

Rapid and Chemically Selective Nicotine Quantification in Smokeless Tobacco Products using GC-MS

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Abstract

In recent years, there has been a rapid proliferation of smokeless products with a wide range of nicotine content and flavoring formulations that may appeal to new users and existing cigarette smokers. The CDC nicotine method, which employs gas chromatography-flame ionization detection (GC-FID), provides a robust means for measuring nicotine in smokeless tobacco. However, several compounds, identified in a few flavored smokeless products, interfere with nicotine quantification using GC-FID. In response, the standard nicotine method (26.7 min run time) was modified to use faster GC ramping (3.7 min run time) and detection with mass spectrometry (GC-MS) in selected ion-monitoring mode to reduce signal interferences that can bias nicotine values. Seven conventional smokeless samples ($n = 12$) and blank tobacco samples spiked at three nicotine concentration levels ($n = 5$) were analyzed using the GC-FID and GC-MS methods and found to be in excellent agreement. However, only the GC-MS method provided confirmation of chromatographic peak purity in certain highly flavored products. The GC-MS method is not intended to replace the GC-FID method but to provide a method versatile enough to analyze a wide range of nicotine values in domestic and international samples of varying complexity. Accurate nicotine quantification is important for determining total nicotine content in tobacco and in subsequent calculations of un-protonated nicotine content.

Introduction

An estimated 7.8 million people in the United States use smokeless tobacco products (1), including loose leaf, plug, twist chewing tobaccos, dry snuff, and moist snuff (2). Among the types of domestic smokeless tobacco, moist snuff, which has a high use prevalence among adolescent males (3), accounts for approximately 85% of smokeless tobacco sales (4). The demand for smokeless tobacco products has rapidly grown as evidenced by a 39% increase in the amount of moist snuff produced in the United States between 2000 and 2008 (5,6).

Domestic smokeless products are usually produced from air- and fire-cured tobacco (2) and are often augmented with inorganic salts, sugar, licorice, molasses, fruit juices, spices, essential oils, and individual flavor chemicals to enhance product taste or other physicochemical characteristics. The addition of inorganic salts such as sodium carbonate and ammonium carbonate (7) boost the product pH, resulting in increased nicotine adsorption (8,9). Moreover, globally, smokeless tobacco products are widely used and vary greatly in terms of tobacco type used, curing processes, additive content, moisture, nicotine concentration, and product pH (10).

At pH levels typically found in domestic moist snuff (approximately pH 5.5–8.5) (11), nicotine exists in two forms: a mono-protonated and an un-protonated form (12) that is also known as free nicotine (13). Un-protonated nicotine is the form of nicotine most readily absorbed across oral membranes (8,9). At increasingly higher pH levels, the fraction of nicotine in the un-protonated form increases; for example, at pH 5.5, only 0.3% of nicotine exists in the un-protonated form; whereas, at pH 8.5, 75% of nicotine is un-protonated (12). The relative fraction of un-protonated nicotine and the total nicotine is used to calculate the absolute amount of un-protonated nicotine (14).

In addition to pH, nicotine absorption is influenced by product characteristics, including nicotine content; cut size (i.e., for moist snuff; fine cut, long cut, or straight cut); additive content; moisture content (8,9,15); or whether the tobacco is loose or in a pouch (16). Individual users influence nicotine absorption by brand choice (15) and use parameters, including dip or sachet size choice, number of daily uses, chewing intensity, and residence time of tobacco in the oral cavity, which influences the amount of nicotine absorbed (17). Moreover, clinical researchers have demonstrated that smokeless tobacco products with higher levels of un-protonated nicotine deliver nicotine into the bloodstream more rapidly and contribute to higher concentrations in the blood than products with lower levels of un-protonated nicotine (9).

The existing Centers for Disease Control (CDC) nicotine methodology, published in 1999, was developed to measure total nicotine in domestic smokeless tobacco products using gas chromatography with flame ionization detection (GC-FID). When

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the GC-FID method was introduced (14), considerably fewer smokeless tobacco products were available in the U.S. marketplace, and most of those brands were unflavored or wintergreen flavored (13).

The GC-FID methodology provides a relatively inexpensive and robust means of determining total nicotine in conventionally-flavored smokeless products; however, challenges associated with nicotine analyses have increased with the introduction of newer products with more complex flavoring formulations. At present, moist snuff products are available in a variety of new fruit and exotic flavors (e.g., apple, bourbon, cherry, cinnamon, spearmint, etc.) (18), which are more chemically complex than unflavored and wintergreen-flavored moist snuff (19). In addition, many international smokeless tobacco products contain substantial amounts of other plant-derived materials, including areca nut, piper betel leaf, saffron, cardamom, catechu, camphor eucalyptus, rose, aniseed, and clove (10), which contribute to sample complexity.

Highly augmented domestic and international smokeless tobacco products contain compounds that could potentially co-elute with nicotine or quinoline (used as an internal reference) peaks leading to errors in quantification. Analysis using gas chromatography-mass spectrometry (GC-MS) in selected-ion monitoring mode (SIM) eliminates the vast majority of potentially interfering or co-eluting peaks. In the past, GC-MS has been successfully used to analyze nicotine in natural products (20) and biological matrices (21–23) with run times varying from 7.4 min to 20 min.

