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Changes in Chemical Composition of Burley Tobacco during Senescence and Curing. 2. Acylated Pyridine Alkaloids

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Acylated pyridine alkaloids, 2,3′-bipyridyl, and cotinine were identified in burley tobacco by capillary GC–MS, after comparison with authentic synthesized standards. A method was developed for the quantification of the minor alkaloids, and the method was used to determine influence of curing and plant maturity on accumulation of these alkaloids in burley tobacco. The compounds quantified were 2,3′-bipyridyl (**1**), cotinine (**2**), *N*′-formylanatabine (**3a**), and *N*′-formyl- (**4a**), *N*′-acetyl- (**4b**), *N*′-butanoyl- (**4c**), *N*′-hexanoyl- (**4d**), and *N*′-octanoylnornicotine (**4e**). Plant maturity had more influence on accumulation of acylated nornicotines than curing temperature. Data have also shown there is specificity for accumulation of acylnornicotines vs acylanatabines, even though the concentrations of nornicotine and anatabine were equivalent.

In recent years there has been increased interest in the chemical changes occurring during senescence and death of plants. This is particularly true for tobacco since the consumable product is obtained after vegetative growth, senescence, and curing. Recent reviews (Burton et al., 1983; Long and Weybrew, 1981; Enzell et al., 1977) summarize many of the chemical changes that occur during senescence and air-curing. During air-curing there is a decrease of nicotine (Burton et al., 1983, 1985; Enzell et al., 1977). Some of the decrease is due to oxidation of nicotine to cotinine and other oxidation products (Frankenburg et al., 1952). Nicotine decrease may be partially explained by formation of nornicotine via demethylation reaction (Bush, 1981; Enzell et al., 1977). There is no apparent increase of nornicotine between harvest and air-curing (Burton et al., 1985); however, the absence of net changes in nornicotine do not necessarily imply that nornicotine is stable but may undergo secondary reactions. Several nornicotine derivatives (Figure 1) [*N*′-formyl- (**4a**), *N*′-acetyl- (**4b**), *N*′-butanoyl- (**4c**), *N*′-hexanoyl- (**4d**), and *N*′-octanoylnornicotine (**4e**)] have been isolated from burley tobacco (Enzell et al., 1977; Matsushima et al., 1983; Strunz and Findlay, 1985). *N*′-Formyl- and *N*′-acetylanatabine and *N*′-formyl- and *N*′-acetylanabasine are known tobacco constituents (Miyano et al., 1979). Very little is known about the formation of these secondary tobacco alkaloids during senescence and curing. Therefore, this study was initiated to identify the minor alkaloids, develop a method for their quantification, and determine whether senescence and curing temperatures influenced their accumulation.

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EXPERIMENTAL SECTION

Plant Materials. Commercially available burley tobacco (*Nicotiana tabacum* L. cv. KY 14) plants were grown at the Kentucky Agricultural Experimental Farm near Lexington in 1985. Recommended fertilization and cultural practices were followed during the growing season (Atkinson et al., 1982). After topping, tobacco was sprayed with a contact chemical (Offshoot T) to control sucker growth. Tobacco was harvested 1, 4, and 7 weeks (immature, mature, over-mature) after topping. At harvest, plants were stalk cut and stalks were speared on sticks. After wilting for 1 day in the field, half of the harvested tobacco (90 plants) was placed in a controlled environmental chamber and cured at 24 °C and 70% RH. The remaining harvested tobacco was placed in a controlled environmental chamber and cured at 32 °C and 83% RH. The use of 70 and 83% RH at 24 and 32 °C, respectively, was to maintain a constant drying rate during the curing process (Walton et al., 1982). Three replicate samples were taken from the top third of the plant at 0, 1, 2, 3, 5, 7, 9, 11, 14, 16, 19, and 21 days after each harvest to determine the changes in acylated pyridine alkaloids that occured during curing. Top stalk position was sampled because it cures more slowly. Therefore, chemical changes occurred over a longer time period. The midveins were removed, lamina were weighed, and leaf area was determined. The lamina were freeze-dried and reweighed to determine moisture content. Samples were ground to pass a 40-mesh screen and stored at −40 °C until analysis.

