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Determination of Nicotine and Other Minor Alkaloids in International Cigarettes by Solid-Phase Microextraction and Gas Chromatography/Mass Spectrometry

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Nicotine, nornicotine, anabasine, and anatabine are the most abundant alkaloids in tobacco. Along with the addictiveness of nicotine, other properties, including their occurrence in tobacco at relatively high concentrations, and as the primary precursors for the highly carcinogenic tobacco-specific nitrosoamines, make these chemicals important from a public health standpoint. Therefore, developing a fast and accurate quantitative method to screen large numbers of cigarette samples for these alkaloids was important. This report describes the first use of headspace analysis using solid-phase microextraction combined with gas chromatography/mass spectrometry for the unambiguous detection of tobacco alkaloids. Detection and confirmation of each analyte is established by both chromatographic retention times and the ratio of reconstructed ion chromatogram peak areas from characteristic quantitation ion and confirmation ion. Twenty-eight cigarette brands from 14 countries were analyzed. Surprisingly, the minor alkaloids' response factors varied considerably among different styles of cigarettes. Accurate quantification was achieved using a three-point standard addition protocol. The standard addition approach was essential to obtain accurate measurements by minimizing matrix effects that would otherwise have contributed to quantitation bias. Significant differences in the alkaloid profiles were measured in the different cigarette brands. These results strongly suggest that such differences reflect variations associated with blend compositions, tobacco quality, and manufacturing practices.

Tobacco consumption remains the leading cause of preventable death in the United States,[1] accounting for approximately 430 000 deaths per year.[2] Globally, tobacco use contributes to approximately 4 million deaths each year.[3] The World Bank[4] has estimated that continued tobacco consumption threatens to claim 7 million deaths per year in developing nations. Among more than 20 different alkaloids found in tobacco,[5] nicotine is the most abundant and accounts for widespread human use of tobacco products throughout the world. The minor alkaloids, including nornicotine, anabasine, and anatabine, are also pharmacologically active. However, they are less potent than nicotine.[6]

Many reports have focused on the analysis of nicotine and its major metabolite, cotinine, in various matrixes, including biological fluids and tissue samples, by employing high-performance liquid chromatography (HPLC),[7,8] gas chromatography (GC),[9,10] or gas chromatography/mass spectrometry (GC/MS).[11−13] However, only a limited number of methods for measuring alkaloid levels in tobacco have been reported. The concomitant presence of high levels of nicotine and lower levels of other minor alkaloids in the tobacco, along with numerous other chemical compounds in the tobacco, impart a significant challenge for developing a fast and accurate quantitative method.

Several methods for the analysis of tobacco alkaloids have been reported. In 1980, Piade and Hoffmann[14] reported a HPLC method measuring these four alkaloids after an extensive tobacco extraction process. In 1998, a high-performance capillary electrophoresis method to quantitatively analyze nicotine levels and possibly quantification of other alkaloids in tobacco products was reported.[15] The use of a solid-phase microextraction (SPME) technique[16] combined with GC/nitrogen−phosphorus detection (NPD), to quantify alkaloid levels in tobacco, was also reported

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in 1998.[17] In each of these reports, the alkaloids were detected with a nonspecific type of detector such as UV and NPD. Given the complexity of the tobacco matrix, with about 3000 compounds having been reported and about 4000 constituents associated with cigarette smoke,[18] we chose to use mass spectral analysis to obtain compound-specific information for unambiguous detection.

We report here the first implementation of a headspace SPME technique combined with isotope dilution GC/MS for the analysis of alkaloid levels present in cigarette tobacco filler. This method combines the advantages of SPME (high throughput, minimal solvent use, unattended operation) with the high chromatographic resolution of capillary GC and high specificity and sensitivity afforded with mass spectral detection. This technique has been applied to the analysis of 28 brands of international cigarettes to simultaneously determine the minor alkaloids in the presence of abundant nicotine. This method is completely automated, uses minimal amounts of solvents, and provides high throughput. In addition, the method achieved high sensitivity and very good linear response.

EXPERIMENTAL SECTION

Instrumentation and Apparatus. GC/MS analysis was carried out on an Agilent mass-selective detector 5973N (Newark, DE) connected to an Agilent gas chromatograph 6890 equipped with a Leap solid-phase microextraction autosampler (Carrboro, NC). A hematology mixer obtained from Oxis Instruments (Warnington, PA) was used for sample preparation. SPME fibers were purchased from Supelco, Inc. (Bellefonte, PA).