In response to the increased sample complexity associated with smokeless products, we developed a versatile GC-MS method that optimizes analytical speed, specificity, and provides unambiguous quantification of nicotine levels in conventional and highly flavored products. This publication presents the parameters for the new GC-MS method and compares the new method to GC-FID in conventional and highly flavored products.

Experimental

Chemicals

Nicotine (purity, 99.97%) was purchased from Fluka Chemical Company (Milwaukee, Wisconsin). Nicotine calibration standards were prepared in isopropanol and purchased from Tedia (Fairfield, OH). Quinoline (purity, 98%), used as the internal reference for all nicotine analyses, was purchased from Aldrich Chemical Co. (Milwaukee, WI). Sodium hydroxide (2N NaOH) was purchased from Lab Chem, Inc. (Pittsburgh, PA). HPLC-grade (purity, 99.8%) methyl *tert*-butyl ether (MTBE) was purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals were used without further purification. Standards and samples were weighed using an analytical balance (Sartorius AG; Göttingen, Germany) with an accuracy of ± 0.01 mg; tobacco samples were weighed to within ± 0.05 mg of 1 g.

Smokeless samples

Commercial U.S. moist snuff products, purchased at various retail outlets or from websites, were stored at -70°C until needed. International smokeless tobacco samples were provided by research partners in the country of origin. Prior to mixing, tobacco tins were equilibrated in a Lab-Line bench-top humidity chamber (Barnstead International, Dubuque, Iowa) at 23°C and at 95% humidity overnight. For each product, five tins or packages of each smokeless tobacco type [quality control (QC) materials and smokeless samples] were homogenized using a RS12V Robot Coupe batch processor (Jackson, Mississippi), and each were transferred into a 6-oz. amber bottle and sealed with a Teflon-lined lid. The bottles containing tobacco were stored long term at -70°C . Samples were equilibrated to room temperature prior to use.

Quality control and blank materials

Two QC materials were used: Copenhagen Snuff, purchased in Atlanta, Georgia in 2005 and a moist smokeless reference tobacco (2S3) obtained from the Tobacco Analysis Laboratory at North Carolina State University (Raleigh, NC). A nicotine-free tobacco blank was produced from the tobacco filler of the Quest 3 nicotine-free cigarettes (Vector Tobacco Inc., Research Triangle Park, NC). The tobacco was extracted (with aspiration) in a large Buchner funnel by rinsing with three 250-mL aliquots of methanol with 10% aqueous NaOH (2N).

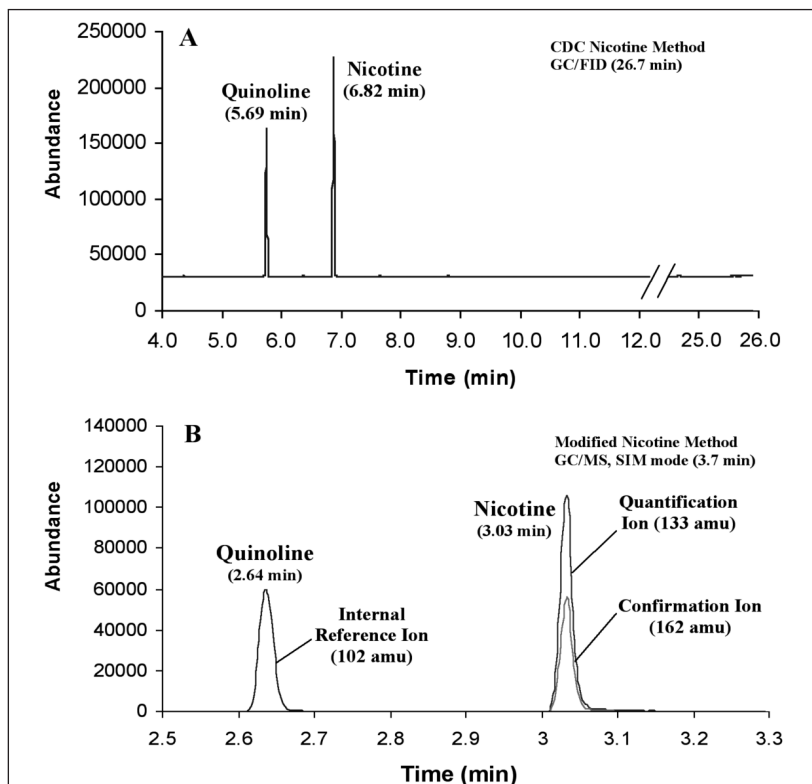


Figure 1. Typical GC-FID chromatogram for an unflavored fine cut moist snuff obtained using the standard CDC nicotine method (A). A reconstructed ion chromatogram for the same sample analyzed by GC-MS using a faster GC ramping (3.7 min run time) and selected-ion monitoring detection is shown in (B).

The solvent-washed tobacco was then dried in an oven at 80°C. Subsequent analysis of the blank tobacco yielded nicotine levels of approximately 0.06 mg/g, which is below the method limit of detection (0.16 mg/g) as determined by GC-MS. This blank material was used for preparation of analytical blanks and calibration curves for both methods.