Preparation of Standards. *N*′-*Formylnornicotine* (***4a***). Nornicotine (Glenn and Edwards, 1978) (100 mg) was heated with an excess of formic acid (1 mL) for 8 h. Excess formic acid was removed under reduced pressure, the residue made alkaline with 10 mL of a 10% NaOH solution and extracted with 3 × 10 mL portions of ether. The ether extract was dried (Na_2SO_4) and the solvent removed to yield a straw-colored oil. After the residue was

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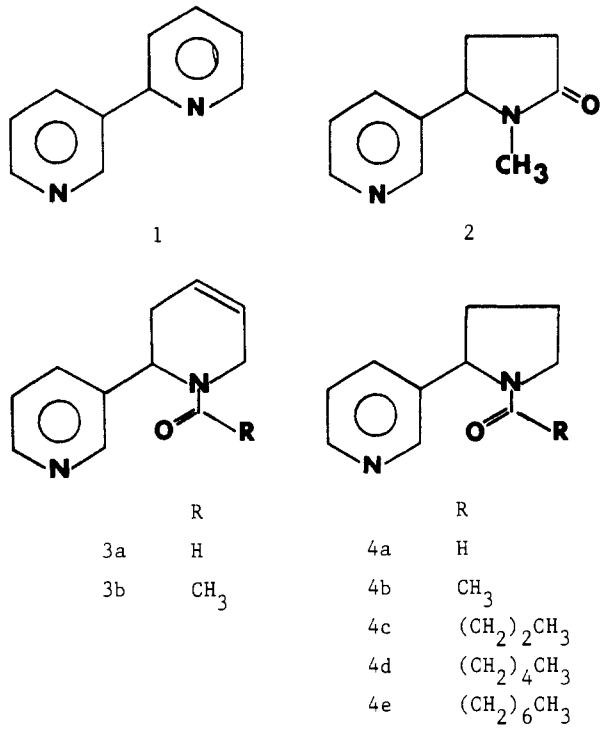

Figure 1. Minor alkaloids analyzed in burley tobacco.

Table I. Mass Spectral Data for Acylated Pyridine Alkaloids

compd	major ions, m/z
3a	188 (M^+, 4.1), 54 (100), 159 (21.9), 55 (15.1), 105 (14.2), 53 (13.2)
4a	176 (M^+, 43.6), 147 (100), 119 (89.7), 105 (68.9), 70 (56.0), 148 (43.6)
4b	190 (M^+, 7.8), 70 (100), 147 (89.5), 120 (71.6), 119 (34.3), 105 (22.5)
4c	218 (M^+, 8.9), 70 (100), 147 (73.7), 106 (61.6), 120 (50.1), 119 (34.5)
4d	246 (M^+, 5.0), 70 (100), 106 (93.1), 120 (73.5), 147 (54.6), 189 (42.5)
4e	274 (M^+, 18.4), 106 (100), 70 (73.8), 147 (57.2), 189 (55.2), 120 (51.1)

transferred to a micro-short-path distillation apparatus, it was distilled to yield 60 mg of *N'*-formylnornicotine. The purity and structure of **4a** were confirmed by GC–MS using a capillary gas chromatograph interfaced to a Finnigan Model 705 ion trap detector (ITD). The spectrum of **4a** (Table I) was identical with that required by Glenn and Edwards (1978).

N'-Formylanatabine (***3a***). This compound was prepared as described above with anatabine (Quan et al., 1965) as the starting material. Mass spectral data are presented in Table I.

N'-Acylnornicotines (***4b–e***). Nornicotine (100 mg), CCl_4, (0.5 mL), triethylamine (0.5 mL), and an excess of the appropriate acid anhydride or acid chloride were allowed to stand at room temperature overnight in a round-bottom flask equipped with a drying tube. After the solvent was removed under reduced pressure, the residue was dissolved in 5 N NaOH (5 mL). The alkaline solution was extracted with ether (4 × 5 mL) and dried over sodium sulfate. After removal of the ether, the residue was transferred to micro-short-path distillation apparatus. Each *N'*-acylnornicotine was purified by distillation under vacuum. The following acylated alkaloids were synthesized by this method: *N'*-acetylnornicotine, *N'*-acetylanabasine, *N'*-butanoylnornicotine, *N'*-hexanoylnornicotine, and *N'*-octanoylnornicotine. Their purity and structure were confirmed by capillary GC/MS.

