2 B	4858 A	17 157	30.8	202.6 A	29.8
HL	55.5	24	12	674.0 A	4163 B	17 275	33.4	176.4 A	30.2
LH	21.9	32	12	16.3 B	4722 A	17 163	35.1	177.5 A	30.9
HH	55.5	32	12	633.6 A	3892 B	17 318	30.9	125.0 B	29.9
		LSD 0.05		96.2	544.7	NS	NS	39.5	NS
LL	21.9	24	24	5.4 C	5900 A	18 801 A	115.0 B	246.8 A	74.8 A
HL	55.5	24	24	1,204 B	4140 B	15 868 AB	85.8 C	129.0 B	73.8 A
LH	21.9	32	24	3.9 C	6009 A	17 200 A	130.5 A	241.3 A	70.9 A
HH	55.5	32	24	1,604 A	1453 C	14 066 B	66.0 D	28.3 C	55.3 B
		LSD 0.05		328.0	881.8	2 982	5.7	11.8	10.8
LL	21.9	24	48	4.3 C	6057 A	16 500 A	112.5 B	219.5 A	71.8 A
HL	55.5	24	48	737.2 A	3379 B	15 943 A	50.9 C	25.7 C	62.6 B
LH	20.9	32	48	4.0 C	5953 A	15 773 A	143.0 A	196.1 B	75.2 A
HH	55.5	32	48	269.6 B	68.3 C	12 557 B	41.6 C	7.4 D	34.5 C
		LSD 0.05		181.9	385.5	1 181	19.7	7.8	6.0
				(B) Dry Snuff					
L	12.3		0	18.3 B	4977	9 956	100.8 B	175.1	90.1
H	51.4		0	14.8 A	4842	10 233	138.1 A	186.4	86.3
		LSD 0.05		2.5	NS	NS	32.7	NS	NS
LL	12.3	24	6	7.0 B	5153 B	9 770 B	130.2 B	176.9	83.9 A
HL	51.4	24	6	11.6 B	5027 B	10 372 AB	162.9 AB	183.9	50.4 B
LH	12.3	32	6	7.0 B	6230 A	10 469 AB	146.6 B	190.2	55.5 B
HH	51.4	32	6	147.7 A	6054 A	10 870 A	196.8 A	174.5	48.9 B
		LSD 0.05		57.1	801.9	713.8	47.2	NS	18.9
LL	12.3	24	12	11.9 B	6642 A	9 746	30.6	137.2 A	18.3
HL	51.4	24	12	33.3 B	6060 A	10 202	26.1	137.1 A	17.7
LH	12.3	32	12	8.1 B	6682 A	12 209	27.9	167.8 A	24.0
HH	51.4	32	12	1,000 A	2242 B	10 090	30.5	84.5 B	20.2
		LSD 0.05		65.9	1138	NS	NS	48.2	NS
LL	12.3	24	24	6.2 B	8117 A	9 724	80.5	165.4 AB	65.3 A
HL	51.4	24	24	120.5 A	6347 B	9 341	123.1	150.3 B	48.4 B
LH	12.3	32	24	4.6 B	8238 A	9 824	116.5	177.5 A	61.7 A
HH	51.4	32	24	31.0 B	59.0 C	9 719	133.3	71.4 C	45.4 B
		LSD 0.05		39.7	241.5	NS	NS	20.4	10.6
LL	12.3	24	48	6.1 B	8030 A	9 674	82.8 B	154.7 A	66.0 A
HL	51.4	24	48	1,228 A	1650 B	9 653	30.2 C	19.1 B	34.4 B
LH	12.3	32	48	5.7 B	8857 A	9 655	110.6 A	155.5 A	63.3 A
HH	51.4	32	48	24.3 B	23.1 B	8 382	85.2 AB	46.9 B	46.5 B
		LSD 0.05		199.7	1118	NS	25.5	38.9	14.9
				(C) Chewing Tobacco					
L	22.3		0	1.9	1712	6 057	115.9	185.2	15.1
H	49.3		0	1.9	1568	5 967	101.6	182.0	14.1
		LSD 0.05		NS	NS	NS	NS	NS	NS
LL	22.3	24	6	12.9 A	1764 B	5 726	151.1 B	184.8	17.4
HL	49.3	24	6	4.4 B	1747 B	5 835	160.6 B	184.6	17.1
LH	22.3	32	6	4.8 B	2118 A	6 465	152.3 B	196.3	17.7
HH	49.3	32	6	5.5 B	1967 AB	5 922	190.7 A	192.4	17.9
		LSD 0.05		4.6	257.3	NS	25.0	NS	NS
LL	22.3	24	12	2.9 D	2139 A	4 877 A	47.0 AB	132.2 A	9.8 B
HL	49.3	24	12	4.5 C	1852 B	4 061 B	48.8 A	110.8 B	8.2 C
LH	22.3	32	12	7.1 A	2034 AB	4 940 A	29.5 C	131.6 A	11.6 A
HH	49.3	32	12	5.8 B	2159 A	5 091 A	38.1 BC	132.1 A	12.0 A
		LSD 0.05		1.0	235.3	590.5	10.3	14.8	1.3
LL	22.3	24	24	1.1	2417 B	5 692	63.0 B	140.3 B	18.2
HL	49.3	24	24	0.9	2546 AB	5 619	77.8 AB	164.6 AB	18.6
LH	22.3	32	24	1.0	2797 AB	5 865	90.3 A	184.5 A	19.3
HH	49.3	32	24	1.2	2969 A	5 852	89.1 A	181.4 A	18.9
		LSD 0.05		NS	426.9	NS	16.7	27.2	NS
LL	22.3	24	48	2.6 A	2541	6 298	87.6 AB	169.2 AB	13.4 B
HL	49.3	24	48	2.1 AB	2493	5 768	77.3 B	158.9 B	22.3 A
LH	22.3	32	48	1.9 B	2485	5 934	100.6 A	180.5 A	18.9 A
HH	49.3	32	48	2.0 AB	2538	6 078	72.6 B	168.0 AB	17.5 AB
		LSD 0.05		0.6	NS	NS	17.1	19.4	5.2