Preparation of extraction solution

To facilitate comparison of the methods, quinoline was used as an internal standard for nicotine quantification for both the GC-FID and GC-MS approaches. An extraction solution was prepared by mixing ~ 500 mg of quinoline into a freshly opened 4-L bottle of MTBE. For larger sample batches, the MTBE extraction solution was prepared by mixing ~ 1.5 g of quinoline into 12 L of MTBE held in a large carboy (Nagle Nunc International; Rochester, NY) connected via flexible tubing to a BrandTech dispenser mounted on a ring-stand.

Protocol for measuring total nicotine

Nicotine concentration levels (mg/g) in tobacco samples were determined by adding 50 mL of extraction solution (MTBE containing quinoline) and 5 mL of 2N NaOH solution to 1.0 g of tobacco in a screw-top amber sample bottle; extraction and NaOH solutions were added using a Brand Tech bottle-top dispenser (BrandTech Scientific Inc.; Essex, CT). Following addition of MTBE and NaOH, the tobacco suspension was agitated on an orbital shaker (Lab-line Instruments, Inc.; Melrose Park, IL) at 160 rpm for 2 h. Subsequently, an aliquot was transferred to a 2-mL autosampler vial for GC-MS or GC-FID analysis.

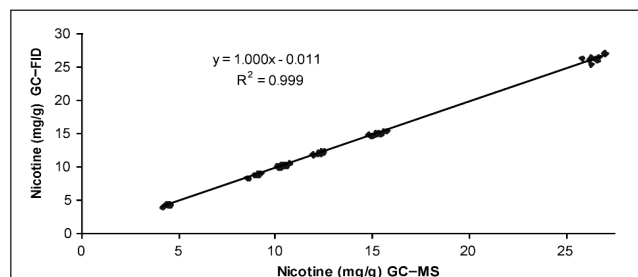


Figure 2. Regression analysis of total nicotine (mg/g) values for seven moist snuff samples ($n = 12$) measured using GC-MS and GC-FID. The least squares linear regressions and correlation coefficients correspond to the nicotine data (mg/g) measured from the same vial by the two methods.

Table I. Method Precision and Accuracy Determined by Spiking a Set of Five 1-g Nicotine-Free Tobacco Samples with a Solution Containing Nicotine at 4.02, 9.07, or 14.01 mg/g Tobacco*

Nicotine Spike (mg)	GC-MS Method ($n = 5$) [†]			GC-FID Method ($n = 5$) [‡]			
	Nicotine Mean \pm Standard Error (mg/g)	Method Precision (CV,%)	Method Accuracy (%)	Nicotine Spike (mg)	Nicotine Mean \pm Standard Error (mg/g)	Method Precision (CV,%)	Method Accuracy (%)
4.02	4.04 \pm 0.02	1.17%	100.6%	4.02	4.08 \pm 0.03	1.77%	101.6%
9.07	9.21 \pm 0.03	0.75%	101.6%	9.07	9.17 \pm 0.06	1.51%	101.2%
14.01	14.17 \pm 0.05	0.73%	101.2%	14.01	13.92 \pm 0.04	0.69%	99.4%

*Following spiking, samples were extracted with methyl tertiary butyl ether, and the resulting extract was analyzed.

[†] Analysis method presented in this manuscript.

[‡] Analysis method published in Federal Register. 1999. pp.14085 – 14096. FR Doc. 99-7022.

GC-FID quantification instrumentation and parameters

Nicotine analyses using GC-FID were performed on an Agilent 6890 GC (Agilent Technologies; Avondale, PA) equipped with a standard flame ionization detector. The GC was equipped with an Ultra2 GC column (25 m \times 0.32 mm \times 0.52 μ m) (Agilent Technologies, Avondale, PA). The GC inlet was maintained at 230°C with a constant flow (1.7 mL/min) of ultrapure helium (99.9999%) as the carrier gas. Ultra zero air and ultra high purity hydrogen were used for the flame ionization detector. An injection split ratio of 50:1 was used for these analyses. The GC oven ramp was as follows: initial temperature, 110°C; ramp at 10°C/min to 185°C; ramp at 6°C/min to 240°C, hold 10 min. Total GC run time was 26.7 min. The operational parameters from the nicotine GC-FID method are published in the Federal Register (14). All GC-FID analyses were performed in triplicate. Nicotine extracts from seven moist snuff products and spiked samples at three concentrations were analyzed in parallel by both GC-FID and GC-MS.

GC-MS quantification instrumentation and parameters

Nicotine quantification by GC-MS was performed by injecting 1- μ L aliquot from each sample vial onto an Ultra2 GC column (25 m \times 0.32 mm \times 0.52 μ m) (Agilent Technologies Inc., Palo Alto, CA) housed in an Agilent 6890 GC with a 5973N Mass Selective Detector. The GC inlet was maintained at 230°C with a constant flow (1.7 mL/min) of ultrapure helium (99.9999%) as the carrier gas. An injection split ratio of 50:1 was used. The GC oven ramp used the following sequence: hold at 175°C for 1 min; ramp at 5°C/min to 180°C; ramp at 35°C/min to 240°C. Total GC run time was 3.7 min. The heated transfer line from the GC oven to the MS ion source was maintained at 280°C. Selected ion-monitoring (SIM) parameters (mass and dwell) were quinoline, 102 amu (10 msec; internal reference) and nicotine, 133 amu (10 msec; quantification), 162 amu (35 msec; confirmation). Two additional ions (quinoline, 129 amu, 10 msec; nicotine, 161 amu, 35 msec) provided alternative ions in case interferences were encountered. Sample injections were made using a CTC CombiPAL autosampler (Leap Technologies, Carboro, North Carolina) equipped with a 10- μ L syringe. All GC-MS analyses were performed in triplicate.

