Improved Methodology for the Quantitative Assessment of Tobacco-Specific *N*-Nitrosamines in Tobacco by Supercritical Fluid Extraction

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This paper describes an analytical method for the assessment of tobacco-specific *N*-nitrosamines (TSNA) in tobacco which involves supercritical fluid extraction and quantification by gas chromatography with thermal energy analyzer detection. Extraction of tobacco with supercritical carbon dioxide, to which 10% methanol is added, is followed by a cleanup on silica cartridges which separates the TSNA from the bulk of other tobacco components present in the extract. Gas chromatographic separation is performed on a DB-5 fused silica capillary column, and quantitative results are ascertained by using 4-(ethylnitrosamino)-1-(3-pyridyl)-1-butanone as an internal standard. This method is quantitative and well reproducible and meets the qualifications to be chosen as a standardized analytical method.

Keywords: *Tobacco-specific N-nitrosamines; TSNA; supercritical fluid extraction; SFE*

INTRODUCTION

In 1991, an estimated 5.3 million adults in America were current users of smokeless tobacco and more than 46 million adults in America were cigarette smokers (U.S. Department of Health and Human Services, 1993; Flach, 1993). The usage of smokeless tobacco is causally associated with cancer of the oral cavity and pharynx; cigarette smoking is the cause of cancer of the lung, larynx, oral cavity, pharynx, esophagus, pancreas, kidney, and urinary bladder (International Agency for Research on Cancer, 1985, 1986; U.S. Department of Health and Human Services, 1986, 1989). Snuff is carcinogenic in rats (Hecht et al., 1986; Johansson et al., 1989); cigarette smoke causes tumors in the lung of mice and rats and in the upper respiratory tract of hamsters (U.S. Department of Health and Human Services, 1989). Processed tobacco contains more than 3000 components; about 30 of these are animal carcinogens. Cigarette smoke contains more than 4000 components, of which more than 50 are animal carcinogens (Roberts, 1988; Hoffmann and Hecht, 1989; Brunnemann and Hoffmann, 1992). Bioassays as well as analytical and biochemical studies have shown that the tobacco-specific *N*-nitrosamines (TSNA) are the most abundant, strong carcinogens in smokeless tobacco (Figure 1). Swabbing of the oral cavity of rats with a solution of the TSNA, *N'*-nitrosonornicotine (NNN), and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induces tumors at the site of application and also in the lungs (Hecht et al., 1986). In rats NNN induces tumors of the esophagus, nasal cavity, and liver, in mice tumors of the lung, in hamsters tumors of the respiratory tract, and in minks tumors of the nasal cavity. NNK always induces tumors in the lungs of mice, rats, and hamsters, independent of the site and form of application, and it also elicits tumors of the exocrine pancreas in rats when it is given with the drinking water (Hoffmann et al., 1994).

In 1988, the World Health Organization recommended that the analysis and regulation of harmful substances in tobacco products should be controlled by governments. This would require the availability of a fast, quantitative, reproducible method for the determination of TSNA in various tobacco products. TSNA are typically extracted from tobacco samples with an aqueous solution of a citrate buffer to which ascorbic acid (Hoffmann et al., 1974; Hecht et al., 1974, 1978; Chamberlain and Arrendale, 1983; Andersen et al., 1989) or ammonium sulfamate is added (Djordjevic et al., 1989). Alternately, a tobacco suspension is sonicated in an aqueous citrate buffer with toluene (Chortyk and Chamberlain, 1991). However, these extraction techniques have several disadvantages. They require extensive handling of samples, which is undesirable for trace analysis because it increases the possibility of major experimental error. Also, large volumes of solvents are needed, the disposal of which is becoming increasingly more expensive. The extractions are also time-consuming. More importantly, they often produce stable emulsions, thus yielding variable results (Chortyk and Chamberlain, 1991). Additionally, the extracts produced are usually of high volume and low concentrations. As an alternative, supercritical fluid extraction (SFE) has attracted our attention because it is less dependent on large volumes of liquid solvents and it is rapid (Prokopczyk et al., 1992a). This paper describes an optimized analytical procedure that utilizes supercritical carbon dioxide as an extracting solvent prior to the determination of TSNA in various tobacco samples by gas chromatography and thermal energy analysis.