[a] Mean values are for three replicated samples and are corrected for Ca content departures from those at 0-time storage. [b] LSD values are least significant differences between mean values of the same tobacco product at an equivalent storage duration. LSD values are significant at the 0.05 level of probability. NS, not significant at $P = 0.05$. Mean values in a vertical column subset for a given tobacco product and storage duration followed by no corresponding letter are significantly different at $P = 0.05$.

as follows: cotinine at 12 weeks and 2,3′-dipyridyl at 24 and 48 weeks.

Nitrite N concentrations (Table IIA) in moist snuff of high water content increased in seven of eight cases, compared to moist snuff counterparts of low water content. The increases were of large magnitude, ranging from 40- to 411-fold. Temperature change affected nitrite N levels in three of eight comparisons, and the differences were generally smaller than those associated with different moistures. Moisture–temperature interactions occurred at 6, 24, and 48 weeks. Maximal nitrite levels in the snuff stored at 55.5% moisture were reached at the 24-week storage duration corresponding to earlier results (Andersen et al., 1989a).

Nitrate N concentrations were at lower levels in the higher water content compared to lower water content moist snuff in seven of eight cases at equivalent temperature and storage durations (Table IIA). The magnitude of decreases supports the view that concomitant nitrite increases result from nitrate reduction. Higher storage temperature effected large decreases of nitrate concentrations in two of eight cases; lower amounts were observed at the longer storage durations. Moisture–temperature interactions were found at 6, 24, and 48 weeks. Nitrate N in moist snuff prior to storage was about 4000 μg/g compared to concentrations in dry snuff and chewing tobacco, which were 4900 and 1600 μg/g, respectively. At longer storage durations there were increases of nitrate concentrations in low water content snuff stored at both temperatures compared to 0-time levels. The cause for this is unknown, but bound nitrate may be released during storage.

A decrease in nitrite from earlier concentrations during storage occurred in each smokeless tobacco product. This trend was consistent with earlier results obtained for smokeless tobaccos (Andersen et al., 1989a), homogenized leaf-cured tobacco (Andersen and Kasperbauer, 1984), and burley tobacco (Burton et al., 1989b). Nitrite in post-harvest tobacco is believed to accumulate from the action of bacterial nitrate reductase on nitrate. The reduction usually occurs under specific conditions, e.g., presence of specific bacteria (Parsons et al., 1986; Calmels et al., 1988), high nitrate levels, and a slightly acid pH (Parsons et al., 1986). The nitrite may be further reduced by bacterial nitrite reductase in denitrification reactions during post-harvest processing of tobacco (Parsons et al., 1986; Calmels et al., 1988), and denitrification may compete with nitrosation of parent alkaloids during formation of NNN, NNK, and NAT.

