Reversed Phase Ion-Pair Liquid Chromatographic Determination of Nicotine in Commercial Tobacco Products. 1. Moist Snuff

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The official methods for the determination of nicotine in commercial tobacco products (AOAC, CORESTA) are based on approaches that are not selective for nicotine (colorimetric measurement, steam distillation, perchloric acid titration), and the availability of published methods based on state-of-the-art chromatographic methods is limited. Reversed phase ion-pair liquid chromatography has been established as a viable alternative for the analysis of basic analytes. A reversed phase ion-pair liquid chromatographic method for the determination of nicotine in commercial tobacco products was developed and optimized in separate experiments (Ciolino, L. A.; Turner, J. A.; McCauley, H. A.; Smallwood, A. W.; Yi, T. Y. *J. Chromatogr.* **1999a**, *852* (2), 451−463). An extensive within-laboratory performance study of the optimized method was subsequently conducted, and results are presented here for the determination of nicotine in commercial moist snuff. Results for the determination of nicotine in commercial cigarettes are presented in a subsequent paper (Ciolino, L. A.; Fraser, D. B.; Yi, T. Y.; Turner, J. A.; Barnett, D. Y.; McCauley, H. A. *J. Agric. Food Chem.* **1999b**, *47*, 3713−3717).

Keywords: *Nicotine; moist snuff; tobacco; alkaloids; reversed phase ion-pair liquid chromatography*

INTRODUCTION

In 1994, our laboratory was searching for a reliable method for the determination of the nicotine content in commercial tobacco products. Our review of the literature showed that the established methods for the determination of nicotine content involved a variation of one of the following analytical approaches: colorimetric measurement of the reaction product of nicotine with cyanogen bromide or chloride after extraction or distillation (Sadler et al., 1960; Collins et al., 1969; Harvey et al., 1969, 1976; Davis, 1976; Harvey and Handy, 1981; Long et al., 1988); steam distillation followed by spectrophotometric or gravimetric analysis of the distillate (Griffith, 1957; De Vries et al., 1976); gas chromatographic (GC) analysis of an organic extract obtained by direct extraction or after liquid−liquid extraction of an aqueous tobacco extract (Lyerly and Green, 1976; Severson et al., 1981; Gottscho et al., 1988; Sisson et al., 1990); or liquid chromatographic (LC) analysis of an aqueous or methanolic tobacco extract (Piade and Hoffmann, 1980; Saunders and Blume, 1981; Murthy et al., 1986). More recently, both free zone capillary electrophoresis (CE) and micellar electrokinetic capillary chromatography (MECC) have also been used (Yang and Smetena, 1995; Yang et al., 1996), but their suitability for routine use has not been established. For a more comprehensive review, the reader is referred to Green et al. (1996).

The colorimetric and steam distillation methods determine the total alkaloids content and are not selective for nicotine. Although the chromatographic methods are selective for nicotine, two of the published GC methods are based on older technology (i.e., packed columns) and generate significant amounts of halogenated solvent waste (Lyerly and Green, 1976; Gottscho et al., 1988). The published LC methods are inconvenient due to time-consuming and/or cumbersome sample preparation procedures (Piade and Hoffmann, 1980; Saunders and Blume, 1981; Murthy et al., 1986) and are based on chromatographic conditions that are now known to be less than optimal for basic analytes on reversed phases (i.e., mobile phase pH values near 7). The AOAC and CORESTA have adopted methods based on steam distillation (AOAC, 1995a,b; CORESTA, 1968) or the colorimetric approach (CORESTA, 1994a) as official methods, as well as a method based on titration with perchloric acid (AOAC, 1995c).

Because of the limited availability of recently published methods based on state-of-the-art chromatographic methods, we elected to adapt one of our in-house methods to the determination of nicotine in tobacco. Our laboratory routinely applies a reversed phase ion-pair liquid chromatographic method for the screening and determination of alkaloids (Smallwood et al., 1997). The method uses pH 3.0 citric acid buffer containing an ion-pairing reagent (sodium octanesulfonate) as both the sample extraction solvent and the buffer portion of the mobile phase. Spike/recovery experiments using this method in conjunction with C_{18} solid-phase extraction (SPE) cleanup have demonstrated that the extraction conditions provide good recovery (78−95%) of added nicotine from milk, orange juice, vegetable juice, and processed squid in a single extraction (Smallwood et al., 1997).