Table II. Percent Recovery of Minor Alkaloids[a]

alkaloid	% recovery
2,3'-bipyridyl (1)	69.6 ± 3.5
cotinine (2)	21.6 ± 0.7
N'-formylnornicotine (**4a**)	23.5 ± 0.9
N'-formylanatabine (**3a**)	63.2 ± 1.4
N'-acetylnornicotine (**4b**)	27.2 ± 1.1
N'-butanoylnornicotine (**4c**)	71.2 ± 2.1
N'-hexanoylnornicotine (**4d**)	100.1 ± 7.0
N'-octanoylnornicotine (**4e**)	108.1 ± 8.2

[a] Means and standard deviations of five replicate analyses.

Analyses of Acylated Alkaloids. The method of analysis was adapted from a procedure published by Andersen and Kemp (1985). Burley tobacco (1.0 g) in a 0.1 M citrate–phosphate buffer, pH 4.5, containing 5 mM ascorbic acid was shaken at room temperature for 45 min. The solution was adjusted to pH 5.0 with HCl, and the amides were partitioned into ethyl acetate (30 mL). After the phases separated, an aliquot (25 mL) was removed and extracted with HCl (3 × 5 mL). The aqueous phase was adjusted to pH 5.0 with 10 N NaOH and extracted with chloroform (3 × 5 mL). Combined extacts were diluted to 25 mL and dried over Na_2SO_4. A 2.0-mL aliquot was taken to dryness and redissolved in 100 μL of chloroform containing 4.8 μg/μL azobenzene as the internal standard. Conditions for GC analyses were identical with those reported by Andersen and Kemp (1985). Quantification was carried out by internal standardization with azobenzene after calibration of retention times and response factors with standards of cotinine (**2**), 2,3'-bipyridyl (**1**), *N'*-formylnornicotine (**4a**), *N'*-acetylnornicotine (**4b**), *N'*-formylanatabine (**3a**), *N'*-butanoylnornicotine (**4c**), *N'*-hexanoylnornicotine (**4d**), and *N'*-octanoylnornicotine (**4e**) from a five-point calibration equation and corrected for the recoveries of the authentic compounds carried through the entire analytical procedure (Table II). Peak identities on selected tobacco extracts were verified by a Finnigan Model 705 ITD interfaced to a Varian 3700 GC equipped with an on-column injector and a 60 m × 0.31 mm DB-5 column.

Analyses of Major Alkaloids. Major alkaloids were quantified by capillary chromatography according to a procedure described by Severson et al. (1981).

RESULTS AND DISCUSSION

Identification of Acylated Pyridine Alkaloids. The presence of acylated pyridine alkaloids in tobacco has been reported. For example, Warfield et al. (1972) reported the presence of both **4a** and **4b** in tobacco, Matsushita et al. (1979) identified **4c**, Bolt (1972) isolated both **4d** and **4e** in tobacco, and Miyano et al. (1979) also identified **3a** in tobacco. However, confirmation of the structures could best be determined by GC–MS of authentic compounds. Synthesis of these compounds and determination of their fragmentation patterns have confirmed their identity (Table I). Since **4a–e** are homologues, they exhibited similar fragmentation patterns. The predominant mass ions of m/z 70, 140, 119, 120 105 are due to fragmentation of the nornicotine moiety (Glenn and Edwards, 1978). In all cases, the intensity of the molecular ion was sufficient to identify each acylnornicotine in the tobacco isolates. Because of their similar fragmentation patterns, *N'*-propionylnornicotine and *N'*-pentanoylnornicotine were tentatively identified in tobacco. However, they were present in trace quantities, and no attempt was made to quantify them or synthesize the authentic compounds. The fragmentation pattern for *N'*-formylanatabine (**3a**)