Among the parent alkaloids in moist snuff, levels of individual alkaloids were lower in 17 of 32 cases for higher water content tobacco compared to lower moisture counterparts at the same temperature and storage duration; no increases occurred, and results of remaining comparisons were not significantly different (Table IIA). Temperature-mediated changes in parent alkaloid levels occurred in 11 of 32 cases. The higher storage temperature caused nine decreases and two increases in parent alkaloid levels. Moisture–temperature interactions for parent alkaloids in moist snuff occurred as follows: nicotine at 24 and 48 weeks, nornicotine at 48 weeks, anatabine at 6, 12, and 24 weeks, and anabasine at 6, 24, and 48 weeks.

Summations of total parent alkaloids in moist snuff prior to storage were about 17 500 μg/g compared to lower levels in dry snuff and chewing tobacco. Decreased levels of parent alkaloids concomitant with increased levels of alkaloid derivatives in moist snuff at higher moisture and temperature conditions during storage support the view that parent alkaloids served as precursors of the alkaloid derivatives.

Dry Snuff. Concentrations of NNN and NAT in dry snuff were significantly larger in the higher water content dry snuff materials at the same temperature and duration than in lower water content counterparts in 12 of 16 cases (Table IB). This trend became more marked at the longer storage durations. All other comparable pairs were not significantly different. Contents of NNK showed a different trend, decreasing in higher water content dry snuff samples in three of eight cases used for the comparisons and not differing in the other cases. Among 24 dry snuff comparisons, storage at 32 vs 24 °C resulted in higher levels of nitrosated alkaloids in six cases and lower levels in four when stored at the same moisture and duration. Nonsignificant differences were found in the other 14 cases. Moisture–temperature interactions for nitrosated alkaloids in dry snuff were as follows: NNN and NAT at all durations, NNK at 24 and 48 weeks. Maximal levels of NNN and NAT occurred at the longest storage duration but were at 24 weeks for NNK. The highest level of total nitrosated alkaloid determined among all samples for dry snuff was about 900 μg/g, while that for moist snuff was 770 μg/g.

The effects of moisture on concentrations of acylated compounds in dry snuff varied among the individual derivatives (Table IB). Thus, shorter chain acyl derivatives were significantly elevated in five and decreased in three cases, whereas longer chain (C > 2) acyl compounds were lowered in four instances in snuff stored at the higher water content. Temperature effects on acylated alkaloids in dry snuff produced 8 cases each of increased and decreased levels among the 56 pairs compared at the same moisture and duration. Moisture–temperature interactions occurred in several cases.

Oxidized alkaloids in dry snuff were increased in 6 of 16 cases in the higher water content tobaccos compared at the same temperature and duration (Table IB). Oxidized alkaloids stored at 32 vs 24 °C at the same moisture and duration were increased in 8 of 16 cases; no decreased levels attributable to higher temperature were observed. Moisture–temperature interactions for oxidized alkaloids were as follows: cotinine at 6, 12, and 48 weeks and 2,3′-dipyridyl at 24 and 48 weeks. Maximal levels of both oxidized alkaloids occurred at 48 weeks in high moisture–high temperature stored dry snuff and moist snuff, but this trend was not observed in chewing tobacco (Table I).

Dry snuff tobaccos stored at higher (compared to lower) water content had increased concentrations of nitrite N in four of eight comparisons (Table IIB). Storage at higher temperature resulted in increased levels of nitrite at the 6- and 12-week durations and decreased levels at 24 and 48 weeks. Moisture–temperature interactions occurred at all durations. Maximal nitrite concentration among all the dry snuff samplings was 1228 μg/g in high water content tobacco. It is likely that high water content promotes the growth of nitrite-accumulating bacteria. The significant difference in nitrite contents at the two moisture levels at 0-time storage can be attributed to omission at 0-time only of normalization of results based on Ca content differences.

Higher storage moisture content caused decreased concentrations of nitrate N in five of eight cases compared to lower water content counterpart dry snuff tobaccos at the same temperature and duration (Table IIB). Remaining cases were not significantly different in this respect. Storage at increased temperature resulted in increased nitrate contents in two and decreased contents in two of eight cases. There were moisture–temperature interaction effects at 12 and 24 weeks. Large decreases in nitrate from 0-time levels occurred in high water content–high temperature tobacco stored 24–48 weeks. These decreases were accompanied by a large increase in nitrite at 12 weeks but not at 24 and 48 weeks; denitrification reactions may have occurred at the longer durations.