Reagents. (−)-Nicotine standard was obtained from Fluka (Milwaukee, WI). Nornicotine, anabasine, and anatabine were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Methyl-$^2H_3$-nicotine and 3′,4′,5′-$^{13}C_3$-DL-nornicotine were obtained from Cambridge Isotope Laboratories (Andover, MA). All other chemicals were of analytical grade and obtained through Sigma (St. Louis, MO) unless otherwise indicated.

Samples. To help assess the current public health risk associated with tobacco use, the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) formulated a joint program to collect and analyze cigarettes collected in at least two nations from each of the six WHO regions. Countries were selected on the basis of their total population. Other than the United States, the 10 most populous countries were China, India, Indonesia, Brazil, Pakistan, the Russian Federation, Japan, Bangladesh, Nigeria, and Mexico. In addition, cigarettes from three other countries, Germany, Egypt, and Kenya, were added to ensure representation in each of the six WHO regions. Multiple packs of two cigarette brands were purchased in each country and forwarded for analysis. The tobacco fillers from these cigarettes were analyzed for a variety of chemical components, including the four tobacco alkaloids reported here.

CDC personnel who were either permanently stationed abroad or on temporary travel status purchased the international cigarettes samples from retail stores in selected countries. The cigarettes were immediately forwarded to the CDC by overnight carrier or diplomatic pouch or were hand carried by the purchaser.

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Table 1. Selective Ion-Monitoring Mode Setup for Alkaloid Analysis in Tobacco Samples

compound	relative RT[a] (min)	quantitation ion (m/z)	confirmation ion (m/z)
nicotine	3.84	133	162
$^2H_3$-nicotine	3.84	87	N/A
nornicotine	4.25	70	119
$^{13}C_3$-nornicotine	4.25	150	N/A
anabasine	4.43	133	106
anatabine	4.56	160	131

[a] RT, retention time.

Upon arrival, the cigarette packs were assigned unique ID numbers, sealed into a plastic bag in their original packaging, and stored in a −70 °C freezer until needed. Only authorized personnel were allowed access to the samples.

Sample Preparation and Analysis Procedure. The tobacco filler was removed from the cigarettes and ground to a fine powder. After conditioning at 22 °C and 60% humidity in a humidity chamber for 24 h, a 30-mg sample of the tobacco filler was removed and transferred into a 10-mL glass SPME vial. Aliquots of 5 μL of pyrrolidine, 4 mL of 0.01 N sodium tribasic phosphate, and the internal standard mixture containing 100 μg of methyl-$^2H_3$-nicotine and 20 μg of 3′,4′,5′-$^{13}C_3$-DL-nornicotine were added into vials. Comparative GC/MS analysis of the new and older alkaloid standard solutions yielded reproducible results for at least 4 months when the alkaloid solution was stored in an amber volumetric flask at 4 °C. Standard addition samples were prepared as follows: a "zero" point was prepared just as described above; the first standard addition sample was spiked with a mixture of 0.349 mg of nicotine, 12.8 μg of nornicotine, 6.90 μg of anabasine, and 15.5 μg of anatabine; the second standard addition samples was spiked with a mixture of 0.698 mg of nicotine, 25.6 μg of nornicotine, 13.8 μg of anabasine, and 31.0 μg of anatabine. All vials were then crimped shut with an aluminum top containing a Teflon-lined septum and rotated overnight on a hematology mixer.

A 100-μm poly(dimethylsiloxane) (PDMS) SPME fiber was used to extract the headspace above the tobacco samples for 2 min at 40 °C. The SPME fiber was desorbed in the GC inlet for 4 min at 260 °C. A J&W (Folsom, CA) DB 1701 (30 m × 0.25 mm i.d. × 0.25 μm film) chromatography column was used for the separation of the alkaloids. The helium carrier gas was maintained at a flow of 1 mL/min through the column in a constant-flow mode. The GC inlet was operated in splitless mode for the first 45 s and then the split vent was opened. The GC oven temperature started at 100 °C for 0.5 min, ramped at 40 °C/min to 280 °C, and held for 1 min. A lower initial oven temperature did not significantly improve the separation of the alkaloids.

The effluent from the GC was directed to an Agilent 5793 mass spectral detector. The alkaloids underwent electron impact ionization (−70 eV) and were detected in a multiple-event single ion-monitoring mode. The electron multiplier voltage was time-programmed to reduce the nicotine signal to achieve a response similar to that of the other alkaloids. The retention times, and masses for the quantitation and confirmation ion for each alkaloid, are shown in Table 1. The labeled nicotine was used as internal standard for the nicotine quantification, and the labeled nornicotine

was used as internal standard for minor alkaloids, nornicotine, anabasine, and anatabine quantification.