Table III. Changes in Minor Alkaloids during Curing When Harvested at Three Stages of Maturity and Cured at 24 °C/70% RH[a]

day[b]	1	2	4a	4b	3a	4c	4d	4e	total[c]
				Harvest 1: 1 Week after Topping					
0	1.80	8.44	7.17	2.35	1.50	0.00	0.29	1.40	11.21
1	3.11	19.65	13.69	6.81	2.45	0.04	0.17	0.52	21.24
2	2.16	18.49	15.24	4.04	2.93	0.01	0.16	0.52	19.97
3	0.45	9.91	9.17	2.21	1.86	0.04	0.29	1.35	13.07
5	1.40	10.22	11.57	4.48	2.02	0.00	0.22	0.89	17.17
7	1.65	11.87	11.46	2.43	2.03	0.07	0.36	1.10	15.42
9	1.13	10.31	7.39	2.18	1.23	0.05	0.35	1.81	11.78
12	1.37	8.52	7.85	2.44	1.69	0.00	1.00	5.50	16.79
14	1.46	13.94	11.17	3.34	2.69	0.04	1.16	6.31	22.03
16	3.68	19.69	27.26	8.09	4.35	1.96	5.92	18.76	62.00
19	3.83	22.27	22.68	3.40	4.19	0.20	3.21	13.08	42.58
21	4.45	24.18	29.84	6.45	4.26	0.40	3.54	12.37	52.60
LSD (05)	1.10	5.44	6.69	2.46	1.18	1.28	1.15	2.25	11.11
				Harvest 2: 4 Weeks after Topping					
0	2.47	25.62	17.90	2.84	4.72	0.00	1.16	7.49	29.39
1	3.35	29.80	30.31	4.37	6.09	0.00	10.66	48.37	93.71
2	1.47	26.55	24.79	5.58	5.20	0.07	3.35	9.63	43.87
3	2.73	36.18	27.82	6.15	5.34	0.55	6.77	23.79	65.08
5	3.48	27.16	29.46	6.08	6.18	0.57	10.84	38.21	85.15
7	5.60	40.44	31.06	5.45	5.12	0.00	11.82	39.37	87.70
9	4.24	31.14	24.36	3.25	4.92	0.00	10.72	38.03	76.36
12	12.20	63.17	40.73	24.90	9.88	0.38	17.52	63.16	146.69
14	28.81	118.13	61.23	66.65	23.27	1.90	36.27	102.19	268.24
16	20.32	103.67	59.43	72.21	21.08	1.60	33.13	94.35	260.72
19	18.16	73.94	119.75	55.06	19.14	3.60	33.55	90.19	302.15
21	14.89	57.23	99.53	51.85	16.44	2.56	35.13	98.35	287.42
LSD (05)	4.37	21.10	33.18	16.14	5.27	1.92	13.27	28.78	82.67
				Harvest 3: 7 Weeks after Topping					
0	4.61	66.22	19.07	3.30	3.09	0.25	9.78	38.16	70.55
1	9.13	110.60	39.10	7.89	9.63	2.44	40.57	164.30	217.79
2	2.91	96.88	34.67	7.97	7.24	1.64	26.62	118.17	189.07
3	9.67	154.95	69.01	33.93	10.68	6.64	71.58	219.71	400.87
5	6.53	96.10	64.71	17.21	7.25	8.49	56.17	151.72	298.30
7	15.48	144.15	73.30	33.76	16.03	2.34	36.29	145.97	291.66
9	12.80	93.17	44.68	10.32	9.41	1.70	26.37	92.35	175.42
12	20.32	138.98	71.13	20.35	16.65	1.75	26.20	100.68	220.11
14	22.51	189.21	134.58	40.67	17.03	8.24	69.32	216.17	468.99
16	27.74	135.40	108.10	37.31	21.28	3.54	38.40	112.34	299.69
19	24.11	129.49	114.40	39.88	20.01	2.38	32.98	99.12	288.76
21	20.83	140.83	96.91	33.77	20.01	1.62	32.55	103.31	268.16
LSD (05)	8.05	51.78	45.28	24.94	6.34	6.45	45.28	97.42	198.60
harvest x date LSD (05)	10.25	43.69	44.67	28.24	8.17	4.18	30.88	78.10	163.76

[a] Micrograms/gram. [b] Days after harvest. [c] 4a + 4b + 4c + 4d + 4e.

was dissimilar to the acylnornicotines, but its pattern was similar to anatabine fragmentation pattern (Burton et al., 1985). This characteristic fragmentation pattern has also led to the tentative identification of *N'*-acetylanatabine in cured tobacco lamina.