Measurement of total nicotine

Nicotine levels were determined from the relative response ratio of the nicotine chromatographic peak area to that from the quinoline internal standard (i.e., response factor). A least squares fit of known nicotine amounts (mg) and their respective response factors yielded calibration equations for quantification. In addition to having the appropriate retention time, nicotine's detection by the GC-MS method was also confirmed by comparing relative areas of the reconstructed ion chromatograms for the quantification (133 amu) and confirmation ions (162 amu) with a known standard. Mass spectral data were deemed valid if the quantification to confirmation ion peak area ratios were within $\pm 5\%$ of the known standard.

Assessment of precision and accuracy

To examine possible matrix effects, we performed standard addition assays by spiking aliquots of nicotine-containing solution on the blank tobacco. Three nicotine levels (4.02 mg/g, 9.07 mg/g, and 14.01 mg/g) were prepared by adding appropriate nicotine amounts to a 1.0-g portion of blank tobacco; five samples

were prepared for each spike level along with five unspiked blank samples. Following spiking, extraction solution was added and the samples were shaken for 2 h. Extracts of the spiked samples were measured by both GC-FID and GC-MS. To assess method repeatability from injection-to-injection variation, samples from one vial from each of the three spiked concentrations (4.02 mg/g, 9.07 mg/g, and 14.01 mg/g) were injected 10 times over several days.

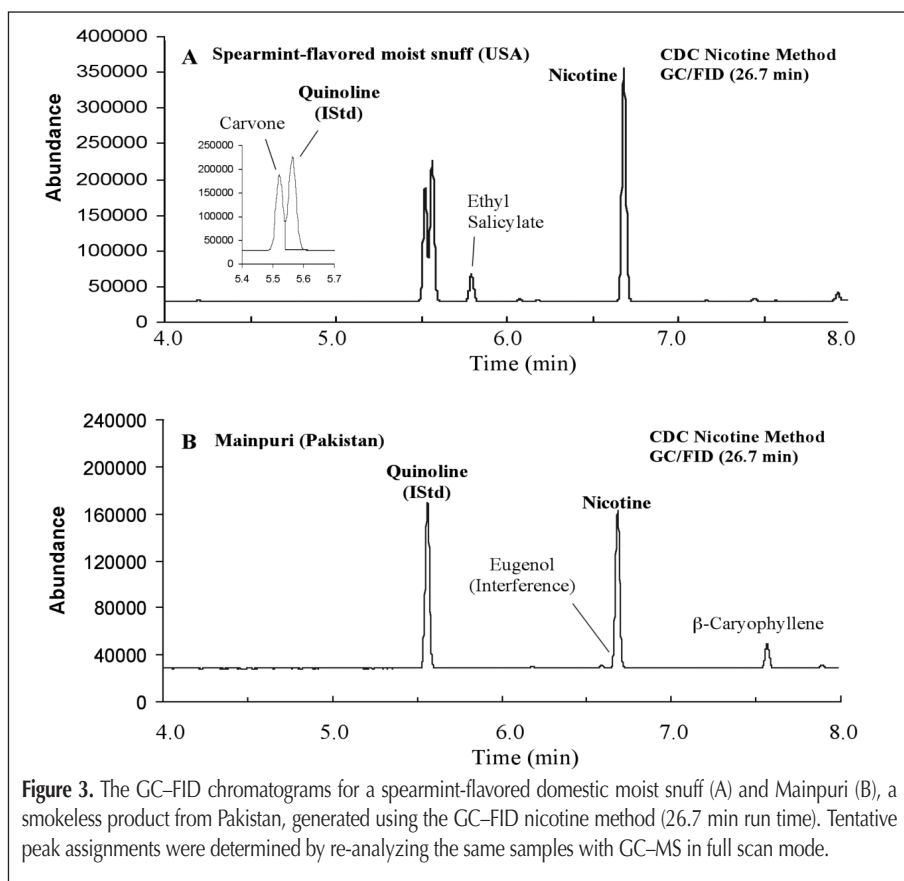


Figure 3. The GC-FID chromatograms for a spearmint-flavored domestic moist snuff (A) and Mainpuri (B), a smokeless product from Pakistan, generated using the GC-FID nicotine method (26.7 min run time). Tentative peak assignments were determined by re-analyzing the same samples with GC-MS in full scan mode.