We investigated and optimized this approach for determining nicotine in commercial moist snuff and commercial cigarettes (Ciolino et al., 1999a). Validation

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10.1021/jf990049s
Published on Web 08/07/1999

data were generated and are presented here for one reference moist snuff (University of Kentucky Moist Snuff 1S3) and eight commercial moist snuff composite samples. The nicotine contents of the nine moist snuff composite samples were also determined using two other methods for comparison: the AOAC steam distillation method for the determination of total alkaloids in tobacco (AOAC, 1995a) and a GC method for the determination of the nicotine content of commercial moist snuff (Gottscho et al., 1988). This latter method has been proposed as a candidate to replace the older AOAC methods. Validation data for commercial cigarettes are presented in the subsequent paper (Ciolino et al., 1999b).

MATERIALS AND METHODS

Composite Preparation. Eight different brands of commercial moist snuff were purchased from stores in the Greater Cincinnati area. Reference Moist Snuff 1S3 was obtained from the University of Kentucky Tobacco Health Research Institute (THRI, 1986). Each composite was prepared in a 32 oz glass jar using the contents of 10 tins (1.0−1.2 oz/tin net weight) of moist snuff except for composite 7, which used 15 tins (20 pouches/tin with ∼0.5 g of moist snuff/pouch; pouches discarded). Product from every other tin was sampled for individual nonvolatile determination immediately after the tin was opened and prior to its being added into the composite. After all of the product was placed in the jar, the contents were thoroughly mixed and additional samples were taken for a second nonvolatile determination. When not in use, the composite samples were sealed in the glass jars and stored in a refrigerator. Additional nonvolatile determinations were conducted on the composites at 2 or 3 week intervals throughout the study. Total composite weights ranged from 270 to 360 g except for composite 7, which weighed ∼160 g.

Nonvolatile Determination. Percent nonvolatiles was determined by drying a weighed portion of moist snuff (sample weight = 1.5−3.0 g) in a laboratory oven for 3 h at 105 °C, cooling the samples in a desiccator, and reweighing. The percent by weight volatile material (100% − % nonvolatiles) was used as an estimate of percent by weight moisture.

Extraction Volume Experiment. Approximately 0.1−0.2 g of moist snuff was accurately weighed into a glass scintillation vial or volumetric flask. The specified extraction volume of the citric acid/sodium octane sulfonate buffer (pH 3.0) was added, and the vial was sonicated for 30 min. After extraction, ∼1.5 mL of the extract was removed and filtered through a 0.45 μm nylon filter into a LC autosampler vial for analysis using the ion-pair LC method. Two sets of extraction volume experiments were conducted on different days: extraction volumes of 4, 6, 8, 15, 25, and 50 mL were tested in the first set; extraction volumes of 5, 10, 50, 100, and 250 mL were tested in the second set. Five replicates were prepared at each extraction volume.

Multiple Extraction Experiment. Approximately 0.1 or 0.2 g of moist snuff was accurately weighed into a glass scintillation vial. A specified volume (5 or 10 mL) of the citric acid/sodium octane sulfonate buffer (pH 3.0) was added, and the vial was sonicated for 30 min. After extraction, ∼1.5 mL of the extract was removed and filtered through a 0.45 μm nylon filter into an LC autosampler vial. The remaining liquid extract was filtered through Whatman No. 2 filter paper to recover the solid tobacco. The solid tobacco was returned to the original scintillation vial, and two additional extractions (5 or 10 mL volumes) were subsequently conducted, recovering the solid tobacco between extractions and sampling the liquid extract after each extraction. Each of the three extracts was analyzed individually for nicotine content using the ion-pair LC method. Five replicates were conducted using both the 5 and 10 mL extraction volumes.

Ion-Pair LC Method. *(a) Instruments and Conditions.* Each of the four analysts used one of the following liquid chromatograph systems or component combinations: Hewlett-Packard Series II 1090 with diode array detector; Hewlett-Packard 1050 with multiwavelength detector; Hewlett-Packard 1050 with diode array detector; Waters 600 E pump, Waters 712 WISP autosampler, and Waters 996 diode array detector.

Zorbax Rx C_{18} (analysts 1 and 4) or Supelco PKB-100 C_{18} columns (analysts 2 and 3), both 5 μm, 15 cm × 4.6 mm i.d., were used for all determinations. The mobile phase was 65:35 buffer/methanol for all determinations except composite 3, for which the buffer/methanol ratio was adjusted to 70:30 to resolve a coeluting peak. The buffer comprised 50 mM citric acid and 10 mM sodium octanesulfonate adjusted to a pH of 3.0. The flow rate used by the four analysts was either 1.00 or 1.25 mL/min. The injection volume was 10 μL. Detection was at 259 nm.