MATERIALS AND METHODS

Chemicals. Methanol and methylene chloride were commercial grade solvents purchased from J. T. Baker, Phillipsburg, NJ; they were used as received. Carbon dioxide, SFE grade, was obtained from Cryodyne Technologies Inc., Chester, CT. The 200-mg, 3-mL silica cartridges were bought from Alltech (Deerfield, IL, catalog no. 209150). The reference compounds NNK, NNN, *N*-nitrosoanabasine (NAB), *N'*-ni-

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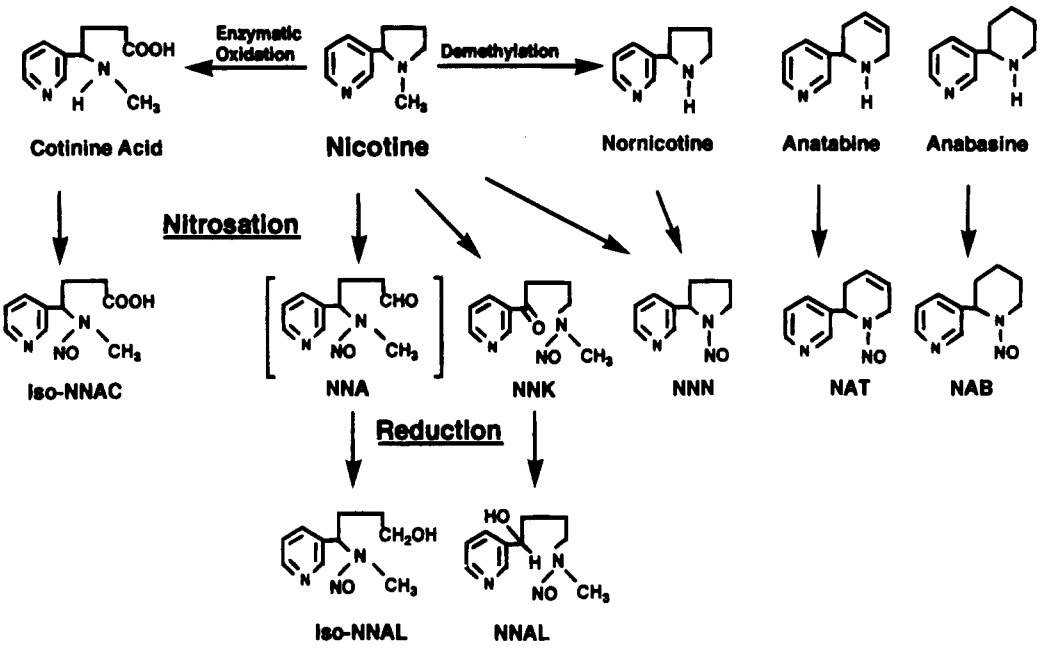

Figure 1. Formation of tobacco-specific *N*-nitrosamines.

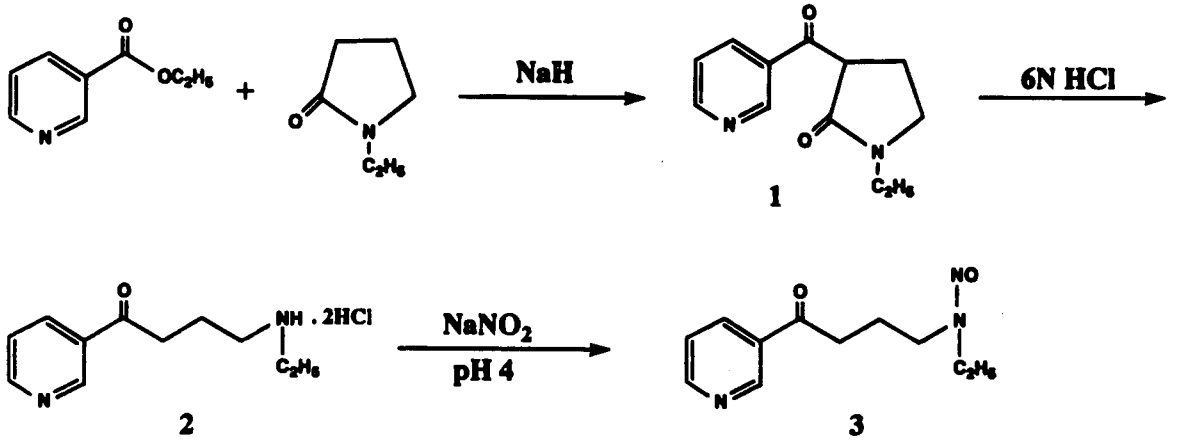

Figure 2. Synthesis of 4-(ethylnitrosamino)-1-(3-pyridyl)-1-butanone.

trosoanatabine (NAT), 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) as well as *N*-nitrosoguvacoline [methyl 1-nitroso-1,2,5,6-tetrahydronicotinate (NG)] were synthesized according to published methods (Hecht et al., 1977; Hu et al., 1974; Christensen and Krogsgaard-Larsen, 1977). The purity of the synthesized compounds, verified by capillary GC and high-performance liquid chromatography (HPLC), was >99%.