Isolation and Quantification of Minor Alkaloids. There has been little progress toward the development of a method for the quantification of the minor alkaloids in tobacco. Matsushima et al. (1983) reported the isolation of these compounds by extraction with dichloromethane. Their extraction procedure also isolated the major alkaloids in tobacco, nicotine,nornicotine, anatabine, and anabasine. These major components interfered with the quantification of the minor alkaloids (Burton et al., 1985), and therefore the development of a convenient method for the enrichment of the minor alkaloids in tobacco was needed. Extraction of a buffered tobacco extract (pH 5) with ethyl acetate offered a means to separate less basic acylated amines from the more basic nicotine, anatabine, nornicotine, and anabsine. The more basic alkaloids remained in the buffered aqueous solution, thereby enriching the ethyl acetate fraction with less basic 2,3'-bipyridyl, cotinine, and the *N'*-acylated alkaloids. This enrichment reduced the background of the chromatogram due to overloading of the column with nicotine and other contaminants. This procedure simplified the isolation of the minor alkaloids, although the recoveries from the authentic standards were not all quantitative (Table II). However, recoveries of the more lipophilic *N'*-hexanoyl- and *N'*-octanoylnornicotine were quantitative. Even though the recoveries for the more hydrophilic acylated nornicotines were low (**4a–c**), their small standard deviations indicated the precision of analyses was acceptable. Even though there was a wide range in recoveries of the individual alkaloids, the isolation procedure for enrichment of these alkaloids allowed for their convenient quantification.

Influence of Curing on Minor Alkaloid Accumulation. Minor alkaloid contents of the lamina from the top stalk position of burley tobacco harvested at 1, 4, and 7 weeks after topping and cured at a constant 24 °C and 70% RH are presented in Table III. Curing tobacco at constant temperature and relative humidity eliminated indeterminant variables that would be encountered if cured at ambient temperature and humidity (Burton and Kasperbauer, 1985). Therefore, it was possible to compare only the influence of plant maturity on accumulation of the

Table IV. Concentrations (mg/g) of Major Alkaloids from the Top Third of the Plant at Harvest

harvest[a]	nicotine	nornicotine	anatabine	anabasine
1	18.00 ± 1.80	0.45 ± 0.06	0.78 ± 0.06	0.06 ± 0.01
2	38.04 ± 4.30	0.83 ± 0.15	1.89 ± 0.28	0.19 ± 0.02
3	49.50 ± 0.44	1.85 ± 0.04	1.80 ± 0.54	0.25 ± 0.01

[a] 1, 3, and 7 weeks after topping.

minor alkaloids. Data presented not only reflect changes that occurred during curing but the influence of immature, mature, and over-mature tobacco on the accumulation of these minor alkaloids during the air-curing process. 2,3′-Bipyridyl and cotinine were included in Table III because they are pyridine alkaloid oxidation products. 2,3′-Bipyridyl (1) arises from the aromatization of anatabine, cotinine (2) is derived from the oxidation of nicotine, and both minor alkaloids are formed during the fermentation of tobacco (Frankenburg et al., 1952). For immature tobacco, there was only a small but significant increase of bipyridyl during curing; however, in the mature and over-mature tobacco, there was significant increase of 1. This, in part, is reflected in the higher concentration of anatabine in the mature tobaccos (Table IV). The twofold increase of anatabine in the mature tobacco levels cannot account for the over fivefold increase observed for 1 in the cured mature tobacco. Therefore 2,3′-bipyridyl formation is favored when tobacco is harvested at normal maturity or over-maturity.

Cotinine values for these samples follow essentially the same trend except that over-mature tobacco contained higher amounts of cotinine at harvest. At the 21 days, there were large differences between cotinine levels and time of harvest. Cotinine concentration increased slightly during curing for immature, mature, and over-mature tobacco. From these data it was apparent that maturity contributed to cotinine formation.

During curing there was an increase of *N*′-formylnornicotine (**4a**) for all harvest dates. For immature tobacco, the increase occurred 2 weeks after harvest, whereas in mature tobacco lamina the increase occurred approximately 2 days earlier and the increase for over-mature tobacco occurred within 2 weeks after harvest. By day 21 after harvest, the level of **4a** in the mature was 3 times higher than in the immature tobacco. This shows that lamina maturity influence the final concentration of **4a**. *N*′-Formylnornicotine may be formed via formylation of nornicotine or oxidation of the *N*-methyl group of nicotine. This latter mechanism was proposed by Leete (1977) to explain the conversion of nicotine to nornicotine. Data obtained from this study cannot differentiate the source of *N*′-formylnornicotine; however, if one examines the profiles for acetylnornicotine (**4b**), there were similarities with the profiles for **4a** and also *N*′-formylanatabine (**3a**). These similar profiles indicate they were formed via acylation of the secondary alkaloid (nornicotine or anatabine).