Parent alkaloids in dry snuff had decreased concentrations when stored at the higher water content compared to the lower moisture level in 10 of 32 cases (Table IIB). It is noteworthy that there were no moisture- or temperature-mediated changes observed for nicotine content. Effects of moisture–temperature interactions occurred for nicotine at 6 weeks, nornicotine at 6 and 48 weeks, anatabine at 12 and 24 weeks, and anabasine at 6 weeks. The significant difference in nornicotine at the two moisture levels at 0-time storage can be explained as in the case for nitrite in dry snuff at 0 time (preceding paragraph). The summation of total parent alkaloids in dry snuff prior to storage was 10 500 μg/g.

Chewing Tobacco. There was only one case of a small change in nitrosated alkaloid concentration that resulted from a moisture–temperature interaction in chewing tobacco during storage (Table IC). The results contrast sharply with those for moist and dry snuff tobaccos in which there were observed changes (often of large magnitude) in nitrosated alkaloids in the majority of paired low vs high water content comparisons. Nitrosated alkaloids in chewing tobacco were significantly changed in only 3 of 24 cases as a result of storage temperature change. There were moisture–temperature interactions for NNK and NAT at 48 weeks. The maximum sum of nitrosated alkaloids among the chewing tobacco samples was 11.2 μg/g and was much lower than corresponding summations for moist snuff and dry snuff. This was in close agreement with our earlier study (Andersen et al., 1989a). The relatively low concentrations of nitrosated alkaloid precursors, i.e., nitrate, nitrite, and parent pyridine alkaloids, present in chewing tobacco compared to snuffs can partly account for the low accumulations of NNN, NNK, and NAT. The sugar content of chewing tobacco (ca. 30%) is another factor that may inhibit bacterially mediated nitrite accumulation and subsequent nitrosation reactions. High concentrations of sugars, which tie up moisture in natural products and make it unavailable to microorganisms, are effective preservatives (Frazier, 1958).

The effects of moisture on acylated alkaloids in chewing tobacco were noteworthy, because unlike the mixed direction of responses to increased storage moisture and greater frequencies of change with snuffs, only 3 cases of 56 showed significant changes (all decreases) of acylated compounds in chewing tobacco (Table IC). Temperature effects on accumulations of acylated alkaloids occurred in 8 of 56 cases with higher storage temperature associated with higher levels of acylated alkaloids in 3 cases and lower levels in 5. There were several moisture–temperature interactions. Levels of total acylated alkaloids at 0-time storage were approximately 90 μg/g compared to sums of levels in moist snuff and dry snuff of 180 and 140 μg/g, respectively. Significant differences between FNN and ANN levels at the two moisture levels at 0-time storage can be attributed to the omission of normalization of results based on Ca content differences at this duration only.

There was no significant effect of moisture on levels of oxidized pyridine alkaloids in chewing tobacco during storage (Table IC). Five of 16 cases, however, showed a temperature-mediated change for cotinine and 2,3-dipyridyl. Moisture–temperature interactions were noted for cotinine at 12 weeks and for 2,3′-dipyridyl at 6 and 12 weeks. Summations of oxidized alkaloids in chewing tobacco at 0-time storage were about 190 μg/g compared to levels in moist snuff and dry snuff which were 530 and 440 μg/g, respectively. Prestorage processing of these tobaccos (snuffs were fermented, chewing tobacco is not) may account for these differences. The maximal sum of concentrations of cotinine and 2,3′-dipyridyl in chewing tobacco (250 μg/g) was present in the high moisture–high temperature storage treatment at 12 weeks; by comparison, maximal levels of the sums of these oxidized alkaloids in moist snuff (1560 μg/g) and dry snuff (1390 μg/g) were found in the tobaccos stored 48 weeks at high moisture and high temperature. Several tobacco matrix compositional differences besides moisture undoubtedly had effects on the storage-related changes in chemical composition among the smokeless tobacco blends.

Storage of chewing tobacco at the higher moisture level caused three cases of change in nitrite N concentration (two decreases and one increase) among eight comparisons made at a given temperature duration (Table IIC). Storage at high temperature resulted in two increases and two decreases of nitrite at low moisture. Moisture–temperature interactions affecting nitrite occurred at 6, 24, and 48 weeks. These changes in nitrite concentrations were small compared to those in snuffs and may be explained by the much higher sugar content of chewing tobacco, which may retard the growth of nitrate-reducing bacteria.