The internal standard, ethyl-NNK, was synthesized as outlined in Figure 2. Unless stated otherwise, ^{1}H NMR spectra were determined in $CDCl_3$ with a Bruker AM 360 spectrometer. The chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane (Me_4Si). All assignments were confirmed by decoupling experiments. MS were determined with a Hewlett-Packard Model 5988A instrument. High-resolution MS were determined with Kratos-Profile, at New York University Mass Spectroscopic Resources, New York. All chemicals used for the synthesis were purchased from Aldrich Chemical Co. (Milwaukee, WI), unless noted otherwise.

1-Ethyl-3-nicotinoyl-2-pyrrolidinone (1). In a nitrogen atmosphere, sodium hydride (1.98 g, 49.6 mmol, of a 60% suspension in mineral oil) was washed twice with hexane and suspended in dry toluene (100 mL). While the suspension was stirred, a solution of ethyl nicotinate (5 g, 33 mmol) and 1-ethyl-2-pyrrolidinone (3.74 g, 33 mmol) in dry toluene (100 mL) was added dropwise. The mixture was then refluxed for 48 h, cooled to room temperature, and poured over cold, dilute hydrochloric acid (2 N, 100 mL).

The toluene layer was separated; the aqueous layer was adjusted to pH 4 and extracted with chloroform (5 × 100 mL). The combined organic layers were dried ($MgSO_4$), filtered, and evaporated to give **1** as a dark brown oil. The lactam **1** was purified on a silica gel column and eluted by EtOAc to yield (6.1 g, 85%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.09 (t, 3H, CH_3, J = 7.15 Hz), 2.15–2.28 (m, 1H, CCH_2), 2.6–2.72 (m, 1H, CCH_2), 3.24–3.60 (m, 4H, NCH_2 and NCH_2CH_3), 4.40 and 4.43 (dd, 1H, CH, J = 5.29 Hz), 7.34–7.44 (m, 1H, pyridyl H5), 8.40 and 8.42 (dt, 1H, pyridyl H4, J = 1.95 Hz), 8.74 (dd, 1H, pyridyl H6, J = 4.83 and 1.67 Hz), 9.27 (d, 1H, pyridyl H2, J = 1.67 Hz); MS m/z (relative intensity) 218 (M^+, 100), 192 (6), 147 (10), 112 (40), 78 (10).

4-(Ethylamino)-1-(3-pyridyl)-1-butanone Dihydrochloride (2). A solution of 1-ethyl-3-nicotinoyl-2-pyrrolidinone (**1**) (6 g) in 40 mL of 6 M HCl was heated and refluxed with stirring for 96 h. The reaction mixture was cooled and adjusted to pH 12 with concentrated NaOH (the reaction mixture was kept at 0 °C during this addition). The alkaline solution was extracted with chloroform (5 × 100 mL). The combined organic layers were extracted with 2 N HCl solution (4 × 100 mL). This aqueous extract was then concentrated to give 4.0 g (yield 55%) of hydrochloride **2** which was recrystallized from ethanol: mp 171–173 °C; MS m/z (relative intensity) 264 (M^+, 6), 254 (10), 218 (14), 192 (6), 174 (52), 159 (100), 130 (12), 117 (12), 105 (14), 78 (6).

4-(Ethylnitrosamino)-1-(3-pyridyl)-1-butanone (Ethyl-NNK) (3). A cold solution of hydrochloride **2** (3.0 g, 11.4 mmol in 30 mL of H_2O) was adjusted to pH 4 with NaOH (2 N). A