Data Analysis. Data from the sample analysis were transferred into an R:Base database (Microrim, Inc., Redmond, WA). Relative response factors for each analyte were calculated using the Colby correction approach[19] to ensure optimal quantitation. Final concentration calculation was carried out in a custom program developed using the Statistical Analysis System (SAS) software (SAS Institute, Cary, NC). In this custom SAS program, quantitation curves were generated by a linear regression of the relative reconstructed ion chromatogram peak areas (native analyte/internal standard) versus analyte spike amount with the best-fit line forced through the native (without addition) sample point. The concentrations of alkaloids in each sample were calculated from the *Y*-intercept divided by the slope obtained from each sample

RESULTS AND DISCUSSION

Method Development. Nicotine is the most abundant alkaloid in tobacco, typically present in the range of 1−3 wt %.[20] The other alkaloids in tobacco generally are present at much lower levels than nicotine with anabasine concentrations often 100-fold less. Thus, to simultaneously determine quantitative levels of analytes whose concentrations differ by orders of magnitude, sample preparation and analytical condition were extensively explored to optimize the quantification of the minor alkaloids in the presence of an abundance of nicotine. The main concern was to prevent nicotine signal saturation while ensuring adequate sensitivity for the minor alkaloids. Because the cigarette tobacco filler is composed of different blend components that vary from brand to brand, grinding the tobacco filler was essential to ensure homogeneous sampling. A 30-mg sample of the ground tobacco filler was found to be an optimal amount for the alkaloid analysis.

Different SPME fiber coatings were evaluated to select the most favorable conditions for headspace analysis of the tobacco alkaloids. The 65-μm Carbowax/divinylbenzene (CW/DVB) provided excellent results in detecting nicotine and maintaining sensitivity for the minor alkaloids. However, the sample-to-sample reproducibility of the 100-μm PDMS fiber was superior. The PDMS fiber, although less sensitive, was more robust for this application, provided more consistent results, had a lower chemical background, and exhibited negligible sample carryover compared with the CW/DVB fiber. The total ion chromatogram from a Kentucky Reference 1R4F cigarette obtained using the PDMS fiber is shown in Figure 1. A 2-min headspace sampling time at 40 °C achieved the best linearity for the alkaloid calibration curves.

Because the tobacco matrix contained numerous compounds, headspace analysis rather than liquid injection or fiber immersion in the tobacco sample solution was adopted to analyze the alkaloid levels. However, a large number of components other than the alkaloids are present even in the "cleaner" headspace. To efficiently separate alkaloids from other constituents in the headspace of tobacco extract and to precisely quantify the alkaloids, a multidimensional analysis approach, such as GC/MS, was critical for the alkaloid analysis. Baseline separation of alkaloids from other compounds could be achieved using either a DB 5ms or a DB 17ms capillary GC column. However, we observed excessive peak tailing for nornicotine with a DB 5ms column. A DB 1701 column provided both good separation and chromatographic peak shape and was selected for the alkaloid analysis.

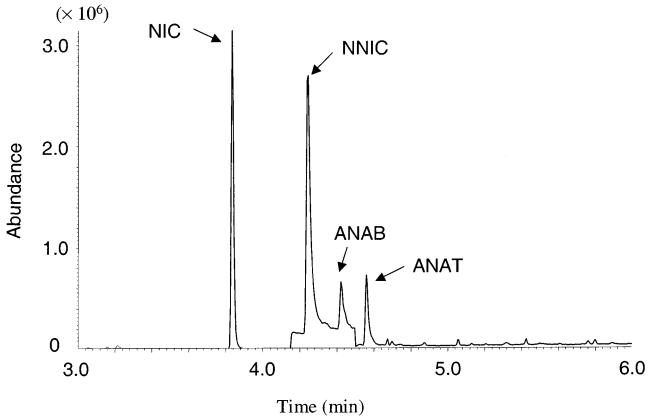

Figure 1. Total ion chromatogram of tobacco alkaloids analysis. Peaks NIC, NNIC, ANAB, and ANAT stand for nicotine, nornicotine, anabasine, and anatabine, respectively. Sample preparation and analysis condition was described in the Experimental Section. Each alkaloid identity was confirmed by the appropriate retention time and the ratio of the quantitation ion to the confirmation ion.

Our initial attempts to quantitatively measure the tobacco alkaloids were based on using denicotinized tobacco containing only trace levels of nicotine and undetectable levels of the other alkaloids as a starting material for spiking to generate calibration curves. At first consideration, denicotinized tobacco should provide an excellent surrogate to mimic the tobacco matrix. The denicotinized tobacco yielded calibration curves for all four alkaloids with excellent linearity, spanning several orders of magnitude. However, when cigarette samples from different countries were analyzed, differences in the relative response factors suggested that the magnitude of the matrix effects was not consistent among the different brands of international cigarettes. This suggested that a more thorough investigation of the tobacco filler matrix effects was needed.