Butanoylnornicotine (**4c**) had the lowest concentration of the quantified minor alkaloids, and the over-mature lamina contained the highest concentration of this alkaloid. The concentration of **4c** increased within 2 weeks after harvest, especially for tobacco harvested at maturity.

The accumulation of *N*′-hexanoyl- (**4d**) and *N*′-octanoylnornicotine (**4e**) were different from **4a–c**, especially for the over-mature tobacco (Table III). At the three levels of maturity there were increases of **4d** and **4e** during curing. However, for the over-mature tobacco, there was a significant increase of **4e** within the first day after harvest. This was not an artifact since these high levels are maintained for both **4d** and **4e** throughout air-curing. By

Table V. Changes in Minor Alkaloids during Curing When Harvested at Three Stages of Maturity and Cured at 32 °C/83% RH[a]

day[b]	1	2	4a	4b	3a	4c	4d	4e	total[c]
				Harvest 1: 1 Week after Topping					
0	1.80	8.40	7.17	2.35	1.49	0.00	0.29	1.40	11.21
1	3.11	19.65	13.69	6.81	2.45	0.04	0.17	0.52	21.24
7	1.96	7.16	0.00	2.38	2.46	0.00	1.30	4.77	8.45
12	3.08	15.82	16.90	0.90	2.25	0.15	0.72	2.18	20.84
14	8.31	27.78	17.71	0.91	3.91	0.00	1.15	2.5	22.30
16	8.93	25.87	13.65	1.20	1.77	0.18	2.01	4.48	64.13
19	33.76	99.33	19.48	19.61	14.10	0.00	4.17	5.50	48.76
21	36.95	90.33	75.31	1.70	10.08	0.00	6.49	12.15	95.65
LSD (05)	5.02	18.18	26.44	9.02	3.05	0.21	1.41	4.69	51.63
				Harvest 2: 4 Weeks after Topping					
0	2.47	25.62	17.90	2.84	4.72	0.00	1.16	7.49	29.39
1	3.35	29.80	30.31	4.37	6.09	0.00	10.66	48.37	93.71
7	8.32	33.32	22.65	*d*	6.41	0.00	6.26	19.08	47.99
12	21.10	62.77	54.77	*d*	11.40	0.00	6.80	12.95	74.52
14	58.51	76.01	198.75	74.17	21.28	3.41	42.10	18.97	337.39
16	53.78	85.66	136.66	31.61	24.36	0.00	25.92	56.41	250.61
19	102.01	144.69	240.32	44.22	45.02	0.00	35.66	60.66	380.26
21	65.62	83.54	81.70	*d*	19.95	0.00	3.07	3.66	88.43
LSD (05)	10.37	42.90	79.21	7.50	8.46	1.78	13.47	21.11	97.44
				Harvest 3: 7 Weeks after Topping					
0	4.61	66.23	19.07	3.30	3.09	0.25	9.78	38.16	70.55
1	9.13	110.60	39.10	7.89	9.63	2.44	40.57	164.30	217.79
7	15.43	76.81	57.48	*d*	14.05	0.00	28.76	67.95	154.19
12	45.37	141.32	162.72	*d*	27.54	0.00	25.16	56.09	243.97
14	74.50	151.26	193.27	*d*	34.56	0.00	26.99	40.76	261.02
16	62.83	128.54	155.78	*d*	24.64	0.00	8.25	15.90	179.93
19	93.31	109.13	89.21	102.14	37.13	0.00	19.35	42.57	253.26
21	163.55	190.49	370.79	171.02	58.59	14.95	99.79	227.63	884.18
LSD (05)	19.79	32.56	123.08	21.35	7.99	7.55	59.53	95.92	228.58
harvest × date LSD (05)	51.02	55.19	125.13	57.61	34.95	5.76	40.78	95.27	147.83

[a] Micrograms/gram. [b] Days after harvest. [c] **4a** + **4b** + **4c** + **4d** + **4e**. [d] Peak was not resolved from *N*-nitrosoanatabine.

the end of curing, there was no significant difference between the concentration of these compounds in mature and over-mature lamina. It is obvious that harvest stress facilitated the accumulation of **4d** and **4e** significantly without dramatically increasing **4a–c**. The specificity for the enhanced accumulation of **4d** and **4e** is not known but is currently being investigated.