Table II. The pH, Total and Un-protonated Nicotine (mg/g) Values for Seven Conventionally Flavored Smokeless Test Samples Analyzed Using GC-MS and GC-FID*

Samples	pH Mean**	Nicotine un-protonated form (%)**	GC-MS Method (n = 12) [†]			GC-FID Method (n = 12) [‡]		
			Total Nicotine Mean ± SE ^{††} (mg/g)	Total Nicotine CV ^{‡‡} (%)	Un-protonated Nicotine (mg/g) ^{§§}	Total Nicotine Mean ± SE (mg/g)	Total Nicotine CV (%)	Un-protonated Nicotine (mg/g)
Sample A	5.54	0.3	4.39 ± 0.04	3.23%	0.01	4.39 ± 0.04	3.20%	0.01
Sample B	7.44	20.8	8.95 ± 0.06	2.32%	1.86	8.92 ± 0.07	2.50%	1.86
Sample C	8.62	79.9	10.24 ± 0.04	1.49%	8.18	10.29 ± 0.03	1.02%	8.22
Sample D	6.64	4.0	10.36 ± 0.06	1.84%	0.41	10.30 ± 0.05	1.68%	0.41
Sample E	7.08	10.3	12.24 ± 0.05	1.54%	1.26	12.23 ± 0.05	1.50%	1.26
Sample F ^{***}	7.41	31.0	15.19 ± 0.09	1.93%	3.00	15.11 ± 0.08	1.83%	2.99
Sample G	5.71	0.5	26.45 ± 0.10	1.29%	0.13	26.46 ± 0.13	1.67%	0.13

* Mean values represent 12 measurements made during a two-month period. Total nicotine values for all seven samples obtained by the two methods were not statistically different at the 95% confidence interval using a pooled two-tail t test.
[†] Analysis method presented in this manuscript.
[‡] Analysis method published in Federal Register. 1999, pp. 14085 – 14096. FR Doc. 99-7022.
[§] Mean values was calculated using triplicate pH measurements
^{**} Nicotine in un-protonated form (%) is calculated using product pH and the pKa value for the pyrrollic nitrogen of nicotine (8.02) substituted into the Henderson-Hasselbalch equation.
^{††} SE = Standard Error.
^{‡‡} CV = Coefficient of Variation
^{§§} Un-protonated nicotine (mg/g) is calculated by dividing fraction of un-protonated nicotine (%) by 100 then multiplying by total nicotine (mg/g). In the table above, un-protonated nicotine values were calculated using the total nicotine measured by the GC-MS and GC-FID methods for comparison.
^{***} Only 11 nicotine measurements were available for the GC-MS method.

Determination of un-protonated nicotine percentage using product pH

Product pH was determined by suspending 2 g of smokeless tobacco products in 10 mL of distilled water and measured using an EA940 expandable ion analyzer (Orion Research; Cambridge, MA). For each sample, pH readings were taken at 5 min, 15 min, 30 min, and 60 min and were averaged; each product was measured in triplicate. Moist snuff products in tobacco-containing sachets were analyzed by removing the tobacco from the sachets, grinding the sachets, and thoroughly mixing the tobacco and the sachets together prior to pH measurement. The total percentage of moisture was obtained by calculating the difference in the weight of a 5-g sample of tobacco before and after drying at $99.5 \pm 0.5^\circ\text{C}$ for 3 h (AOAC method 966.02) (21); measurements were made in triplicate. Substitution of a product's pH value and the reference pKa value of the pyrrollic nitrogen of nicotine (8.02) into the Henderson-Hasselbalch equation yields the fraction of nicotine present in the un-protonated form (α_{nb}). By multiplying α_{nb} by total nicotine, the amount of un-protonated nicotine is calculated; the equations for determining un-protonated nicotine are published in the Federal Register (14).

Characterization of peak co-elution using GC with FID and MS

Characterization of co-eluting compounds was performed by injecting 1- μL aliquot onto an Ultra2 GC column (25 m \times 0.32 mm \times 0.52 μm). The column connects to zero-dead volume connector, which splits the column stream between a 5975 Mass Selective Detector (MSD) and FID detector. The MSD was operated in either full-scan mode (40–350 amu) for compound identification or in selected ion monitoring mode using the same parameters used for GC-MS quantification described earlier in this publication.

Results and Discussion

Overview of nicotine analysis by GC–FID and GC–MS methods

The GC–FID nicotine method includes a 2-h MTBE extraction of a 1-g sample of smokeless tobacco, followed by a 26.7-min GC–FID analysis of a 1- μ L aliquot of the extract (14). A chromatogram showing nicotine in a smokeless tobacco sample analyzed by GC–FID is shown in Figure 1A; when the method was developed, the 26.7 run time was implemented to ensure the GC column had sufficient time to elute all injected compounds before subsequent run were initiated. The nicotine and quinoline peaks are baseline separated, and there are no obvious interferences for conventionally-flavored domestic smokeless products. Nicotine measured in standards and commercial smokeless products using the GC–MS method showed excellent chromatographic separation for all smokeless tobacco products with more rapid elution of quinoline and nicotine (Figure 1B). This chromatogram also shows readily quantifiable baseline-separated peaks. Mass spectrometric detection had sufficient chemical specificity that even with chemically complex smokeless tobacco samples, no interfering contributions to target peak areas were identified for co-eluting peaks.