Six-point standard addition calibration curves were established using tobaccos from two widely different styles of cigarettes. Surprisingly, the slopes of the calibration curves, especially for nornicotine, anabasine, and anatabine, obtained from these two different brands varied by as much as 50%. In contrast, the slopes of the nicotine calibration curves were not significantly different. In light of such differences in the minor alkaloids' response factors, the traditional calibration curve strategy was abandoned in favor of a standard addition approach that compensated for differences in the tobacco matrixes. Therefore, this method incorporated a three-point standard addition approach to accurately assess the minor alkaloid concentrations.

Under these circumstances, the method of standard addition was the best option available. Although standard addition requires more sample runs than a standard calibration curve approach, it remains an efficient technique for this situation. Triplicate analysis using a three-point standard addition resulted in measurement of nine samples for each cigarette brand. Alternatively, measuring the response factor for each brand of cigarettes would have required a minimum of a three-point recovery analysis per brand

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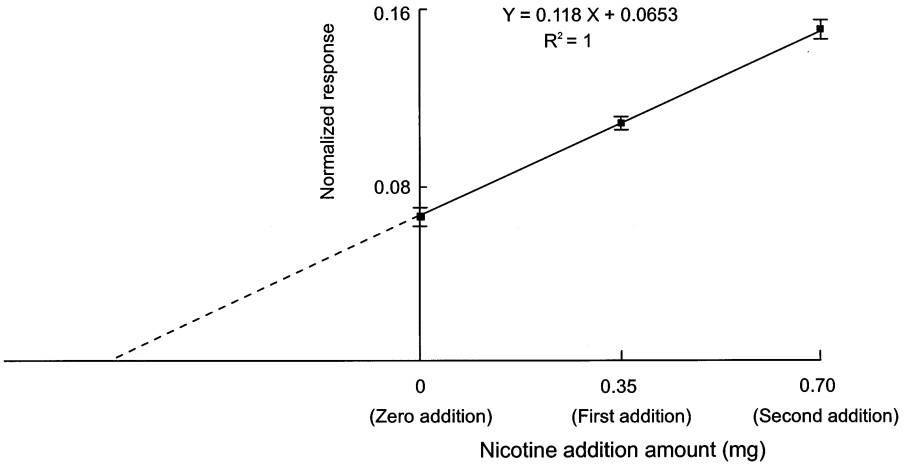

Figure 2. Standard addition calibration curve for nicotine in tobacco filler from Kentucky reference 1R4F cigarette. The data points at zero addition, first addition, and second addition represent the average ± SD of 14 measurements. The nicotine level in the 1R4F tobacco filler was calculated using the *Y*-intercept divided by the slope.

measured in triplicate (9 samples) and triplicate quantitative analysis of each brand (3 samples) yields a total of 12 samples per brand. In addition to those 12 samples per brand, additional standard samples for the calibration curve generation would have required additional sample preparation. Given that the number of quality control (QC) and blank samples remains essentially constant in either approach, we believe that the standard addition approach provided the most efficient and accurate quantification of tobacco alkaloids in cigarette filler.

Another problem noticed during method development was poor precision obtained for anabasine and anatabine analysis. Because nornicotine, anabasine, and anatabine are homologues, the stable isotope-labeled nornicotine should serve as a useful surrogate internal standard for the quantitation of anabasine and anatabine. However, during characterization of QC samples using Kentucky reference 1R4F cigarettes, the coefficients of variation (CVs) exceeded 40%, and the long-term precision of anabasine and anatabine was deemed not acceptable. Anabasine and anatabine are secondary amines and could readily undergo various chemical reactions. We suspected that these chemicals were interacting with other compounds such as aldehydes and ketones also present in the tobacco. To test this possibility, a 5-μL pyrrolidine spike, which is a 100-fold molar excess over the minor alkaloid levels expected in the tobacco, was added into the ground tobacco filler during sample preparation. Improved long-term QC reproducibility (CVs <20%) was obtained for anabasine and anatabine when pyrrolidine was present in excess. This finding confirmed the primary cause of poor precision for anabasine and anatabine quantification most likely resulted from reactive losses, rather than the difference in chemical properties between these two alkaloids and stable isotopically labeled nornicotine internal standard.