(b) Reagents and Standards. Citric acid monohydrate (ACS reagent), sodium octanesulfonate (98% or Ultra grade), and nicotine hydrogen tartrate salt (catalog no. N-5260) were obtained from Sigma (St. Louis, MO). The nicotine content of the nicotine salt was determined at 32.6% at the beginning of the study and 32.6% after the study using CORESTA Recommended Method 39 (CORESTA, 1994b). This corresponds to a purity of 100% (100.3%) as the salt. Nicotine standards were prepared in the mobile phase buffer to cover the calibration range 0−100 μg/mL.

(c) Sample Preparation. Approximately 0.1−0.2 g of moist snuff composite was accurately weighed into a 20 mL glass scintillation vial. Mobile phase buffer (10 mL) was added using a pipet, and the vial was sonicated for 30 min. The vial was subsequently centrifuged for 10 min (*g* force = 1422), and the liquid extract was drawn off using a fine-tip disposable pipet and set aside. Second and third extractions of the original sample were conducted using 10 mL volumes of mobile phase buffer, sonicating, centrifuging, and drawing off the liquid extract after each extraction. The three extraction volumes were combined and filtered through a 0.45 μm nylon filter into an LC vial for analysis. Eight replicate samples were prepared for each composite. All values for nicotine content are based on peak area quantitation and are reported on an "as is" basis (not corrected for moisture content).

(d) Sample and Standard Preparation, Single Extraction Experiments. Approximately 0.1−0.2 g of moist snuff composite was accurately weighed into a 20 mL glass scintillation vial. Mobile phase buffer (10 mL) was added using a pipet, and the vial was sonicated for 30 min. The liquid extract was filtered through a 0.45 μm nylon filter into an LC vial for analysis. Eight replicate samples were prepared for each composite. Nicotine standards were prepared in the mobile phase buffer to cover the calibration range 0−500 μg/mL. All values for nicotine content are based on peak area quantitation and are reported on an "as is" basis (not corrected for moisture content).

(e) Spike/Recovery Experiments Using a Single Extraction. For spike recovery experiments, an appropriate volume of a nicotine hydrogen tartrate solution prepared in mobile phase buffer was spiked onto 0.1−0.2 g of moist snuff to provide an additional nicotine content equal to the previously determined nicotine content of the tobacco sample. The sample was then analyzed according to section d above (single extraction). All spiking experiments were conducted in duplicate.

AOAC Method 967.02 (AOAC, 1995a). *(a) Instrument.* All UV measurements were made using a Shimadzu UV-2101 PC scanning spectrophotometer.

(b) Steam Distillation Apparatus. Steam was generated using a Sussman electric boiler MBA 3 with an operating pressure of 13 psi. The distillation apparatus comprised a 500 mL Kjeldahl flask, a bulb type steam distill head, and a 600 mm Graham condenser. The distillation volume was 2 L. The performance of the apparatus was tested according to ISO 3400 (ISO, 1989) by distilling portions of nicotine free base standard at the beginning and end of the study and obtaining a minimum of 98% recovery.

(c) Standards. Nicotine free base (98% minimum) was obtained from Aldrich (Milwaukee, WI), desiccated in a standard laboratory desiccator using commercial Drierite, and

stored in the refrigerator. The purity of the nicotine free base was determined at 99.7% at the beginning of the study and at 99.1% after the study using CORESTA Standard Method 39 (CORESTA, 1994b). *Caution: Nicotine free base is extremely toxic via inhalation, ingestion, and skin absorption. Do not inhale, ingest, or allow skin contact. Wear gloves and prepare all stock solutions in a hood.*

(d) Samples. Three replicate analyses were conducted for each moist snuff composite using sample weights in the range 2−5 g. The reported nicotine contents were not corrected for the purity of the nicotine free base standard. All values for nicotine content are reported on an "as is" basis (not corrected for moisture content).

GC Method (Gottscho et al., 1988). *(a) Instrument and Conditions.* All GC determinations were made using a Hewlett-Packard 5890 Series II gas chromatograph with an FID detector and an Alltech catalog no. C6089 glass column (2% KOH and 10% Carbowax 20 M, support 80−100 mesh Chromosorb WHP, 6 ft length, 4 mm i.d., 0.25 in. o.d.). The column was preconditioned by the manufacturer. The column temperature (isothermal) was 190 °C; injector and detector temperatures were both 230 °C. The carrier gas flow rate (helium) was 30 mL/min. The injection volume was 5 μL.

