Pyrolysis of Cherry Red Tobacco and 1-Deoxy-1-[(S)-2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructose (Pyranose and Furanose Isomers) Amadori Products of Cherry Red Tobacco

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Nornicotine (NN) reacted with glucose and malic acid as a catalyst in dry methanol produced two NN Amadori rearrangement isomers (NDF). 1-Deoxy-1-[(S)-2-(3-pyridyl)-1-pyrrolidinyl]-β-D-(fructopyranose) was the major isomer and -(fructofuranose) the minor isomer. Pyrolysis of NDF at 500 °C produced nicotine, NN, myosmine, pyridine, β-picoline, 3-vinylpyridine, substituted NN, and compounds that produced pleasant and sweet aromas. Eleven percent of the NN content from "cherry red" (CR) tobacco in this study was in NDF form. CR tobacco pyrolyzed at 500 °C produced 10 of the same pyrolytic products as the NDF isomers. This suggests that NDF in CR tobacco possibly contributes the same pyrolytic products as the synthetic NDF. Pyrazines, lactones, furan derivatives, and short-chain carboxylic acids were also produced from pyrolysis of CR tobacco.

Keywords: *Nornicotine Amadori compounds; cherry red tobacco; pyrolysis; alkaloids; nicotine; aroma*

INTRODUCTION

Nornicotine (NN) is a secondary alkaloid produced in the leaf tissue of *Nicotiana tabacum* L. from enzymatic demethylation of nicotine during senescence and curing. Conversion of nicotine to NN is regulated by a single gene (Mann et al., 1964). Although NN is found in all cultivated tobacco, flue-cured cultivars producing NN in excess of 13% of the total alkaloid concentration are considered alkaloid converters. Alkaloid converters are considered undesirable for smoking and other use. Cherry red (CR) color in flue-cured tobacco results from NN and o-diphenols (Weeks et al., 1993). Although breeding lines are thoroughly screened to eliminate converters before they are released as commercial varieties, at least 0.8% of each flue-cured crop produces CR tobacco (Bowman and Rawlings, 1985).

Leffingwell (1976) reported that cured tobacco leaves containing both nitrogenous compounds and reducing sugars react to form isolable Amadori compounds that produce pyrazines upon combustion. Koiwai et al. (1979) isolated 150 mg of crystalline 1-(1′-2′S-nornicotino)-1-deoxy-β-D-fructofuranose (NDFF), an Amadori rearrangement product, from 1.5 kg of CR tobacco strain TR 261, which produced 1% NN by weight. Siddiqui et al. (1981) isolated the Amadori compound 1-deoxy-1-[(S)-2-(3-pyridyl)-1-pyrrolidinyl]-β-D-fructopyranose (NDFP) from flue-cured lamina of Delhi tobacco and showed evidence for the structure of this compound from GC/MS and NMR spectroscopy.

This study was undertaken to (1) synthesize an Amadori rearrangement compound, (2) confirm the structure of the synthetic product by obtaining spectral data, (3) quantitate the Amadori product in CR tobaccos, (4) identify the pyrolysis products of the Amadori product, (5) characterize the aromas produced from pyrolysis of the Amadori product, and (6) compare the pyrolysis products formed from CR tobacco and the Amadori rearrangement compound.

MATERIALS AND METHODS

Synthesis and Purification of NN Amadori Rearrangement Compound. A modified method of Koiwai et al. (1979) was used to synthesize and purify the NN Amadori rearrangement product (NDF). Nornicotine [synthesized in the laboratory using the method of Brandange et al. (1976) and Hu et al. (1974)] (1.92 g, 0.013 M) and 1.98 g (0.011 M) of D-glucose (Fisher Scientific, Fair Lawn, NJ) were refluxed in 25 mL of dry methanol for 1 h. A catalytic amount of malic acid (150 mg) was added to the reaction mixture, and the solution was refluxed for an additional 2 h. The reaction mixture was concentrated under vacuum at 45 °C, and 25 mL of deionized, distilled water was added to the reaction mixture. The aqueous solution was passed through a Rexyn 101 (H+) 50 × 8 cm resin column (75–150 mesh) (Fisher Scientific). The column was washed with 500 mL of deionized water to remove the catalyst and unreacted material. The column was eluted with 1 L of 2 N NH_4OH, and the eluant was retained in 100 mL fractions. The first 500 mL of the eluant contained the Amadori product plus impurities. Ammonia was removed from the solution, and the impure solution was concentrated by freeze-drying. The freeze-dried mixture was further purified on a 2.5 × 30 cm, 210–400 mesh silica gel column (Whatman Laboratory Division, Clinton, NJ) using 30% aqueous ethanol as the mobile phase. NDF was eluted from the column in two 100 mL fractions, and the composition of each fraction was determined by using 5 × 20 cm silica GF gel TLC plates (Fisher Brand, Fisher Scientific). $CHCl_3$/MEOH/NH_4OH (60:30:10 v/v/v) was used as the developing solvent. The thin-layer plates were sprayed with 3% *p*-aminobenzoic acid (Fisher Scientific) in 95% ethanol, dried, and reacted with CNBr (Eastman Kodak, Rochester, NY) vapor in a closed chamber in a fume hood to detect the NN complex. The two fractions were combined, and the aqueous solution was concentrated by freeze-drying.