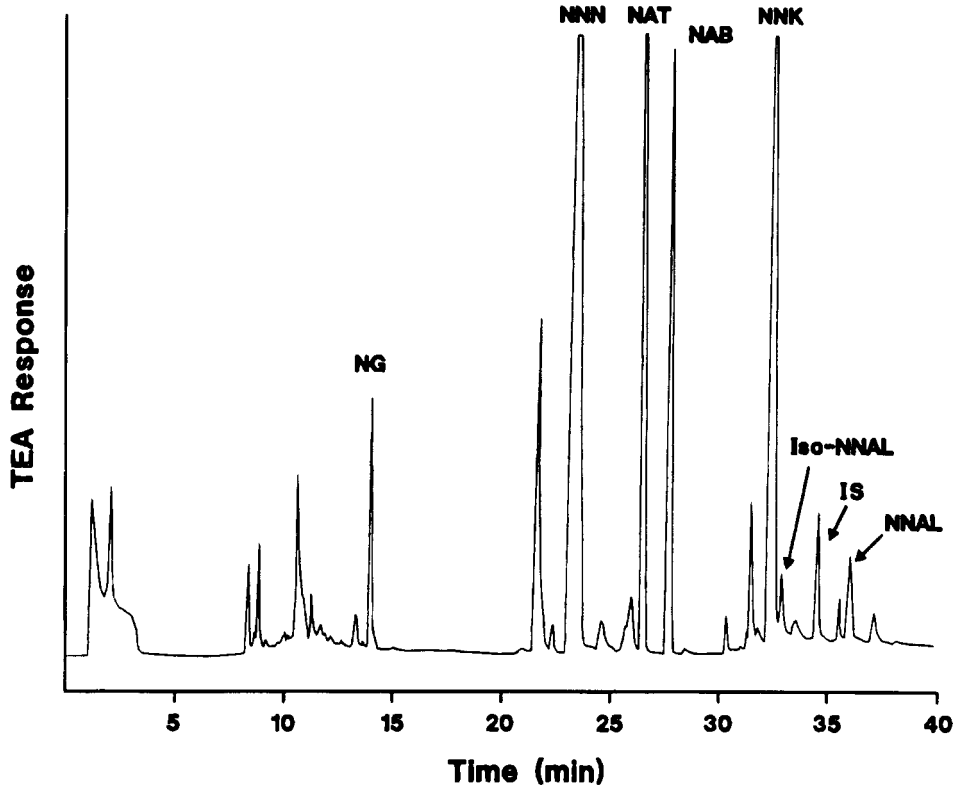

Figure 3. Representative GC-TEA chromatography of *N*-nitrosamines extracted from Sudanese snuff (toombak).

solution of $NaNO_2$ (1.17 g, 17 mmol, in 5 mL of H_2O) was added dropwise at 0 °C. The mixture was stirred at room temperature for 18 h while pH 4 was maintained.

The reaction mixture was then extracted at pH 4 with $CHCl_3$ (2 × 20 mL), adjusted to pH 10 with 2 N NaOH, and extracted with $CHCl_3$ (2 × 20 mL). The combined $CHCl_3$ layers were dried (over $MgSO_4$), filtered, and evaporated. The crude product was purified on a silica gel column using EtOAc as an eluent to give **3** (1.1 g, 44%) as an oil: ^{1}H NMR ($CDCl_3$) δ 1.12 (t, 1.6H, E-CH_3, J = 7.21 Hz), 1.43 (t, 1.4H, (*Z*)-CH_3, J = 7.25 Hz), 1.92–2.05 (m, 1H, (*E*)-CH_2C, J = 7.27 Hz), 2.20–2.30 (m, 1H, (*Z*)-CH_2C, J = 6.88 Hz), 2.97 (t, 1H, (*E*)-$COCH_2$, J = 6.8 Hz), 3.09 (t, 1H, (*Z*)-$COCH_2$, J = 6.88 Hz), 3.58–3.7 (m, 2H, NCH_2 and (*E*)-NCH_2CH_3), 4.12–4.24 (m, 2H, (*Z*)-NCH_2 and (*Z*)-NCH_2CH_3), 7.38–7.49 (m, 1H, pyridyl H5), 8.21 (dd, 1H, pyridyl H4, J = 7.98 and 1.78 Hz), 8.79 (bs, 1H, pyridyl H6), 9.16 (bs, 1H, pyridyl H2); MS m/z (relative intensity) 221 (M^+, 2), 191 (74), 148 (28), 134 (6), 106 (100), 78 (62). High-resolution MS Calcd for (M^+ − NO), $C_{11}H_{15}N_2O$: 191.1180. Found: 191.1182.

Safety. TSNA are established animal carcinogens; therefore, they were handled in accordance with the NIH Guidelines for the Laboratory Use of Chemical Carcinogens (NIH Publication 81-2385).

Sources of Tobacco Products. Leading U.S. moist snuff and cigarette brands were purchased from retailers in Westchester County, NY. Sudanese moist snuff samples (toombak) were bought in the city of Khartoum, Sudan, packed on dry ice, shipped to our laboratory, and stored in a cold room (4 °C) until they were analyzed.

Instrumentation. A Suprex Prepmaster stand-alone SFE system equipped with a Suprex Model MPA-1 solvent modifier pump and a DuraFlow restrictor (Suprex Corp., Pittsburgh, PA) was used for all extractions described in this study. These extractions were carried out in a 5-mL volume extraction vessel.