(b) Standards. The internal standard octadecane (97%) was obtained from Fisher (Pittsburgh, PA, Acros division). See section c under the AOAC Method 967.02 for a description of the nicotine free base standard.

(c) Samples. Five replicate analyses were conducted for each moist snuff composite using 2 g samples. Sample extractions were conducted as specified in the reference (Gottscho et al., 1988). The reported nicotine contents were not corrected for the purity of the nicotine free base standard. All values are reported on an "as is" basis (not corrected for moisture content).

Experimental Design for Validation Studies. Four analysts participated in the ion-pair LC portion of the validation study. To compare results between and among analysts in an efficient design, the analysts were assigned to the composites as shown in Table 4. This design provided for two separate determinations (two different analysts) for each of the commercial composites and for a comparison of results from all four analysts based on the reference moist snuff composite (composite 9). This design also allowed results from each analyst to be compared to results from every other analyst at least once. The analyst assignments were the same for the single extraction experiments.

The AOAC method and the GC method were conducted by two additional analysts (one per method) who did not participate in the LC portion of the study.

RESULTS AND DISCUSSION

Extraction of Nicotine from Tobacco. For analytical purposes, aqueous solutions of acids (Collins et al., 1969; Davis, 1976; Gottscho et al., 1988; Harvey et al., 1969, 1976; Long et al., 1988; Piade and Hoffmann, 1980; Saitoh et al., 1985) or bases (Lyerly and Greene, 1976; Saunders and Blume, 1981; Yang and Smetena, 1995; Yang et al., 1996) have been successfully employed for the extraction of nicotine from tobacco. The ability of either acidic or basic solutions to extract nicotine from tobacco makes sense because both nicotine free base and nicotine salts are highly water soluble under ambient conditions (Jackson, 1941; *Merck Index*, 1995). For GC analysis, the extraction of nicotine using aqueous solvents represents the first step in a two-phase extraction. The aqueous solution is made strongly basic and the nicotine free base is subsequently extracted into a nonpolar organic solvent such as chloroform or dichloromethane alone or in combination with hexane (Gottscho et al., 1988; Lyerly and Greene, 1976; Saitoh et al., 1985). For LC, CE, or colorimetric analysis, the single-phase aqueous-based extract is frequently used for analysis after filtration and/or dilution (Collins et al., 1969; Davis, 1976; Harvey et al., 1969, 1976; Long et al., 1988; Saunders and Blume, 1981; Yang and Smetena, 1995; Yang et al., 1996).

Table 1. Nicotine Content of a Commercial Moist Snuff Product Determined as a Function of Extraction Volume in a Single Extraction

first set		second set	
extraction vol[a] (mL)	nicotine content[b] (% by wt)	extraction vol[a] (mL)	nicotine content[b] (% by wt)
4	1.28 (0.02)	5	1.33 (0.01)
6	1.26 (0.01)	10	1.33 (0.01)
8	1.28 (0.01)	50	1.26 (0.01)
15	1.33 (0.02)	100	1.29 (0.01)
25	1.34 (0.01)	250	1.39 (0.07)
50	1.39 (0.03)		

[a] All extractions with volumes of ≤15 mL were conducted in glass scintillation vials. Extractions with volumes of ≥25 mL were conducted in glass volumetric flasks. [b] Each value is the average of five trials. Standard deviation is shown in parentheses.

Table 2. Nicotine Content of a Commercial Moist Snuff Product Obtained in Three Extractions Using a Constant Extraction Volume

	5 mL extraction vol		10 mL extraction vol	
extrn no.	nicotine content[a] (% by wt)	% of total extracted	nicotine content[a] (% by wt)	% of total extracted
1	1.31 (0.02)	91	1.34 (0.01)	93
2	0.12 (0.02)	8	0.08 (0.01)	6
3	0.02 (0.003)	1	0.01 (0.001)	1
total	1.45		1.44	

[a] Each value is the average of five trials. Standard deviation is shown in parentheses.