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The product was crystallized from $CHCl_3$. After desiccation under N_2, 1.58 g of the crystalline NDF product was recovered. Three milligrams of the crystallized Amadori product was dissolved in 3 mL of HPLC grade dry methanol for HPLC and UV analysis.

The UV spectrum of NDF was obtained using Waters 990 HPLC equipped with a diode array detector and a 4.6 × 250 mm Bio-Rad Bio-Sil Amino 5S column (Bio-Rad, Richmond, CA). Operating conditions were isocratic using CH_3CN/HOH (90:10 v/v) at 1 mL/min, scanning from 195 to 320 nm. The HPLC chromatogram was obtained at 260 nm.

A mass spectrum was obtained from a 3 μL injection from a 3 mL solution of recrystallized Amadori product containing 1 mg/mL using a thermospray interface between a VG-20-253 MS and the HPLC. A mass spectrum was also obtained from on-column injection of TMS derivatives of the NN Amadori rearrangement product. Five milligrams of recrystallized Amadori product was mixed with 100 μL of pyridine and 100 μL of BSTFA with 1% TMCS (Supelco Co., Bellefonte, PA). The mixture was stored in the dark at 25 °C. One microliter of the silylated solution was injected (on-column) to a Supelco DB-17 (5% phenyl methyl silcone) 30 m (WCOT), 0.32 i.d., 0.5 μm film, using a HP-5895 MS (EI 70 eV). Helium was used as carrier at 10 psi. The oven temperature was programmed from 40 to 180 °C at 50 °C/min and from 180 to 280 °C at 5 °C/min. The ^{13}C NMR spectrum was recorded at 25 °C with a Bruker AC-300P instrument (75.47 MHz, 5 mm tube). Chemical shifts were measured in D_2O relative to 3-(trimethylsilyl)propionic-d_4 acid, sodium salt, used as internal standard.

Pyrolysis of Amadori Product. Pyrolysis of NDF crystals was performed using a CDS Pyroprobe Model 100, equipped with a quartz boat, attached directly to the injection port of a Hewlett-Packard 5890 GC. Different amounts of NDF crystals (0.1, 0.55, and 1 mg) were weighed in a dry atmosphere and pyrolyzed for 10 s at 500 °C. Pyrolysates were separated using a 0.32 mm i.d. (WCOT) 60 m Supleco DB-Wax fused silica capillary column (Supelco); column temperature was programed from −40 to 220 °C at 2 °C/min, with helium carrier (1 mL/min), to obtain a total ion chromatogram. A postcolumn fused silica splitter was attached to the VG 20-253 MS and a sniffing port. The 0.32 mm fused silica tubing attached to the sniffing port was wrapped with heating tape to maintain the temperature high enough to avoid condensation of column effluent. This allowed a professionally trained sniffer employed by Brown and Williamson Tobacco Co. to perceive aroma at the sniffing port as the pyrolysates eluted.

The sniffing port was located in the proximity of a personal computer programmed to allow the sniffer to produce a chromatogram of the eluting aromas from the column effluent. A peak was generated for each aroma that was detected. Peak height and peak width of individual peaks of the aroma chromatogram were dependent on the intensity of each aroma generated that was detected from the column effluent, similar to the procedure of Acree et al. (1984). At the same time, the sniffer described the characteristic aroma produced to a person recording the description of the aroma. When the MS chromatogram was terminated, retention times of the aroma chromatogram and the MS chromatogram were compared. Tentative identification of the perceived aromas of the pyrolysates were assigned to the peaks of the aroma chromatogram using the MS spectra.