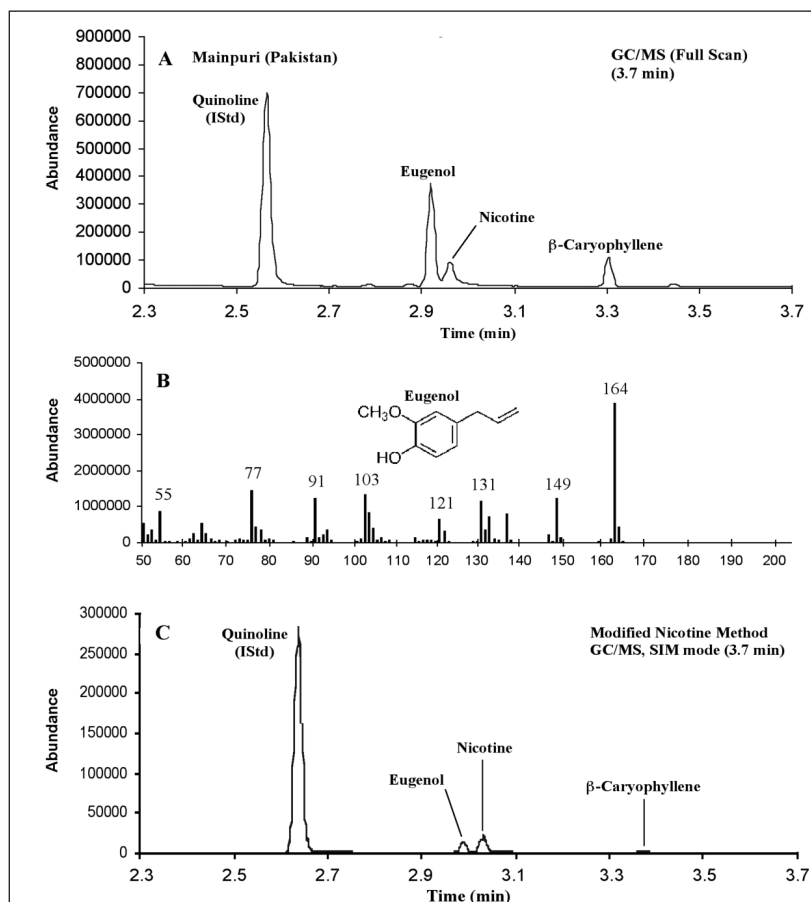


Figure 4. Modified GC ramping (3.7 min run) yields chromatographic separation of eugenol and nicotine. A GC–MS analysis (full scan mode) of Mainpuri (A) using the modified GC ramping is shown below. The mass spectrum for eugenol resolved from nicotine in Mainpuri is shown in (B). The analysis of Manipuri with the new GC–MS method using faster GC ramping coupled with SIM-MS detection is shown in (C).

Evaluation of GC–MS method: quantification range

Peak areas from chromatograms were used to generate calibration curves spanning a concentration range from 0.05–65.62 mg/g tobacco with excellent linearity ($R^2 > 0.990$). All domestic smokeless tobacco samples had nicotine levels within this range. The extended calibration curve was useful for analyzing certain international and non-traditional products with higher nicotine levels. The GC–MS method has excellent sensitivity and can easily quantify nicotine in smokeless tobacco samples as low as 0.16 mg/g, which is the method's analytical limit of detection (LOD). The LOD was calculated as three times the y-intercept of the regression line of standard deviation of calculated concentration versus concentration for repeated measurements of low calibrators (24).

Evaluation of GC–MS method: repeatability and reproducibility

Injection-to-injection variation for the GC–MS analysis was examined using 30 injections of each spike concentration extract (4.02 mg/g, 9.07 mg/g, and 14.01 mg/g). A series of 10 injections from each standard solution were analyzed on three non-consecutive days for a total of thirty injections for each concentration. The coefficient of variation (CV) values were calculated for each concentration ($n = 10$) on each day. The CV values for the first 10 injections at low (4.02 mg/g), medium (9.07 mg/g), and high (14.01 mg/g) concentrations were 0.38%, 0.63%, and 0.24%, respectively. The average CV values for a total of 30 injections at low, medium, and high concentrations were 0.38%, 0.32%, and 0.33%, respectively.

Evaluation of GC–MS method: analytical stability

To determine the analytical stability for commercial samples, 18 individual samples of a commercial smokeless tobacco product were analyzed by GC–MS during a seven-day period. The measured nicotine values ranged from 12.62 mg/g to 13.55 mg/g with a standard deviation of 0.27 mg/g. The mean value for the samples analyzed by the GC–MS method was 13.11 mg with a CV of 2.05%, which was comparable with a value of 13.12 mg/g ($n = 3$) obtained by independent measurements made by LabStat Inc. (Kitchener, Ontario, Canada) using the standard GC–FID nicotine method. Smokeless reference material 2S3 (1-g samples), obtained from North Carolina State University, was analyzed using the GC–MS method in our laboratory 81 times over a 24-month period generating a CV value of 1.83% and demonstrating excellent analytical stability over an extended period of time.