Method Characterization. Figure 2 shows a typical standard addition curve for nicotine in the 1R4F cigarettes as determined in this study. Calibration curves for other alkaloids are similar to that shown for nicotine. In 98% of the samples, the three-point standard addition curves had correlation coefficients greater than 0.99. Standard deviations calculated from multiple 1R4F measurements were used to estimate the limit of detection (LOD), set equal to $3S_0$,[21] where S_0 is the standard deviation of the analyte concentration at zero concentration. The LODs for nicotine, nornicotine, anabasine, and anatabine were estimated as 1.80, 0.134, 0.475, and 0.209 μg, respectively.

Using the standard addition approach described above, tobacco from Kentucky reference 1R4F cigarettes was used as a QC material and was repeatedly analyzed to determine the reproducibility, accuracy, acceptable criteria for the linearity of the calibration curve, concentration range of alkaloid levels, and suitable limits for the ratio of the peak areas of the quantitation ion to the confirmation ion. In 1 g of 1R4F tobacco, the average levels of nicotine (n = 14), nornicotine (n = 13), anabasine (n = 12), and anatabine (n = 14) were determined to be 18.7, 0.841, 0.101, and 0.554 mg, respectively. The relative standard deviations (RSDs) for the measurement for nicotine (n = 14), nornicotine (n = 13), anabasine (n = 12), and anatabine (n = 14) in the Kentucky reference 1R4F cigarette tobacco filler were 10.3%, 11.7%, 18.6%, and 12.9%, respectively. To determine the recovery of the measurement, a mixture of known amounts of nicotine, nornicotine, anabasine, and anatabine was spiked into the 1R4F tobacco sample, which was treated as an unknown sample for measurement. The recoveries (n = 3) for nicotine, nornicotine, anabasine, and anatabine are calculated as (mean calculated concentration/nominal concentration) × 100% and were determined to be 108%, 100%, 103%, and 108%, with RSDs of 6.7%, 9.9%, 17.6%, and 20.6%, respectively.

Measurements were made to determine how much native analyte contributed to the isotope-labeled internal standard and vice versa. This is necessary when stable isotope dilution mass spectrometry is used because an internal standard may not be 100% pure or may contain impurities that could contribute to the quantitation ion of the native analyte.[19] For example, although the nicotine standard claimed to have a purity of greater than 99%, GC/MS analysis showed a 0.5% nornicotine contamination. Therefore, the nornicotine level in the nicotine standard was quantified and the final concentration of nornicotine added into each addition sample was adjusted accordingly.

The use of analyte/confirmation ratios to rule out interferences followed standard practices except in the case of nornicotine. All

(21) Taylor, J. K. Quality Assurance of Chemical Measurement; Lewis Publishers: Chelsea, MI. 1987; pp 79−82

Table 2. Alkaloid Levels (mg/g) in Various Brands Obtained from Different Countries[a]

country	brand name	nicotine	nornicotine	anabasine	anatabine
Bangladesh	JP Gold Leaf	16.4 ± 0.7	1.03 ± 0.29	0.101 ± 0.017	0.56 ± 0.15
Bangladesh	Marlboro	14.1 ± 1.6	0.71 ± 0.07	0.069 ± 0.027	0.42 ± 0.11
Brazil	Derby	16.1 ± 2.8	1.06 ± 0.16	0.125 ± 0.053	0.59 ± 0.04
Brazil	Marlboro	18.0 ± 1.0	0.77 ± 0.08	0.082 ± 0.011	0.97 ± 0.41
China	Hongtashan	22.1 ± 0.6	1.02 ± 0.22	0.143 ± 0.013	1.12 ± 0.07
China	Marlboro	16.4 ± 0.9	0.85 ± 0.07	0.100 ± 0.026	0.51 ± 0.07
Egypt	Cleopatra	16.5 ± 2.4	1.12 ± 0.74	0.089 ± 0.034	0.67 ± 0.20
Egypt	Marlboro	21.0 ± 0.5	0.76 ± 0.24	0.094 ± 0.019	0.59 ± 0.03
Germany	West	16.5 ± 1.7	0.67 ± 0.12	0.060 ± 0.008	0.61 ± 0.22
Germany	Marlboro	15.3 ± 1.3	1.16 ± 0.72	0.076 ± 0.012	0.54 ± 0.11
India	Gold Flake	13.6 ± 0.6	0.51 ± 0.03	0.089 ± 0.029	0.31 ± 0.07
India	Marlboro	18.9 ± 3.4	0.61 ± 0.09	0.096 ± 0.022	0.92 ± 0.37
Indonesia	Ardath	16.0 ± 0.5	0.39 ± 0.02	0.073 ± 0.011	0.69 ± 0.10
Indonesia	Marlboro	17.0 ± 1.1	0.95 ± 0.12	0.092 ± 0.023	0.54 ± 0.06
Japan	Mild Seven	16.7 ± 1.4	1.30 ± 0.38	0.094 ± 0.021	0.86 ± 0.39
Japan	Marlboro	17.7 ± 1.9	1.10 ± 0.50	0.085 ± 0.008	0.68 ± 0.20
Kenya	Sportsman	12.5 ± 0.8	0.50 ± 0.10	0.051 ± 0.003	0.49 ± 0.11
Kenya	Marlboro	15.6 ± 1.1	0.82 ± 0.28	0.084 ± 0.011	0.44 ± 0.08
Mexico	Boots	16.3 ± 0.9	1.27 ± 0.14	0.071 ± 0.012	0.55 ± 0.20
Mexico	Marlboro	21.2 ± 0.8	1.50 ± 0.48	0.106 ± 0.003	0.56 ± 0.15
Nigeria	High Society	8.1 ± 0.4	0.36 ± 0.14	0.044 ± 0.013	0.13 ± 0.02
Nigeria	Marlboro	17.6 ± 1.9	0.77 ± 0.28	0.077 ± 0.013	0.69 ± 0.16
Pakistan	Embassy	18.1 ± 0.6	0.63 ± 0.03	0.163 ± 0.062	0.31 ± 0.10
Pakistan	Marlboro	18.2 ± 0.8	0.84 ± 0.30	0.089 ± 0.009	0.58 ± 0.10
Russia	Prima	15.3 ± 2.8	0.73 ± 0.06	0.060 ± 0.006	0.43 ± 0.04
Russia	Marlboro	16.2 ± 1.6	0.85 ± 0.19	0.071 ± 0.019	0.76 ± 0.29
United States	Doral	13.6 ± 0.6	0.66 ± 0.12	0.074 ± 0.009	0.50 ± 0.10
United States	Marlboro	18.1 ± 1.5	0.75 ± 0.01	0.071 ± 0.011	0.55 ± 0.12