The TSNA were separated and quantified on a Hewlett-Packard Model 5890 Series II gas chromatograph with pressure programmable injection port, interfaced with a Model 543 thermal energy analyzer (Thermedics Incorp., Waltham, MA) and a Hitachi Model D-2500 chromatointegrator (Hitachi Instruments Inc., San Jose, CA).

Sample Preparation. The ground, freeze-dried tobacco sample (about 700 mg of cigarette tobacco, 500 mg of snuff, or 350 mg of toombak) containing 4.0–4.5% water was placed in the 5-mL extraction vessel and spiked with 2 μg of ethyl-NNK as internal standard (IS). After sealing, the extraction vessel was placed in the Prepmaster oven and was heated to 60 °C while pressurized to 350 atm. These conditions were maintained for 15 min. Then the extractions were performed in the dynamic mode at 60 °C and 350 atm for 30 min with CO_2 that contained 10% methanol at a flow rate of 1.5 mL/min. Analytes were collected in a vial containing 2 mL of *n*-hexane. Upon completion of the extraction, most of the organic solvent was removed by a stream of nitrogen to yield a concentrate of about 0.5 mL. Extracts were then purified on the 200-mg silica cartridges preconditioned with *n*-hexane. After the samples were applied, cartridges were washed first with 3 mL of *n*-hexane and then with 3 mL of methylene chloride. The TSNA fraction was then eluted with 3 mL of methylene chloride containing 2% of methanol; the eluate was concentrated to about 150 μL for cigarette tobacco samples, to 350 μL for snuff samples, and to 1000 μL for toombak samples. Then 2 μg of the chromatographic standard, NG, was added. Aliquots of 2 μL were analyzed by GC-TEA.

Gas Chromatography. The TSNA were analyzed on a DB-5 fused silica capillary column (30 m × 0.32 mm, 0.25-μm film thickness, J&W Scientific, Folsom, CA). The column head pressure was kept constant at 5 psi throughout the analysis. The injection port was kept at 220 °C. The programmed temperature runs consisted of an isothermal hold for 5 min at 40 °C, followed by a 30 °C/min ramp, and then a 10-min hold at 140 °C. This isothermal hold was followed by a 1 °C/min ramp, a hold at 145 °C for 5 min, and a final ramp of 20 °C/min with the final isothermal hold at 180 °C for 10 min. Under these chromatographic conditions NG (chromatographic standard) eluted at 13.96 min, NNN at 22.39 min, NAT at 26.50 min, NAB at 27.71 min, NNK at 32.42 min, iso-NNAL at 32.83 min, ethyl-NNK at 34.57 min (IS), and NNAL at 36.00 min (Figure 3).

RESULTS AND DISCUSSION

Our earlier experiments had indicated that SFE can be applied successfully to the analysis of TSNA in

smokeless tobacco samples (Prokopczyk et al., 1992a,b). The original experiments were performed with a simple, self-assembled apparatus with limited means of flow control. Consequently, analytes were trapped in cartridges filled with Tenex GR, and TSNA were then introduced into the gas chromatograph by thermal desorption.

The objective of this study was to apply and optimize this method with commercial instrumentation and to evaluate its applicability to the analysis of various types of tobacco products containing different levels of nitrosamines. When the method was first evaluated with the commercially available instrument (Prepmaster, Suprex Corp.), the TSNA values determined in tobacco samples were not reproducible from one extraction to the other (standard deviations ranged up to 35%). The poor reproducibility was caused by problems with restrictors that became plugged during the extraction procedure.

Restrictor Evaluation. In the first part of this study, a mixture containing TSNA and ethyl-NNK was applied to filter paper and then extracted using either a Suprex DuraFlow restrictor or an ISCO stainless steel capillary restrictor, both at a flow rate of 1.5 mL/min. The filter paper was chosen since earlier experiments had indicated that there was no difference in recoveries of TSNA between this matrix and the analyte-free tobacco (Prokopczyk et al., 1992a). Figure 4 shows graphically how nitrosamines are recovered with restrictors, namely that there is initially no noticeable difference between them (panel A). However, when tobacco samples were extracted, stainless steel restrictors clogged up much more quickly than DuraFlow. About 20 reproducible extractions can be carried out with the DuraFlow restrictor and only about 10–12 with stainless steel capillary tubing. Additionally, DuraFlow restrictors can be easily rebuilt using inexpensive peek tubing. Thus, DuraFlow restrictors were used exclusively for all analyses described here.