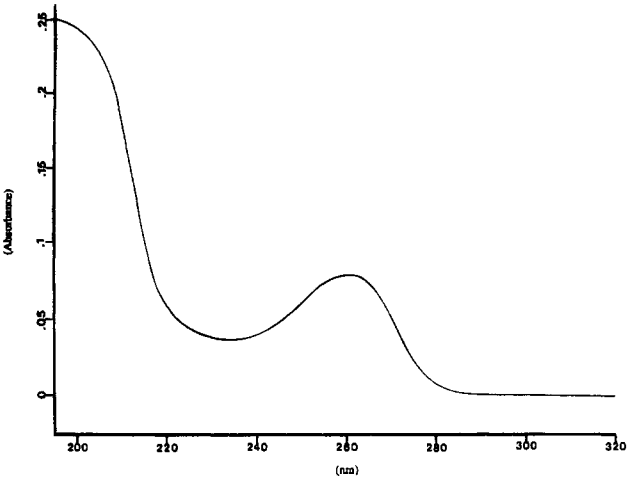


Figure 1. UV scan of NDF products.

Extraction and Analysis of a NN Amadori Compound from Cherry Red Tobacco. Five grams of aged CR tobacco obtained from Export Leaf Co., Wilson, NC, was ground to pass a 200 mesh screen and extracted with 250 mL of *n*-hexane for 5 min at room temperature to remove wax and other apolar constituents. The *n*-hexane was removed by filtration and the tobacco air-dried under the hood and further treated with N_2 to remove *n*-hexane residue. The tobacco was further extracted with 100 mL of methanol using a magnetic stirrer for 30 min at room temperature. The extract was filtered, the tobacco residue was washed with 50 mL of methanol, and the combined filtrates were evaporated to dryness under vacuum in a 250 mL evaporating flask at 35 °C. The extract residue in the flask was dissolved in 30 mL of $CHCl_3$ and treated in a dry ice/acetone bath for 15 min. The sticky solid that formed in the cold solution was removed by filtration. The filtrate was concentrated to dryness under vacuum at 35 °C and resuspended in 3 mL of CH_3OH for HPLC analysis. The solution was analyzed with a Waters 990 HPLC using a Bio-Rad Bio-Sil Amino 5S (4.6 × 250 mm) column isocratically with CH_3CN/HOH (90:10 v/v) at a flow rate of 1 mL/min.

Pyrolysis of CR Tobacco. Five grams of aged CR tobacco was dried at 60 °C overnight in a crucible and ground to pass a 200 mesh screen. From the dry sample, 1, 1.25, and 1.55 mg of ground tobacco was weighed into a clean quartz boat and pyrolyzed at 500 °C for 10 s. Each sample was duplicated to ensure reproducibility of pyrolysis.

RESULTS AND DISCUSSION

Identification of Synthetic NDF Isomers. The CR pigment has not been isolated, nor has the pigment been synthesized. CR color, however, has been produced *in vitro* (Weeks et al., 1993). Since NN Amadori compounds were commercially unavailable, it seemed more practical to synthesize NDF than to isolate and purify NDF from large quantities of CR tobacco containing variable amounts of this compound.

Figure 1 shows the UV spectrum of the synthesized and recrystallized Amadori rearrangement product from 195 to 320 nm using the HPLC UV array detector. An absorption peak detected at 260 nm from the HPLC spectrum was comparable to the UV absorption spectrum reported by Koiwai et al. (1979). Further verification of NDF was provided by HPLC/MS (Figure 2). The MS spectrum was obtained through a thermospray

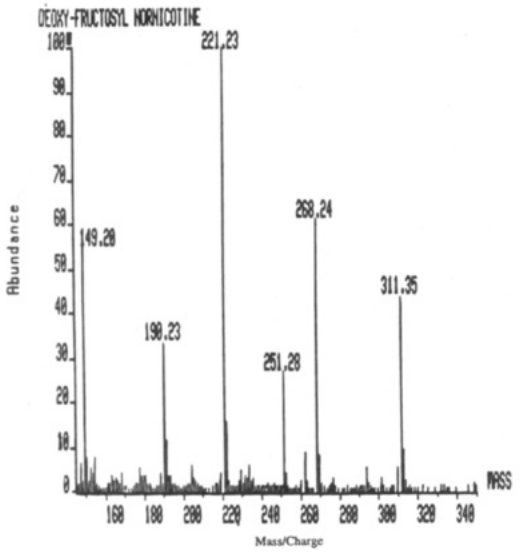


Figure 2. MS of NDF crystals.