[a] Values are means ± SD of three cigarettes from different packs.

measurements with an ion ratio within the 99% confidence range were deemed acceptable for the tobacco samples. For nornicotine, only a limited number of masses had enough intensity to be useful analytically, so mass 119 was chosen as the confirmation mass despite interference at the same nominal mass from the isotopically labeled nornicotine. The absolute contribution of m/z 119 from native nornicotine as the confirmation area was determined by subtracting the total m/z 119 counts from the contribution of internal standard.

Application to Cigarette Samples. The cigarette samples analyzed from 14 countries included both a Marlboro and a locally popular brand. Marlboro was chosen because it is the top-selling international brand and was a common brand available in each of the selected countries. One cigarette from each of 3 different packs for each of the 28 samples was individually ground and analyzed. Mean and between-pack variation of alkaloid levels in the same brand of commercial cigarette from various countries were measured and are summarized in Table 2. Overall, several interesting trends in the total alkaloid concentrations were noted.

It was previously reported that the nicotine content in the tobacco filler present in commercial cigarettes typically varies from 1% to 3% of the total weight of tobacco, depending on the blend composition used in the manufacturing practice.[20] The nicotine concentrations are characteristically higher in air-cured tobacco, followed by flue-cured, Maryland, and oriental tobaccos.[22] Other factors such as growth conditions and leaf stalk position also affect nicotine levels in tobacco.[22] Thus, differences in blend composition yield cigarette brands with different nicotine levels. As shown in Table 2, the nicotine content varied nearly 3-fold, ranging from 8.1 to 22.1 mg/g, in the brands analyzed in this study. Comparing the nicotine levels between the local top-selling brand and Marlboro for each of the countries by means of a two-tailed, two-sample unequal variance *T*-test, we noticed several interesting trends (Figure 3). In five countries (China, Kenya, Mexico, Nigeria, United States), the top-selling local brand and Marlboro had statistically significant differences ($P < 0.05$) in the nicotine levels. Marlboro cigarettes had higher levels of nicotine than local brands in four of these five countries. The exception was China, where the local Hongtashan brand had the highest nicotine level (22.1 ± 0.6 mg/g) measured in this study. The lowest nicotine level, 8.1 ± 0.4 mg/g, was measured in the Nigerian High Society brand (Figure 3).

Nornictoine is generally the second most abundant alkaloid in tobacco and commercial cigarettes.[14,23] However, in selected bright tobacco samples, anatabine levels higher than the corresponding nornicotine levels have been reported.[14] Among the commercial cigarettes analyzed in this work, the nornicotine content varied 4-fold from 0.36 to 1.50 μg/g (Table 2). In most cases, the difference between nornicotine levels in the locally popular and Marlboro brands was not statistically different ($P > 0.05$). A statistically significant difference in the nornicotine levels between the local and Marlboro brands was observed only in Indonesia ($P = 0.01$).