Evaluation of GC–MS method: precision and accuracy

Measured levels of nicotine in conventional smokeless tobacco products using the GC–MS and GC–FID methods agreed well. To compare the

GC-MS and GC-FID methods, solutions were spiked at one of three nicotine concentrations (4.02 mg/g, 9.07 mg/g, and 14.01 mg/g of tobacco) on nicotine-free tobacco (Table I). Data from both methods exhibit excellent method precision ($CV < 2\%$) and accuracy ($\pm 1.6\%$ of expected value). The average CV was similar for the GC-MS method (average $CV = 0.88\%$) and the GC-FID method (average $CV = 1.32\%$). For method accuracy, the average values for the GC-MS and GC-FID methods were 101.1% and 100.7%, respectively.

Evaluation of GC-MS method: determination of total and un-protonated nicotine in test samples

For comparison, nicotine measurements were made on seven conventional moist snuff samples with a wide range of nicotine and pH values using both the GC-FID and the GC-MS methods (Table II). The total nicotine levels for these test samples ranged from 4.39 to 26.45 mg/g. The GC-MS and GC-FID methods generated comparable results when analyzing these smokeless tobacco samples. The results obtained from these two methods were not statistically different ($p > 0.05$) using a pooled two-tailed t-test. Data from both methods exhibited excellent method precision with average CV for both methods of 1.95%. Using total nicotine levels from the GC-MS method and measured pH values, the amount of un-protonated nicotine levels in the seven products used in the comparison were calculated (Table II). The levels of un-protonated nicotine values ranged from 0.01 to 8.18

mg/g using the GC-MS method. Similarly, the calculated levels of un-protonated nicotine in data measured by GC-FID ranged from 0.01 to 8.22 mg/g. Regression analysis of total nicotine values for seven moist snuff products by the GC-MS and GC-FID methods also showed excellent agreement (slope = 1.000; $R^2 = 0.999$) (Figure 2).

Evaluation of Potential Peak interferences using GC with dual FID and MS detectors.

The CDC nicotine method (14) provides a robust means of measuring nicotine in smokeless tobacco using GC-FID. When the GC-FID method was introduced, most domestic moist snuff products were either unflavored or wintergreen (13); wintergreen flavor contains large amounts of methyl salicylate, which does not interfere with nicotine quantification by the CDC nicotine method by GC-FID. Recently, several compounds that interfere with nicotine quantification have been found in new domestic and several international products. A standard GC-FID chromatogram for a flavored moist snuff containing carvone, a major constituent of spearmint (19), overlaps partially with the quinoline peak (Figure 3A). Overlap of carvone with the quinoline internal reference compound tends to result in GC-FID integrations that truncate a portion of the quinoline peak, resulting in a slight increase in relative response and reported nicotine value (Table III).

In another example, the GC-FID chromatogram for Mainpuri, a smokeless product from Pakistan, contains a large peak eluting at the retention time for nicotine (Figure 3B); however, GC-MS analysis revealed a large contribution to the peak area that is attributable to eugenol. To further determine the extent of eugenol contribution to the nicotine peak, the Mainpuri sample was rerun with the faster GC ramp (3.7 min) using full-scan MS detection (Figure 4A). This chromatogram shows the chromatographic separation of a nicotine peak and a large eugenol peak. The mass spectrum for eugenol is shown in Figure 4B. The co-elution of eugenol with nicotine can result in an elevated nicotine peak area that can contribute to an erroneously high value. Figure 4C shows the Mainpuri samples run with the GC-MS quantitation method with the fast ramping and SIM detection; this is the method used to measure nicotine in the smokeless samples.

In the standard GC-FID method, carvone co-elutes with quinoline as a shoulder peak, whereas eugenol co-elutes at the same retention time as nicotine. The co-elution of eugenol with nicotine results in elevated nicotine values by GC-FID. Examples of nicotine values obtained using the standard GC-FID method and the GC-MS method in domestic and international products that contain carvone or eugenol are shown in Table III. Eugenol and nicotine co-elute as a single symmetrical peak; hence, a nicotine peak con-

Table IV. A List of Flavor-Related Compounds that Closely Elute to Quinoline (Internal Standard Compound) or Nicotine When Analyzed by GC-MS (3.7 min run time) and GC-FID (26.7 min Run Time) is Shown Below*

Closely eluting compounds	GC-MS Method (3.7 min)			GC-FID Method (26.7 min)		
	RT* (min)	Potential Peak Co-elution?	Interference resolved with GC-MS?	RT (min)	Potential Peak Co-elution?	Interference resolved with GC-FID?
Quinoline (Internal standard)	2.67	-	-	5.75	-	-
Methyl Salicylate [†]	2.45			5.21		
Pulegone	2.61			5.68	Yes	No
Carvone	2.62	Yes	Yes	5.70	Yes	No
p-Anisaldehyde	2.66	Yes	Yes	5.82	Yes	No
Piperitone	2.67	Yes	Yes	5.84	Yes	No
Ethyl Salicylate	2.70	Yes	Yes	5.97		
Cinnamylaldehyde [‡]	2.72	Yes	Yes	5.99		
Nicotine (Target Analyte)	3.06	-	-	6.88	-	-
Eugenol [§]	3.02	Yes	Yes	6.88	Yes	No
α -Cubebene**	3.04	Yes	Yes	6.86	Yes	No
Valerophenone	3.04	Yes	Yes	6.89	Yes	No
p-Anisaldehyde ^{††} dimethyl acetal	3.06	Yes	Yes	6.97	Yes	No

* The retention time and status of interferences are shown.