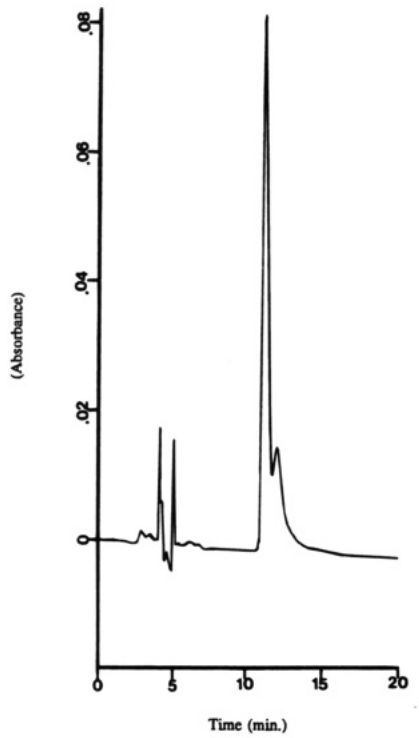


Figure 3. HPLC chromatogram of NDF products.

interface which generates a $(M + 1)^+$ *m/e* 311.35, which also agreed with the mass spectrum data of NDFF presented by Koiwai et al. (1979) showing a FD-MS: *m/e* 311.1607 $(M + 1)^+$. Further evidence of the Amadori compound was obtained by inspection of the ^1H and ^{13}C NMR spectra. The ^{13}C chemical shifts agree with NMR data reported by Siddiqui et al. (1981). These results confirmed a NN Amadori rearrangement product.

Although the UV spectrum showed only one absorption band, the HPLC chromatogram of the crystallized NDF (Figure 3) showed a mixture of compounds in which the minor peak was approximately 20% of the major peak based on peak heights. This prompted an investigation of both peaks. The HPLC/MS showed the same molecular ion and fragments for both compounds from the two peaks indicating the presence of isomers. The ^{13}C NMR spectrum of the recrystallized Amadori product also indicated a mixture of isomers but combined 15 major peaks showing signals (ppm) at (C-1)

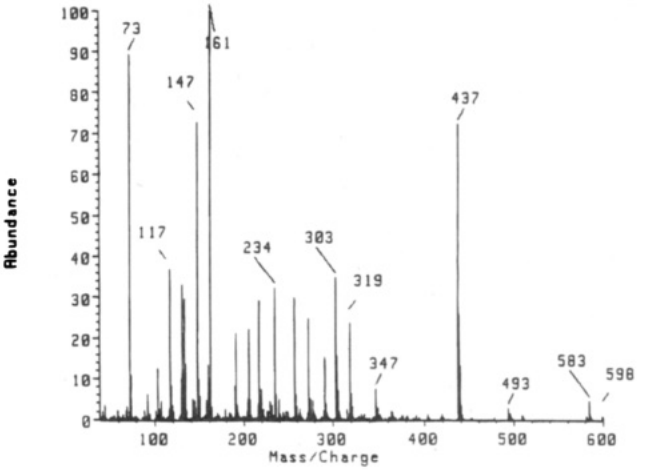
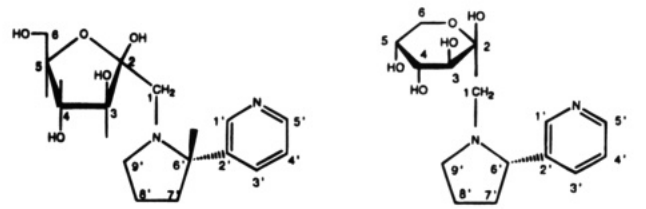


Figure 4. MS of TMS derivatives on column injection.

61.78, (C-2) 99.56, (C-3) 71.92, (C-4) 72.23, (C-5) 73.21, (C-6) 65.89, (C-1′) 151.49, (C-2′ and C-3′) 139.76 and 139.85, (C-4′) 127.39, (C-5′) 150.99, (C-6′) 70.69, (C-7′) 35.45, (C-8′) 25.49, and (C-9′) 58.60. These signals were in agreement with the Amadori compound with a fructopyranose ring as the major isomer in this mixture. Three minor signals were also observed at 77.07, 80.62, and 83.35 ppm, which agree with the signals reported for C-3, -4, and -5 of β-D-fructofuranose, as reported by Siddiqui et al. (1981), suggesting that the major isomer was NDFP and a minor isomer, NDFF.



NDFF **NDFP**

A downfield shift of 1.0−1.5 ppm was observed in all signals compared to data reported by Siddiqui et al. (1979), basically due to the difference in the internal reference used in the two measurements in this study versus that in Siddiqui et al. (1979).