Comparison of the total acylnornicotine concentration from the three harvest dates shows the influence of plant maturity on the accumulation of these minor alkaloids (Table III). The acylnornicotines at 21 days from the later two harvest dates are over 5 times higher than those from the early harvest date. The higher levels of these acyl compounds in the cured lamina were probably due to the higher concentration of their precursor, nornicotine (Table IV). The concentration of nornicotine was 4 times higher in the overmature tobacco than the immature sample (Table IV). Even though there is some relationship between nornicotine concentration and that of its acylated derivatives, the level of acylated nornicotines at day 21 was 21% of the nornicotine concentration, showing these components are in significant concentration in cured burley tobacco. The influence of maturity on the formation of acylated nornicotines was evident. Over 2 weeks were required before significant increases in the acylated compounds were detected in the immature tobacco, whereas the increase of these compounds for the mature tobacco occurred in less than 2 weeks. Over-mature tobacco lamina showed a large increase in these compounds within 1 day after harvest. The LSD for the over-mature tobacco was large and may be due to limited sample size taken (three plants/replicate). This sample size was required since only 90 plants could be cured in the constant-temperature/humidity chamber, and there were 10 sampling dates. Even though the LSD was large, comparison of the levels of total acylated nornicotine showed the over-mature tobacco had the highest concentration of the acylated nornicotines within 1 day after harvest, whereas immature tobacco had the lowest concentration of the acylated alkaloids. The LSD for the harvest × day interaction showed overmature tobacco contain significantly high amounts of the acylated nornicotine.

Concentrations of the minor alkaloids in tobacco cured at 32 °C and 83% RH are presented in Table V. The increase of the minor alkaloids during curing at higher temperature, in general, paralleled those obtained from normal curing temperatures (Table III). In some instances the concentrations of individual minor alkaloids were higher. For example, 2,3′-bipyridyl concentration was greater in the tobacco when cured at 32 °C/83% RH vs 24 °C/70% RH for all harvest dates. This could be predicted since higher temperatures and humidities at the end of curing should facilitate fermentation, thereby enhancing 2,3′-bipyridyl accumulation. Calculation of the LSD (05) for the 32 °C/83% RH vs 24 °C/70% RH curing regime showed there was no statistical differences between curing temperature for the levels of the other minor alkaloids. Higher temperature did not facilitate cotinine or acylnornicotine formation to any significant amount in comparison to normal curing temperature. This indicates that curing temperature did not affect a large increase of the accumulation of cotinine or the acylnornicotines.

There was no effect of temperature on the accumulation of formylanatabine (**3a**), indicating that plant maturity rather than temperature has the greatest influence on the final concentration of this component. Acetylanatabine (**3b**) also was identified in the extract (Andersen et al., 1988); however, no other acylanatabine derivatives (i.e., hexanoyl and octanoyl) were detected. This is of interest since the concentration of anatabine in tobacco is as great as that of nornicotine. Since total acylnornicotine concentrations were generally 10 times greater than the total acylanatabines concentrations, there must be a specificity for the accumulation of the acylnornicotine derivatives. Whether this is due to reactivity of the alkaloids or enzyme-mediated reactions is not known at this time.

In conclusion, a method was adapted for the detection and quantification of the weakly, basic minor alkaloids in tobacco. It was possible to determine the influence of plant maturity and curing temperatures on the accumulation of these minor alkaloids. Data showed that, during curing, mature and over-mature tobacco produced significantly larger quantities of the minor alkaloids than the immature tobacco. Except for the over-mature tobacco, the minor alkaloids increase during the latter stages of curing.

Registry No. 1, 581-50-0; 2, 486-56-6; 3a, 61892-65-7; 4a, 38840-03-8; 4b, 5979-94-2; 4c, 69730-91-2; 4d, 38854-09-0; 4e, 38854-10-3; nicotine, 54-11-5; nornicotine, 494-97-3; anatabine, 581-49-7; anabasine, 494-52-0; formic acid, 64-18-6.