Increased moisture in chewing tobacco during storage resulted in only one case of changed nitrate nitrogen concentration of the eight comparisons made (Table IIC). There were two cases of eight that indicated a temperature-mediated change in nitrate. Moisture–temperature interactions affecting nitrate levels occurred at 6 and 12 weeks.

Concentrations of parent alkaloids in chewing tobacco were changed in 6 of 32 cases after storage at the higher moisture level at the same temperature and duration (Table IIC). Decreased parent alkaloids were found in four cases and increased alkaloids in two cases. Storage at 32 °C caused 10 significant changes in parent alkaloid levels in chewing tobacco compared to 24 °C counterparts at the same level of moisture and for the same duration. The changes included 8 increases and 2 decreases of 32 comparisons. Moisture–temperature interactions affecting parent alkaloid levels in chewing tobacco were nicotine at 12 weeks, nornicotine at 6, 24, and 48 weeks, anatabine at 12 and 24 weeks, and anabasine at 12 and 48 weeks. The summation of total parent alkaloids in chewing tobacco prior to storage was 6500 μg/g, which was less than that in either moist snuff or dry snuff.

Alkaloids, Derivatives, and Nitrite after Prolonged Storage. Sums of parent pyridine alkaloids and their derivatives and nitrite in each smokeless tobacco at 0 time and after 48 weeks of storage under controlled moisture–temperature environments are shown in Figure 1. The ranges of moisture and temperature during the prolonged storage were similar to those that might be encountered in conventional postharvest storage treatments of tobacco materials and products.

After 48 weeks, sums of nitrosated, acylated, and oxidized alkaloid derivatives and nitrite (Figure 1) in moist snuff and dry snuff generally accumulated at higher levels under environmental conditions of high moisture (55.5% and 51.4%, respectively) and high temperature (32 °C)