The MS of the silylated compound from on-column injection (Figure 4) showed the molecular ion *m/z* 598 and 437 $(M - 161)^+$, indicative of a sugar moiety with four $(CH_3)_3SiO_3$ groups, and *m/z* 437 and 257 were indicative of (trimethysilyl)-D-fructose. *m/z* 161, the base peak, showed the presence of NN plus one methylene group, and *m/z* 147 accounted for NN. All other fragments found in the MS obtained from the on-column injection were consistent with the fragmentation patterns reported by Siddiqui et al. (1981), which confirmed the NMR interpertation of two isomers. The major isomer was NDFP and the minor isomer was NDFF in the NDF synthetic mixture.

Evaluation of NDF in CR Tobacco. Alkaloid content of the CR tobacco analyzed using routine GC analysis (Severson et al., 1981) was 29.9 mg of nicotine and 6.6 mg of NN per gram of tobacco. Quantification of NDF from the CR extract was obtained by HPLC following calibration using the synthesized and recrystallized NDF. Since the UV absorption spectra and the molecular ions of the two peaks of the synthesized NDF products were the same, the area of both peaks was used

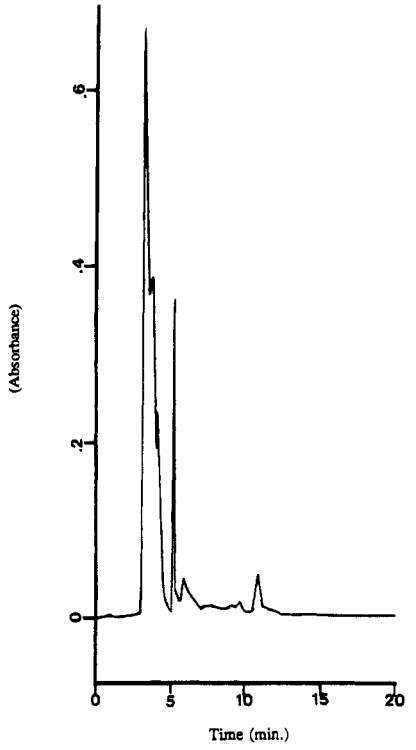


Figure 5. HPLC chromatogram of NDF obtained from CR tobacco.

for a calibration curve for quantification of NDF from the CR extract. HPLC analysis of the CR sample yielded a mean concentration of 1.51 mg of NDF, which contained 11% of the NN quantified from the CR sample after five analyses. The HPLC chromatogram of the tobacco extract (Figure 5) gave one peak with the retention time comparable to the major peak in the HPLC chromatogram of the Amadori product. The same absorption spectrum obtained in Figure 1 was obtained for the compound found in tobacco.

Pyrolysis of NDF and CR Tobacco. Pyrolysis of 0.55 mg of the recrystallized NDF gave the most satisfactory results for sniffing and for MS interpretation (Figure 6). It was not possible to quantitate the individual pyrolysates, nor was it possible to identify the MS spectra of all the pyrolytic products. The compounds obtained from pyrolysis of NDF indicated that the NN moiety, and the pyranose and furanose rings of fructose, were immediate precursors. NN and myosmine were detected (peaks 16 and 17; Table 1 and Figure 6), and the characteristic mousy aroma of these compounds was also detected (peaks 13 and 14; Figure 7 and Table 2). Balasubrahmanyam and Quinn (1962) observed myosmine, β-picoline, 3-vinylpyridine, 3-cyanopyridine, quinoline, and isoquinoline from pyrolysis of NN at 400 °C, but NN was completely destroyed at 500 °C. Surprisingly, nicotine (peak 12; Table 1 and Figure 6) was detected as one of the pyrolytic products of NDF in addition to cotinine, β-nicotyrine, *N*-propionylnornicotine, *N*-furoylnornicotine, *N*-acetylnornicotine, and *N*-formylnornicotine (peaks 14, 19, 24, 25, 27, and 28; Table 1). This is the first report of nicotine appearing as a pyrolysis product from sources other than nicotine salts commonly found in tobacco. However, an inspection of the structure of NDFP and NDFF shows that a break between the C-1 and C-2 bond in either case would likely generate nicotine from pyrolysis at 500 °C. Nicotine produced from pyrolysis of NDF can help explain smoke results reported by Koiwai et al.