Internal Standard (IS). We have evaluated three *N*-nitroso compounds as internal standards, namely *N*-nitrosoguvacoline (NG), 2-[2-(methylnitrosamino)-ethyl]pyridine, obtained by direct nitrosation of 2-[2-(methylamino)ethyl]pyridine (Aldrich Chemical Co., Milwaukee, WI), and ethyl-NNK. The IS selected for our application should be extractable by supercritical fluids to about the same degree as the TSNA and should also have similar gas chromatographic properties. Ideally, its retention time under gas chromatographic conditions should be close to that of NNK. We observed the first symptoms of deterioration of a fused silica capillary column by a broadening of the NNK peak, which causes inefficient integration. To assess the degree of extraction of TSNA vs compounds evaluated as internal standards, initially we purposely used conditions that would not allow for the quantitative recoveries of TSNA. Therefore, at this stage, only a single 15-min extraction in static mode, followed by a single 30-min extraction in a dynamic mode, was performed. Under these conditions more than 82% of 2-[2-(methylnitrosamino)ethyl]pyridine and more than 98% of NG were recovered. Conversely, TSNA were extracted only between 52 and 65%. Therefore, both compounds were regarded as unsuitable as internal standards for the application. However, ethyl-NNK was extracted under these conditions to the same extent as other TSNA (Figure 4A). Two consecutive SFE extractions of the same tobacco sample (15-min static at 60 °C and 350 atm, followed by 30-min dynamic, followed by second static and dynamic extractions) allow for the quantitative recoveries of TSNA and ethyl-NNK (Figure 4B). Additionally, ethyl-NNK under gas chromatographic conditions is retained for about 2 min longer than NNK. This method was used throughout the study for the quantitative analysis of TSNA in tobacco products. Next, we conducted extractions with deliberately partially plugged restrictors. As illustrated in Figure 4B, even though the extraction efficiency for all nitrosamines was lower, the ratio of ethyl-NNK to the naturally occurring TSNA remained the same. This evaluation proved that ethyl-NNK can be used as an internal standard for the determination of TSNA by the method utilizing supercritical CO_2 modified with MeOH as the extracting solvent.

Cleanup on Silica Cartridges. The thermal energy analyzer is a selective detector for nitroso compounds (Fine et al., 1973). Therefore, TSNA can be determined directly in the supercritical fluid extracts of tobacco samples. However, tobacco contains about 3000 other compounds, many of which can also be extracted by SFE. While not necessarily interfering with the determination of TSNA by capillary GC, these compounds can drastically shorten the lifespan of the capillary columns. Therefore, a cleanup procedure was needed to eliminate the bulk of tobacco compounds without impairing the TSNA. Both the ethyl-NNK and TSNA are eluted and separated from the bulk of the other components in the SFE fraction by chromatography on 200-mg silica cartridges with 2% methanol in methylene chloride as solvent.

Comparison of Methods. The new quantitative method for determining TSNA, based on supercritical carbon dioxide extraction, was compared with the conventional procedure consisting of tobacco extraction with an aqueous acidic solution of ammonium sulfamate (Djordjevic et al., 1989).

Four toombak samples were extracted using both methods. A comparison of the results is shown in Table 1. Differences in NNN, NAT, and NAB values most likely reflect the lack of uniformity of the toombak samples. Although neither of these methods resulted in higher recovery of NNN, NAT, or NAB, NNK values obtained by SFE were always higher (18–115%) than those determined by the conventional solvent extraction method. This finding is in agreement with our previously published papers which demonstrated that the levels of NNK in snuff tobaccos currently available on the U.S. market are 2–7 times higher than those determined by the conventional solvent extraction method (Prokopczyk et al., 1992a). Similarly, the quantities of iso-NNAL determined by the SFE method were 16–118% higher than those assessed by the solvent extraction method. Also, the levels of NNAL in samples A and B quantified by the new method were, respectively, 110 and 234% higher than those determined by the classical method. These data suggest that TSNA with an open alkyl chain and a keto or alcohol group might be present in smokeless tobacco at levels higher than previously reported. This finding is of major significance since the saliva of snuff dippers extracts more NNK than the conventional solvent method (Prokopczyk et al., 1992b).