Anabasine is usually the least abundant alkaloid in tobacco,[23] although Yang and Smetena reported a comparable nornicotine

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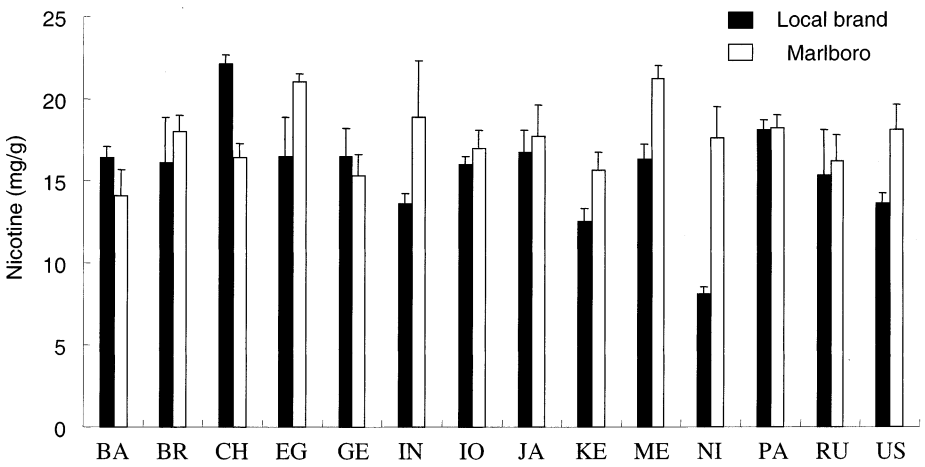

Figure 3. Comparison of nicotine levels between the local brand and Marlboro in all the countries: BA, Bangladesh; BR, Brazil; CH, China; EG, Egypt; GE, Germany; IN, India; IO, Indonesia; JA, Japan; KE, Kenya; ME, Mexico; NI, Nigeria; PA, Pakistan; RU, Russia; US, United States.

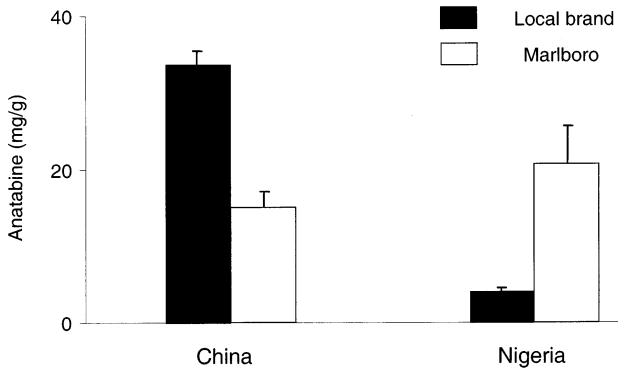

Figure 4. Comparison of anatabine levels between the local brand and Marlboro from China and Nigeria.

and anbasine level in selected tobacco samples.[17] The anabasine content varied approximately 4-fold from 0.044 to 0.163 mg/g among cigarette brands we analyzed. The range of anabasine levels in the Marlboro cigarettes, 0.069−0.106 mg/g (Table 2), showed a smaller variation than in the locally popular brands (0.044−0.163 mg/g). Except for Kenya ($P = 0.04$), Mexico ($P = 0.03$), and Nigeria ($P = 0.04$), the average anabasine content between the Marlboro and the locally popular brand was not statistically significant.

Compared with the other alkaloids, the anatabine levels varied most widely, from 0.13 to 1.12 mg/g (Table 2). Tobacco from the Chinese Hongtashan cigarettes had the highest level of anatabine, which is more than 2-fold over that measured in Marlboro cigarettes purchased in China (Figure 4). In contrast, the lowest anatabine levels were detected in the Nigerian High Society brand, which was only approximately 80% less than that detected in the Marlboros sold in that country (Figure 4). Chinese Hongtashan cigarettes are manufactured primarily from the flue-cured bright tobacco. Therefore, the high anatabine level in Chinese cigarette agrees with the higher anatabine levels previously observed in other bright tobaccos.[14] Pakistan also had average anatabine levels in tobacco that were significantly different between the locally popular brand and Marlboro ($P = 0.03$) (Table 2).