[†] Methyl salicylate is listed because it is the primary flavor-related compound in wintergreen flavored smokeless products; however, it does not interfere with quinoline or nicotine.

[‡] At concentrations normally encountered in smokeless samples, cinnamylaldehyde and quinoline are baseline separated. In samples with extremely high cinnamylaldehyde concentrations (i.e. products with very high cinnamon content) co-elution may occur. The 129 amu peak could be used alternatively in that case.

[§] At concentrations normally encountered in smokeless samples eugenol and nicotine are baseline separated. In samples with extremely high eugenol concentrations (i.e. products with high clove content), mass 161 amu should be used for nicotine quantification instead of 133 amu due to the small 133 amu produced by eugenol fragmentation.

** The data above were taken from various samples run with both GC-FID and GC-MS over a two-month period; slight retention shifts occurred relative to other compounds during that time.

^{††} Reaction or degradation product of p-Anisaldehyde.

taining eugenol is indistinguishable from one containing only nicotine by the GC–FID method (26.7 min). The co-elution of eugenol under the nicotine peak in Mainpuri caused a 243% increase in the nicotine amount determined by GC–FID.

Flavor-related compounds with retention times (25) similar to quinoline or nicotine (Table IV) introduce interfering peaks that can contribute to analytical bias associated with nicotine quantification by GC–FID. Also, as the concentration of these compounds increases, the incidence and severity of chromatographic peak co-elution increases. Using the GC–MS method, the compounds we identified can be resolved from the peaks of interest and accurately quantified. Moreover, the GC–MS method includes additional ions for quinoline (129 amu) and nicotine (161 amu). The area ratio of 102 amu (the internal reference ion) to 129 amu provides a useful check for potential co-elution of interfering compounds with quinoline. Also the ion ratios of the 133 amu ion and 161 amu ion provide an additional check for potential co-elution of interfering compounds with nicotine. In the event of a co-elution in highly complex samples, the 129 amu and 161 amu ions can be substituted for the ions customarily used.

The prevalence of products with complex flavorings (domestic and international) has increased in recent years and introduces an increased potential for analytical interference with quinoline or nicotine. For example, carvone, partially overlaps with quinoline and is found in common flavoring materials derived from spearmint and dill. Eugenol, which co-elutes with nicotine, is a constituent of flavoring materials derived from clove, cinnamon, cananga, geranium, and nutmeg (19), which are added to some domestic smokeless products. Besides being present in essential oils, eugenol and carvone are flavor compounds that are individually added to domestic smokeless products (7). For this reason, these compounds, whether individually added or as essential oil constituents, can be present in domestic smokeless products and if at high enough concentrations can affect nicotine quantification in domestic smokeless samples. Moreover, international products, can contain additional flavoring materials not used in the United States which could contribute potential interfering peaks. Judicious selection of mass fragments for monitoring greatly reduces interferences in highly flavored smokeless tobacco products.

Conclusion

The new GC–MS analysis method offers a more rapid and compound selective means for accurately quantifying total nicotine in the presence of potential interferences, although the GC–MS approach does require a larger initial outlay in capital equipment expenses. Advantages of the GC–MS method include higher throughput and chemical specificity not possible with the GC–FID. The new GC–MS implementation has a rapid 3.7 min run time substantially increasing sample throughput as com-

Table III. A Comparison of Nicotine Quantification by the Existing GC–FID Method and New GC–MS Method in Several Domestic and International Products that Contain Carvone and Eugenol which Co-elute with Quinoline or Nicotine

Product Description	Sample Origin	Interfering Compound	Nicotine (mg/g) GC/MS Method	Nicotine (mg/g) GC/FID Method	% Bias
Spearmint-flavored snuff	United States	Carvone	12.07	12.35	2.32%
Mainpuri	Pakistan	Eugenol (L)*	2.26	7.76	243%
Flavored snuff	Sweden	Eugenol (M)*	8.64	9.74	12.7%
Cinnamon-flavored pouch	United States	Eugenol (S)*	10.97	11.13	1.46%

* L = large; M = medium; S = small.

pared to the established GC–FID nicotine analysis with a 26.7 min run time (15). In the unlikely event of interferences which have common compound specific ions with either nicotine or the internal standard, the sample can be re-analyzed by the GC–MS in full-scan mode for compound identification providing additional information about the sample's chemical composition.

The GC–FID has proven to be an accurate and robust method for analyzing nicotine in conventionally-flavored domestic smokeless tobacco. Thus, the GC–MS method is not meant as a replacement of the GC–FID method but as a more powerful alternative, particularly when analyzing more complex samples (highly flavored domestic or international smokeless products). Use of FID detection with the faster GC ramping regimen (3.7 min) is not advisable due to known flavor compounds that co-elute with quinoline and nicotine (Table IV); the presence of these compounds would compromise quantification. The GC–MS nicotine method has been used successfully to analyze nicotine in domestic smokeless samples (12). In addition to smokeless products, the GC–MS method has utility for analyzing nicotine in other tobacco products including cigarette filler, clove cigarette filler, bidis, cigars, clove cigarettes, hookah (water pipe), and pipe tobaccos (data not shown).

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