Quantification of TSNA. The reproducibility of the method was evaluated using a selected U.S. brand of smokeless tobacco. Five samples were prepared from the same batch of snuff and were extracted by super-

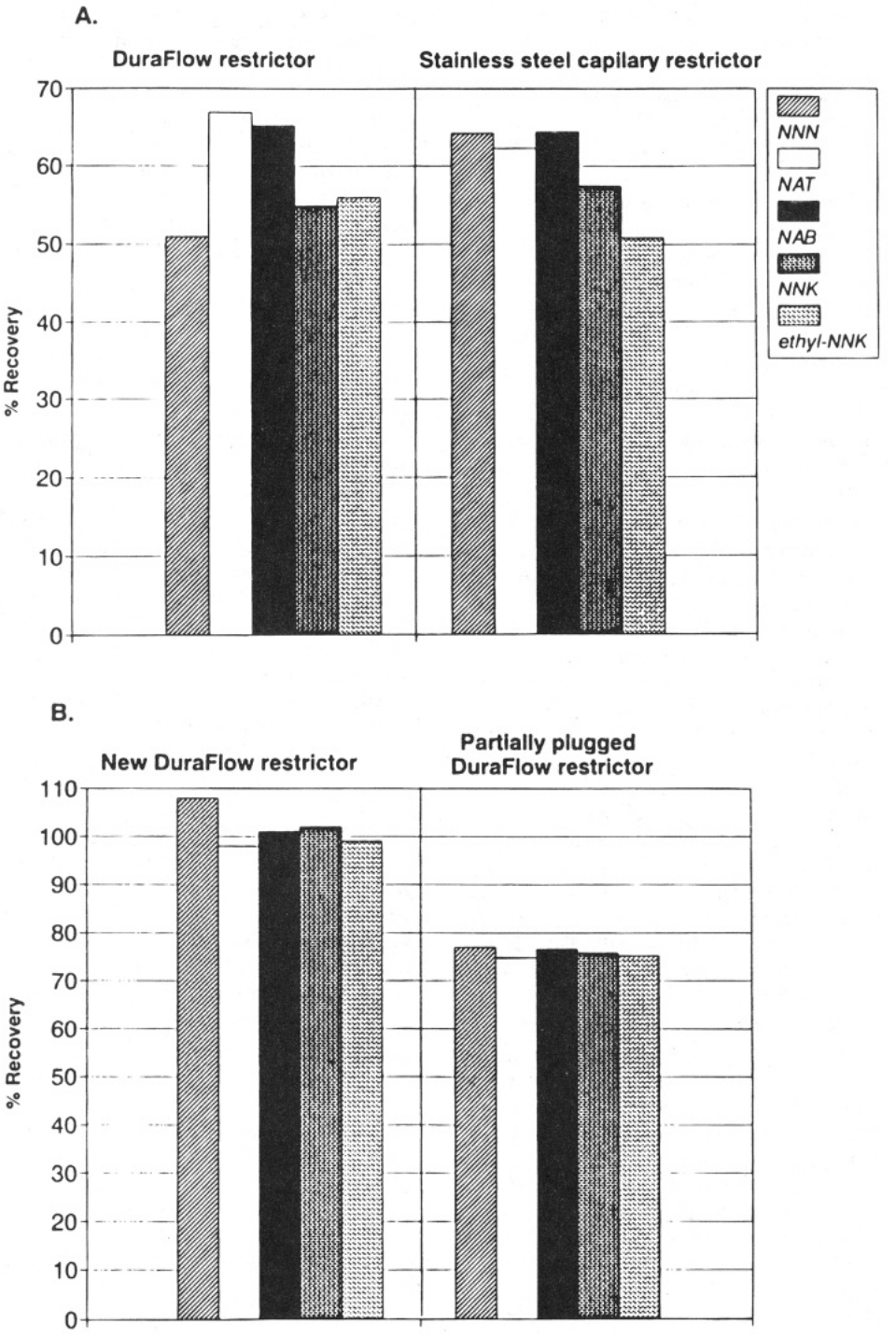

Figure 4. Recovery assays for TSNA using SCF-GC-TEA method. Single extractions at 60 °C and 350 atm first for 15 min in a static mode followed by 30 min in a dynamic mode were performed for the evaluation of restrictors as represented in panel A; for the evaluation of restrictors in panel B the SFE process was repeated twice.

critical CO_2 that contained 10% methanol. After cleanup on silica cartridges, levels of TSNA were determined by capillary GC-TEA. Table 2 demonstrates the reproducibility of the SFE-GC-TEA method. The recovery rate using ethyl-NNK as IS was greater than 90%. Table 3 lists the levels of TSNA in three types of tobacco. As illustrated by these results, the lowest TSNA concentration was determined in cigarette tobacco and the highest in Sudanese toombak. These data are in line with published levels of nitrosamines in cigarette tobacco and U.S. snuff tobaccos as assayed by the conventional solvent extraction method (Brunnemann and Hoffmann, 1991) with the exception of NNK. The levels of TSNA as determined in toombak are 2−10 times lower than those previously reported (Idris et al., 1991). This result is not surprising since toombak is a homemade preparation with no uniformity in fermentation, processing, and storage conditions. Furthermore, directly after we collected the samples, they were kept in vials on dry ice to avoid artifactual increase of TSNA in toombak during its transportation from the Sudan (Anderson et al., 1989; Djordjevic et al., 1993).