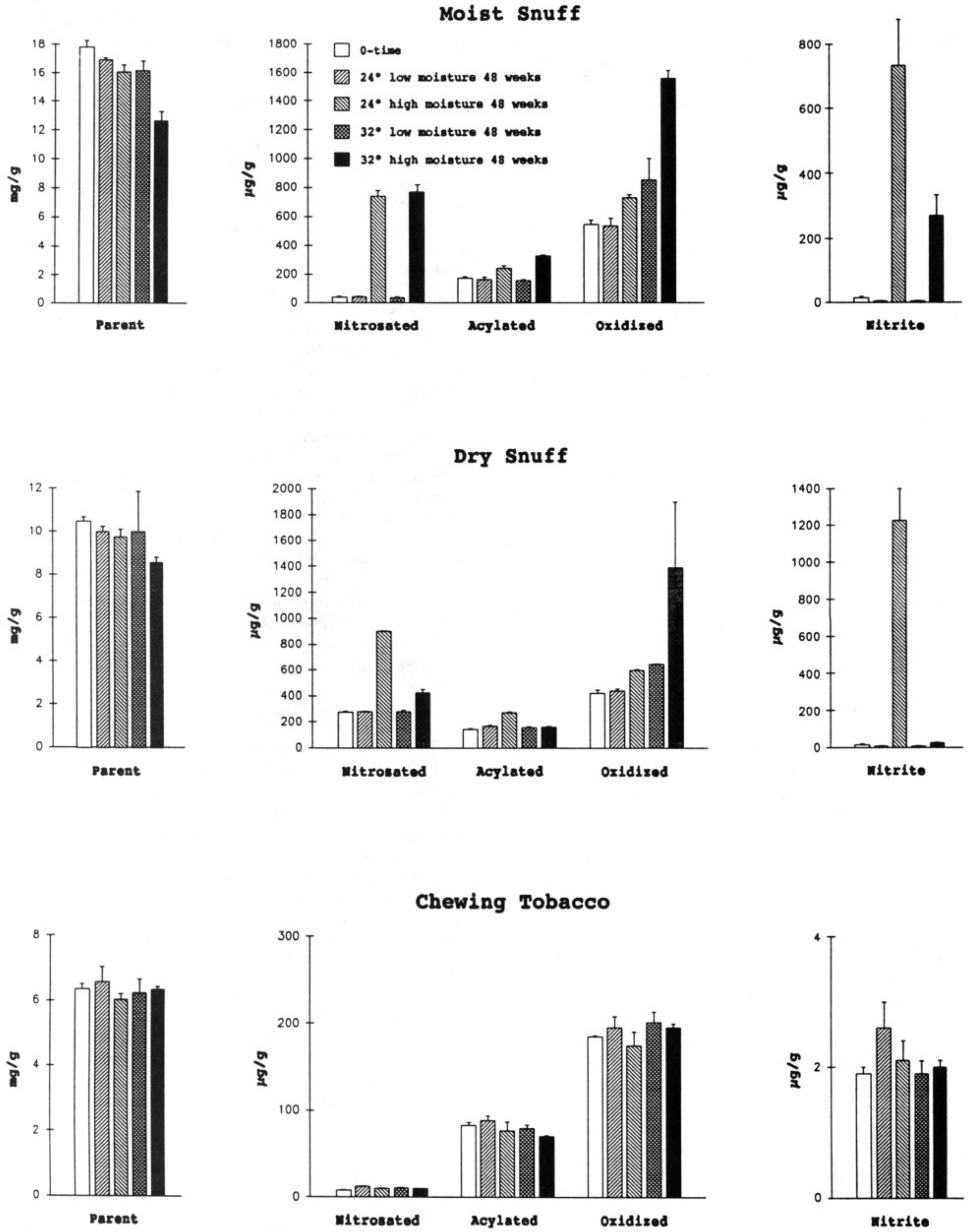

Figure 1. Sums of parent pyridine alkaloids and their derivatives and nitrite (±SD) in smokeless tobaccos at 0 time and after 48 weeks of storage under different moisture–temperature environments.

compared to low moisture (21.9% and 12.3%, respectively) and low temperature (24 °C). In these tobaccos higher moisture resulted in relatively larger increases in nitrosated and acylated alkaloid levels than levels affected by higher temperature. High moisture and high temperature were about equal, however, in their ability to yield higher levels of oxidized alkaloids at the 48-week storage duration in both snuff products. Parent alkaloid summations in moist snuff and dry snuff generally decreased under high moisture and high temperature compared to low moisture and low temperature counterpart storage treatments.

Sums of nitrosated and oxidized alkaloid derivatives, parent alkaloids, and nitrite in chewing tobacco (Figure

1) stored 48 weeks were not significantly different among the four moisture–temperature treatments. It was clear that chewing tobacco had a more satisfactory shelf life than snuff tobaccos. The high sum of sucrose and corn syrup contents of chewing tobacco (ca. 30% at 0 time) may retard the growth of nitrate-reducing bacteria, resulting in lower or unchanged levels of nitrite and less total derivatization of parent alkaloids. Sugars were not major constituents in moist or dry snuff.

Conclusions. Nitrosated, acylated, and oxidized alkaloid derivates and nitrite in moist snuff and dry snuff generally accumulated at higher rates during intervals of storage up to 1 year at high moisture (ca. 50%) and high temperature (32 °C) compared to low moisture (12–22%) and low temperature (24 °C). Parent alkaloids and nitrate in moist snuff and dry snuff generally decreased in their concentrations during intervals of storage, and the magnitudes of the changes were greater under the high moisture–high temperature environments. In these tobaccos, high moisture resulted in larger increases of nitrosated and acylated alkaloids and nitrite during storage than those increases attributed to higher temperature. High moisture and high temperature were about equally effective in causing high levels of oxidized alkaloids and lower levels of parent alkaloids. There were frequent interactions between effects of moisture and temperature during storage.

In general, concentrations of parent, nitrosated and oxidized alkaloids, nitrite, and nitrate in chewing tobacco were not significantly different among the four moisture–temperature treatments. The high sugar content of chewing tobacco (ca. 30% at 0 time) may have retarded bacterial growth required for significant changes to occur.

In the previous investigation on pyridine alkaloids and their derivatives in reference smokeless tobaccos during prolonged storage the influences of moisture were not entirely separable from those of temperature (Andersen et al., 1989a). The present study has attempted to eliminate this deficiency.

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Registry No. NNK, 64091-91-4; NNN, 16543-55-8; NAT, 71267-22-6; FNN, 38840-03-8; FAT, 61892-65-7; ANN, 5979-94-2; BNN, 69730-91-2; HNN, 38854-09-0; ONN, 38854-10-3; AAT, 61892-64-6; nornicotine, 494-97-3; anabasine, 494-52-0; cotinine, 486-56-6; nicotine, 54-11-5; 2,3′-dipyridyl, 581-50-0; anatabine, 581-49-7; nitrate, 14797-55-8; nitrite, 14797-65-0.