This investigation also found marked differences in the levels of alkaloids in two cigarette brands from the same country. For example, the four alkaloids levels in the Nigerian local brand are extremely low compared with Marlboro cigarettes purchased in that country (Figure 5), as well as nearly all other cigarette brands from other countries (Table 2). Such low alkaloid levels in domestic Nigerian cigarettes most likely reflect the use of lower quality tobaccos. However, such differences indicate that the cigarette manufacturers have some control over the alkaloid levels

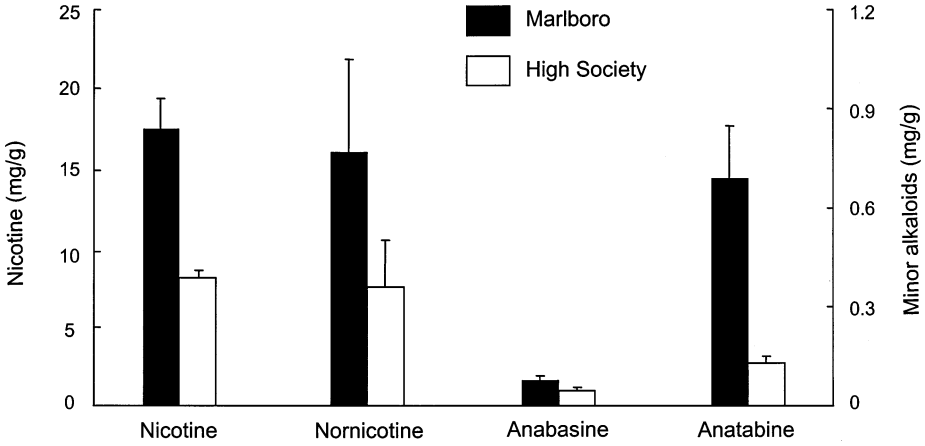

Figure 5. Comparison of four alkaloid levels between the local brand and Marlboro from Nigeria.

and providing a commercially viable product with reduced alkaloid content is possible.

Analysis of these data identified significant differences in the alkaloid levels measured in commercial cigarettes purchased in diverse geographic locales. Given that the composition of tobacco filler can vary tremendously from brand to brand, it is not surprising to observe significant variations in the alkaloid levels. The cigarettes measured in this study ranged from a 100% flue-cured blend to a typical American blend containing flue-cured, burley, and oriental tobaccos. In addition to the type of tobacco used, the use of expanded tobacco, stems, and reconstituted tobacco in the filler also may influence the alkaloid composition. However, on the basis of our observations of the tobacco type and limited information printed on the cigarette packs identifying the cigarettes as either bright (flue-cured, or Virginia blend) or as "American blend" (usually containing a blend composed of bright, burley, and oriental tobaccos), the most significant difference in the alkaloid content among the different brands of commercial cigarettes is attributable to variations in the tobaccos used for manufacture.

CONCLUSIONS

We have developed a headspace SPME/GC/MS method to simultaneously determine nicotine and other minor alkaloid contents in international cigarette tobacco samples. This approach offers distinct advantages over previous methods, including unambiguous analyte identification, ease of sample preparation, high throughput, and good reproducibility. Our experience with tobacco alkaloid analysis leads us to believe that this methodology could easily be adapted for the analysis of other analytes in tobacco, tobacco smoke, and a variety of agricultural products.

In addition to obtaining quantitative information about alkaloid content in the tobacco filler from these 28 brands of cigarettes, one aspect of this work deserves additional consideration. Foremost in any quantitative method is establishing reliable calibration curves that accurately reflect the analyte levels to be measured. Meaningful recovery studies are always critical to access the contribution of any matrix effects that may exist over the entire spectrum of samples. In the current work, dramatic brand-specific analytical response factors were observed. Application of a standard calibration curve to measure relative response ratios for the minor alkaloids could have introduced errors of 50% or higher. It was surprising to find such large differences in analytical response factors in what we believed to be a fairly homogeneous matrix. All the samples in this study were cigarette filler from commercial cigarettes, consisted primarily of cured tobacco leaf, and appeared to be comparable products. The only apparent physical differences observed in the blend was the inclusion of burley and oriental tobaccos used in American blend cigarettes as opposed to the nearly exclusive use of flue-cured tobacco associated with Virginia blend cigarettes.

However, the difference in the analytical response factors for the minor alkaloids did not fall neatly in two categories. A wide range in the magnitude of the brand-specific response factors was observed. Blind application of a method based on using a standard calibration curve approach using either neat standards or spiking standards on a surrogate matrix would have produced significant errors in the measured minor alkaloids concentrations. To minimize differences in the brand-specific analyte response factors, we adopted the technique of standard addition in a stringent QC protocol to ensure accurate quantification. In this situation, the standard addition method provided the best possible approach because any sampling bias associated with the matrix is virtually eliminated when both quantitation and calibration are performed on the same sample. The findings in this work should help reinforce the necessity of including a rigorous and applicable recovery study as an essential component in quantitative application.

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