Table 1. Compounds Formed from Pyrolysis of Synthesized NDF

peak no.[a]	compound	RT
1	water	19.43
2	pyridine	22.70
3	β-picoline	23.95
4	acetic acid	31.30
5	furfural	31.52
6	3-vinylpyridine	32.46
7	propionic acid	35.28
8	protoanemonin	36.85
9	pyridine derivative	44.99
10	3-acetylpyridine	48.10
11	3-methylcyclopentane-1,2-dione	50.97
12	nicotine	51.17
13	maltol	52.11
14	cotinine	54.11
15	2,5-dimethyl-3-methoxy-4-keto-2,3,5-trihydrofuran	59.77
16	nornicotine	65.97
17	myosmine	66.87
18	2-methyl-3,5-dihydroxy-4-keto-2,3-dihydropyran	68.83
19	β-nicotyrine	71.97
20	3-cyanopyridine	74.70
21	3,4,5-trihydro-2,6-dipyridone	77.13
22	2(3*H*)-furanone	79.33
23	2,3-indoledione	81.67
24	*N'*-propionylnornicotine	83.09
25	*N'*-furoylnornicotine	86.66
26	6-methylquinoxaline	100.96
27	*N'*-acetylnornicotine	107.00
28	*N'*-formylnornicotine	107.74

[a] Refer to Figure 6.

(1979) from smoking nicotineless flue-cured cigarettes spiked with NDFF. The sweet tobacco-like aroma (peak 7, Figure 7; Table 2) was identified with 3-acetylpyridine, which produces characteristic tobacco-like aroma. Although the 3-acetylpyridine peak was small, the aroma was readily detected from sniffing.

Pyrolysis products, probably from the pyranose and furanose rings of NDF, gave some very pleasant aromas detected at the sniffing port. The aroma of peaks 8, 10, and 12 (Figure 7 and Table 2) was described as sweet, sweet cake baking, and bread baking aromas. These aromas were assigned to peaks 11, 13, and 15 (Figure 6), which had the same retention times, 50.97, 52.11, and 59.77, respectively, in the pyrolysis chromatogram (Table 1; Figure 6). Acetic acid and propionic acid (peaks 4 and 7, Table 1; peaks 3 and 5, Table 2 and Figure 6) were detected at the sniffing port and from MS. Other aromas were detected, but the sniffer could not distinctly describe the odor of the effluent because the intensity was too low.

Pyrolysis of 1.25 mg of CR tobacco (Figure 8) gave better results than either 1 or 1.55 mg of tobacco. We were unsuccessful in determining the aroma produced from the tobacco pyrolysates, although pyridine, β-picoline, acetic acid, propionic acid, protoanemonin, nicotine, maltol, NN, myosmine, β-nicotyrine, and *N*-propionylnornicotine (Table 1 and Figure 6) were also found in the CR total ion chromatogram (Figure 8 and Table 3). Myosmine was probably a product of NN generated during pyrolysis regardless of the source of the NN complex.

Acetic acid, formic acid, propionic acid, butyric acid, and 2-methyl-2-butenoic acid, commonly found in flue-cured tobacco (Lloyd et al., 1976), were found in the CR chromatogram (peaks 8, 11, 12, 15, and 17; Table 3 and Figure 8).

Pyrazine, 2,3-dimethylpyrazine, and furanylpyrazine (peaks 3, 7, and 29; Table 3) were also found in the total

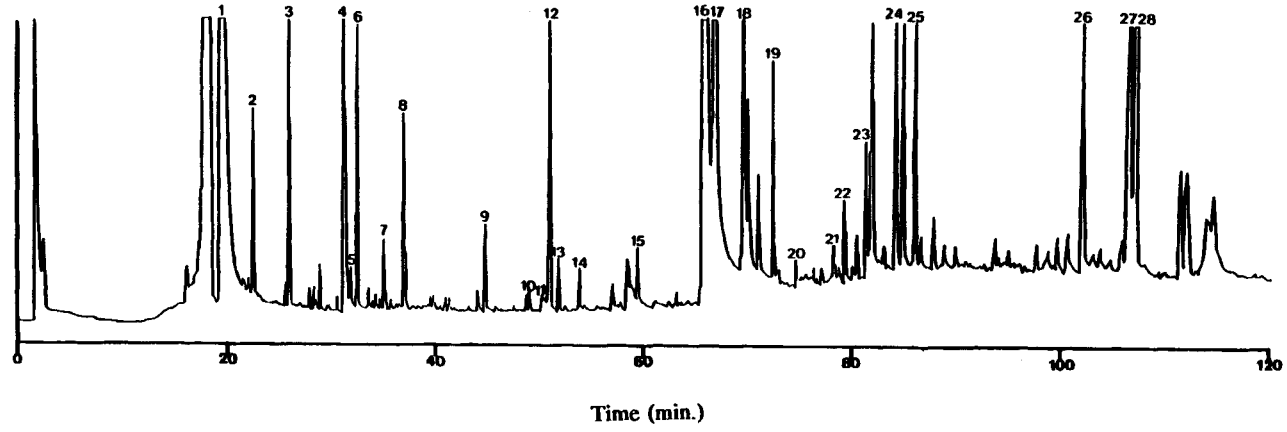


Figure 6. Total ion chromatogram of NDF pyrolysates.