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Olfactive Properties of Alkylpyrazines and 3-Substituted 2-Alkylpyrazines

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Odor profiles were determined for the series of alkylpyrazines and 3-substituted 2-alkylpyrazines. The odor threshold decreases with an increasing number of carbon atoms in the side chain. It is at a minimum in pentylpyrazines and increases again irrespective of the substituents at the 3-position. In general, the odor thresholds of 3-substituted 2-alkylpyrazines increase in the following order: $OCH_3 < OC_2H_5 \leq SCH_3 < SC_2H_5 < OC_6H_5 \leq SC_6H_5$. The alkyl substituents play an important role in the tonalities of the pyrazines.

It has been well recognized that the alkylpyrazines significantly contribute to the flavor of heat-treated food (Fors and Olofsson, 1986). 2-Isobutyl-3-methoxypyrazine was isolated from bell peppers and was shown to possess an extremely low odor threshold (Buttery et al., 1969a,b). The odor thresholds of some alkylpyrazines and 3-substituted 2-alkylpyrazines in water were reported in the literature (Fors and Olofsson, 1985; Calabretta, 1978; Takken et al., 1975; Teranishi et al., 1974; Seifert et al., 1970). In general, the odor thresholds decrease with increasing chain length within each isomer. One should be careful in drawing conclusions from a comparison of threshold values determined by the different groups. In order to derive the relationship between chemical structures and their odor thresholds, we have studied the effects of varying the alkyl chain length of the alkylpyrazine and 3-substituted 2-alkylpyrazines on their olfactive properties.

EXPERIMENTAL SECTION

Instrumentation. IR, NMR, and mass spectral data were obtained on a Jasco IR-S, a JNM-PMX 60, and a Hitachi Model M-80A, respectively.

Materials. All starting chemicals were obtained from reliable commercial sources and used without further purification. Pyrazine and methyl-, ethyl-, and methoxy-, 2-methoxy-3-methyl-, 2-ethyl-3-methoxy-, 2-ethoxy-3-ethyl-, and 2-ethyl-3-(methylthio)pyrazine were commercially available (Pyrazine Specialties).

Synthesis of Alkylpyrazines 1–7. Alkylpyrazines were prepared from the parent pyrazine by alkylation with the appropriate aldehyde in sodium dimethoxyethane solution (Bramwell et al., 1971).

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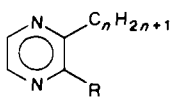
Synthesis of Ethoxy-, Phenoxy-, (Methylthio)-, (Ethylthio)-, and (Phenylthio)pyrazine (15, 20, 24, 29, 36). Chloropyrazine was reacted with sodium ethoxide, sodium phenoxide, sodium thiomethoxide, sodium thioethoxide, or sodium thiophenoxide to obtain **15**, **20**, **24**, **29**, and **36**, respectively (Masuda et al., 1981).

Synthesis of 3-Substituted 2-Alkylpyrazines. A series of alkylpyrazines has been chlorinated specifically in the 3-position with sulfuryl chloride in the presence of *N,N*-dimethylformamide (Bramwell et al., 1972). These chloro derivatives were subsequently allowed to react with the corresponding sodium alkoxide, sodium phenoxide, sodium thioalkoxide, or sodium thiophenoxide to obtain the desired alkoxy-, phenoxy-, (alkylthio)-, and (phenylthio)alkylpyrazines, respectively (Masuda et al., 1981).

Sensory Evaluation. The threshold values and the odor characteristics of the pyrazines were determined as described previously (Masuda and Mihara, 1986).

RESULTS AND DISCUSSION

The yields and mass spectral data of the pyrazines I are shown in Table I. The IR and ^1H NMR spectral data of the new pyrazines were recorded and analyzed. (See paragraph at the end of paper regarding supplementary material.)



I, n = 0–10

Figure 1 shows the relation between log $1/T$, where T is the odor threshold, and the carbon number of the alkyl side chain, n, for various alkylpyrazines and 3-substituted 2-alkylpyrazines. The log $1/T$ increases with increasing carbon number and has a maximum value at 5. It then

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