Currently we are developing a SFE-GC-TEA for the analysis of TSNA in the smoke of cigarettes and cigars.

Conclusion. On the basis of the results presented here, we conclude that the method utilizing the extrac-

Table 1. Comparison of the Conventional Solvent Extraction Method with a New Improved Methodology Utilizing Supercritical Solvent Extraction

	levels of TSNA in toombak[a] (μg/g of dry wt)					
toombak	NNN	NAT	NAB	NNK	iso-NNAL	NNAL
A						
SFE	141	21.7	28.7	245	10.9	22.9
conventional	146	20.9	17.1	114	4.98	10.9
B						
SFE	179	21.2	25.4	291	3.86	11.0
conventional	169	21.4	19.2	145	3.02	3.29
C						
SFE	198	20.9	21.5	195	11.1	ND[b]
conventional	201	28.2	21.4	165	9.59	2.33
D						
SFE	272	28.1	13.9	188	2.57	ND
conventional	255	36.0	12.3	151	2.03	ND

[a] Data determined by single extraction using internal standard. [b] Not detected.

Table 2. Method Reproducibility for TSNA Determinations

	NNN	NAT	NAB	NNK
1	9.8	5.9	0.39	3.4
2	10.3	6.2	0.44	2.6
3	11.8	8.2	0.48	2.7
4	9.0	5.1	0.38	2.4
5	11.3	7.4	0.78	2.7
mean	10.4	6.6	0.49	2.8
SD	1.14	1.23	0.17	0.36

Table 3. TSNA Content in Cigarette Tobacco, Smokeless Tobacco, and Sudanese Snuff (Toombak) Determined by Supercritical Fluid Extraction (SFE)[a]

	μg/g of dry wt					
sample	NNN	NAT	NAB	NNK	iso-NNAL	NNAL
cigarette						
A	1.26	1.36	0.07	1.13	ND[b]	ND
B	1.13	0.91	0.08	0.79	ND	ND
C	1.73	2.72	0.27	1.12	ND	ND
D	0.54	0.62	0.02	0.21	ND	ND
E	1.99	0.87	0.05	0.50	ND	ND
U.S. snuff						
A	17.4	22.5	3.10	5.54	ND	ND
B	7.05	7.09	0.37	1.94	ND	ND
C	6.51	7.33	0.28	3.51	ND	ND
D	0.87	0.89	0.12	1.11	ND	ND
E	10.4	6.6	0.49	2.8	ND	ND
Sudanese snuff						
A	141	21.7	28.7	245	10.9	22.9
B	179	21.2	25.4	291	3.86	11.0
C	198	20.9	21.5	195	11.1	ND
D	272	28.1	13.9	188	2.57	ND
E	240	28.9	24.8	257	20.7	2.15
F	369	37.4	42.5	262	1.4	ND
G	331	42.0	43.0	362	14.7	2.00

[a] Each tobacco sample was extracted once for a total of 90 min. This consisted of a 15-min extraction in a static mode, followed by a 30-min dynamic extraction, then followed by another 15-min static extraction, and a final 30-min dynamic extraction. Extraction parameters: 60 °C and 350 atm. [b] Not detected (<0.02 μg/g of dry weight).

tion with supercritical carbon dioxide and the quantification by GC-TEA provides a favorable alternative for the analysis of TSNA in various tobacco products with widely varying nitrosamine content. The method is simple, quantitative, and reproducible and meets the qualifications of regulatory agencies as suggested by the World Health Organization (1988) to become a standard method for TSNA in smokeless tobacco which is needed for control analysis.

ABBREVIATIONS USED

TSNA, tobacco-specific *N*-nitrosamine(s); NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAB, *N'*-nitrosoanabasine; NAT, *N'*-nitrosoanatabine; ethyl-NNK, 4-(ethylnitrosamino)-1-(3-pyridyl)-1-butanone; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; iso-NNAL, 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol; NG, *N*-nitrosoguvacoline (methyl 1-nitroso-1,2,5,6-tetrahydronicotinate); GC, gas chromatography; TEA, thermal energy analyzer; SFE, supercritical fluid extraction; IS, internal standard.

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