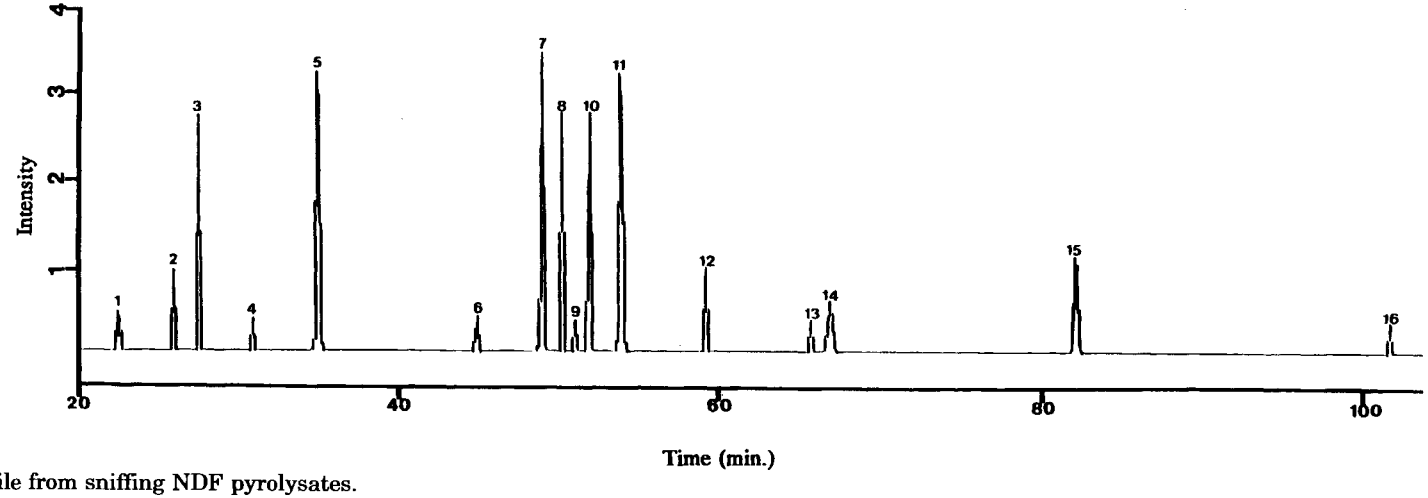


Figure 7. Aroma profile from sniffing NDF pyrolysates.

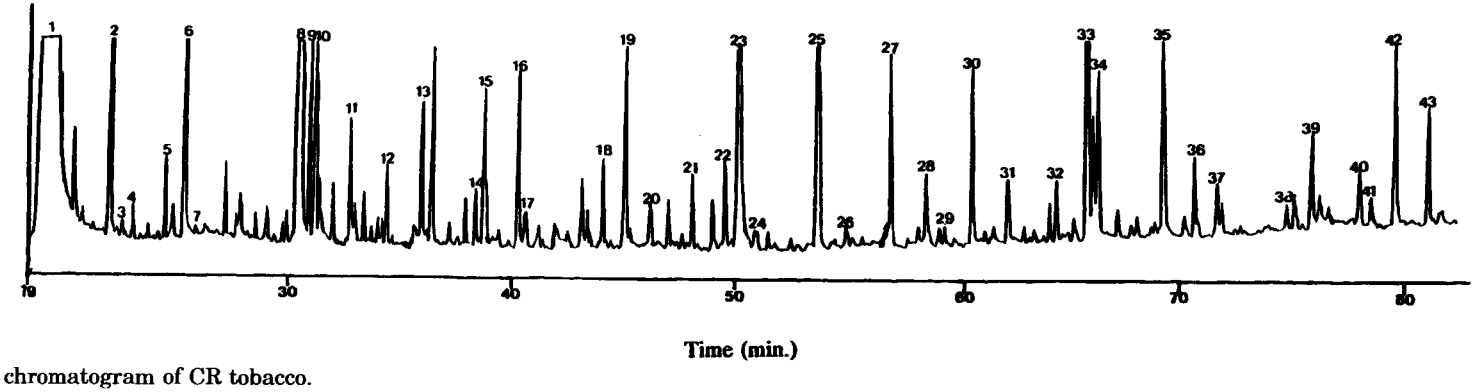


Figure 8. Total ion chromatogram of CR tobacco.

Table 2. Aromas Detected at the Sniffing Port from Pyrolysis of Synthesized NDF

peak no.[a]	compound	aroma	RT
1	pyridine	pyridine	22.70
2	β-picoline	pyridine-like	23.95
3	acetic acid	acetic acid	31.30
4	furfural	caramel-like	31.52
5	propionic acid	dirty socks	35.28
6	pyridine derivative	cyano	44.99
7	3-acetylpyridine	sweet (tobacco-like)	48.10
8	3-methylcyclopentane-1,2-dione	sweet	50.97
9	nicotine	weakly basic	51.17
10	maltol	sweet (cake baking)	52.11
11	cotinine	sweet ketonic	54.15
12	2,5-dimethyl-3-methoxy-2,3,5-trihydrofuran	bread browning	59.77
13	nornicotine	mousy	65.99
14	myosmine	mousy	66.83
15	N′-propionylnornicotine	stale moldy	83.09
16	6-methylquinoxaline	chemical odor	100.96

[a] Refer to Figure 7.

Table 3. Compounds Formed from Pyrolysis of Cherry Red Tobacco

peak no.[a]	compound	RT
1	water	19.43
2	pyridine	22.70
3	pyrazine	23.45
4	β-picoline	23.95
5	3-pentanone	25.36
6	2-hydroxy-2-propanone	26.12
7	2,3-dimethylpyrazine	27.04
8	acetic acid	31.30
9	furfural	31.52
10	1-diethoxyethane	31.82
11	formic acid	33.23
12	propionic acid	35.28
13	5-methylfurfural	36.39
14	protoanemonin	36.85
15	butyric acid	39.16
16	furfuryl alcohol	40.71
17	2-methyl-2-butenoic acid	41.10
18	β-angelica lactone	44.66
19	tetrahydropyrrole	45.69
20	cyclohexanol	46.80
21	cyclotene	48.65
22	2-methyl-3-keto-4-hydroxy-5-methylfuran	50.10
23	nicotine	51.17
24	maltol	52.04
25	neophytadiene	54.08
26	N-acetylpyrrole	55.27
27	phenol	57.24
28	2-pyrrolidone	58.80
29	1-furanylpyrazine	59.62
30	p-cresol	60.93
31	4-hydroxy-5-methyl-2,3-furandione	62.55
32	cyclopentanol	64.82
33	nornicotine	65.97
34	myosmine	66.87
35	1-methyl-3,5-dihydroxy-γ-pyrone	69.47
36	5-methyl-2-pyridinone	70.85
37	β-nicotyrine	71.97
38	benzofuran	74.83
39	3-hydroxypyridine	75.92
40	3,3′-bipyridyl	78.00
41	2,3-dihydro-3,5-dihydroxy-6-methyl-4-pyrone	78.49
42	5-(hydroxymethyl)furfural	79.51
43	N′-propionylnornicotine	83.09

[a] Refer to Figure 8.

ion chromatogram of the CR pyrolysates (Figure 8). Pyrolysis of the furanose and pyranose rings of NDF produced sweet and pleasant aromas previously described. Lactones, furan, and pyran derivatives reported by Leffingwell et al. (1972) as having characteristic sweet aromas and tastes were found in the NDF and CR total ion chromatograms (peaks 8, 13, 18, and 22, Table 1, Figure 6; peaks 14, 16, 18, 19, 29, 31, and 41, Table 3, Figure 8).

Conclusion. Nicotine was produced from the pyrolysis of CR tobacco as expected because the tobacco contained 2.9% nicotine. However, nicotine produced from pyrolysis of NDF was totally unexpected. Pyridine, β-picoline, 3,3′-bipyridyl, NN, myosmine, and N-acylated nornicotine were produced, as well, from pyrolysis of CR tobacco and NDF. These compounds could be responsible for undesirable taste and aroma of smoke detected by some smokers from CR tobacco. This, however, was not confirmed from sniffing the effluent from pyrolysis of NDF. Chromatographically separating the pyrolysates of CR tobacco and NDF allowed detection of individual eluants from CR tobacco and NDF. During smoking, it is difficult for the smoker to identify individual notes from a complex mixture. Sniffing NDF pyrolysates produced pleasant aromas with higher intensity than the unpleasant aromas. Pyrolysis of CR tobacco produced pyrazines, lactones, and furan derivatives that have been described as having pleasant aromas and taste. Results from pyrolysis of NDF and CR tobacco do not refute previous claims that CR tobacco produces off-flavor, but the data showed that pyrolysis products of NDF and CR tobacco produced some pleasant and sweet aromas.

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