We investigated the citric acid/ion-pairing agent buffer for the extraction of nicotine from tobacco. The influence of extraction volume using a single extraction was determined in a series of experiments (Table 1) using a commercial moist snuff product. Extraction volumes ranging from 4 to 50 mL were tested in the first set of experiments; extraction volumes ranging from 5 to 250 mL were tested in the second set of experiments. The sample weight was maintained in the range 0.1−0.2 g, and the sonication time was constant at 30 min. In both sets of experiments, the highest apparent nicotine content (1.39 wt %) was obtained using the largest extraction volume. [The nicotine content of the commercial moist snuff was independently determined at 1.37 ± 0.01% using the published GC method (Gottscho et al., 1988) selected for comparison in this study.] However, it is believed that the transmission of the sonication energy was diminished for extractions which were conducted in glass volumetric flasks (extraction volumes of ≥25 mL) as compared to the extractions conducted in glass scintillation vials (extraction volumes of ≤15 mL) on the baiss of the results from the second set of experiments (Table 1). Separate experiments showed that sonication *time* was not a critical factor, with similar results being obtained for sonication times in the range 15−120 min.

We also investigated the use of multiple extractions using a constant extraction volume. The nicotine content in each of three successive extraction volumes was determined for the same commercial moist snuff product as tested above (Table 2, expressed as percent by weight in the product). Constant extraction volumes of either 5 or 10 mL were used. The results showed that the majority of the nicotine (>90%) was extracted in the

Table 3. Determination of Nonvolatiles for Moist Snuff Individual Tins and Composited Product

composite no.	individual tins		composited product	
	% nonvolatiles	%RSD	% nonvolatiles	%RSD
1	45.3	2.0	45.5	0.6
2	45.0	0.6	44.5	0.4
3	45.4	2.1	45.1	0.4
4	47.1	4.7	47.0	0.5
5	44.6	2.3	45.4	0.4
6	46.7	1.2	46.4	0.5
7	48.0	1.3	48.8	0.4
8	70.7	2.5	71.7	1.4
9	45.8	1.1	45.5	0.4

first extraction and that virtually all of the nicotine has been extracted after three extractions (third extraction represents only 1% of total extracted). Note that the total apparent nicotine content (1.45 or 1.44%) was higher than previously determined (1.39% in extraction volume experiment, 1.37% by independent GC method). Although this experiment was conducted to minimize carry-over of nicotine between extractions (see Materials and Methods), some carry-over probably occurred, making the apparent result higher. To eliminate this problem in actual analysis, the three extracts were combined and the nicotine determination was made on the combined extract.

Nonvolatile Determination and Composite Homogeneity. Commercial moist snuff is typically packaged in "tins" and has a significant moisture content, up to 55 wt %. Once a tin is opened, moisture can be lost due to evaporation. Nonvolatile determinations were conducted on the products immediately after the tins were opened to determine the approximate moisture content of the fresh product. Nonvolatile determinations were also conducted immediately after the products had been composited.

Table 3 provides the average and percent relative standard deviations (%RSD) for both the individual tin and composite product nonvolatile determinations. The moisture content of the commercial moist snuffs ranged from 52 to 55 wt % except for composite 8, which had a moisture content of ∼29%. The precision of the nonvolatile determinations conducted on the individual tins of product gives an indication of the tin to tin product consistency. For all of the commercial moist snuff brands, the %RSD based on the individual tins was <5%, showing a high degree of tin to tin consistency. The precision of the nonvolatile determinations conducted on the freshly composited products gives an indication of the composite homogeneity. In all cases, the %RSD after compositing was <1.5% and was also less than the RSD based on the individual tins, showing that the composited samples were well mixed and homogeneous.

Finally, nonvolatile determinations were also conducted at 2−3 week intervals throughout the study so that if any moisture changes occurred, the nicotine content could be related to that of the fresh product. In all cases, the difference in moisture content as measured on the fresh composites and throughout the study was <3% on a relative basis, with no trends observed in gain or loss of moisture. Therefore, no corrections were made to any of the nicotine determinations based on moisture content.

Ion-Pair LC Method, Chromatographic Performance, Figures of Merit, and Nicotine Content Results. A typical chromatogram obtained using the reversed phase ion-pair conditions is shown in Figure 1A. Nicotine elutes in <10 min and is well resolved from a host of relatively polar compounds that elute near the column void volume. Separate experiments (Ciolino et al., 1999a) demonstrated that the use of the pH 3.0 mobile phase dramatically improved the peak shape of nicotine relative to higher pH mobile phases by minimizing interactions with residual silanols on the stationary phase. This is consistent with established chromatographic practice for basic analytes on reversed phases (Snyder et al., 1995). At a pH of 3.0, the use of the ion-pair additive selectively increased the retention of nicotine away from the column void volume. Nicotine was also well resolved from the minor tobacco alkaloids nornicotine, anatabine, anabasine, and cotinine (Ciolino et al., 1999a).

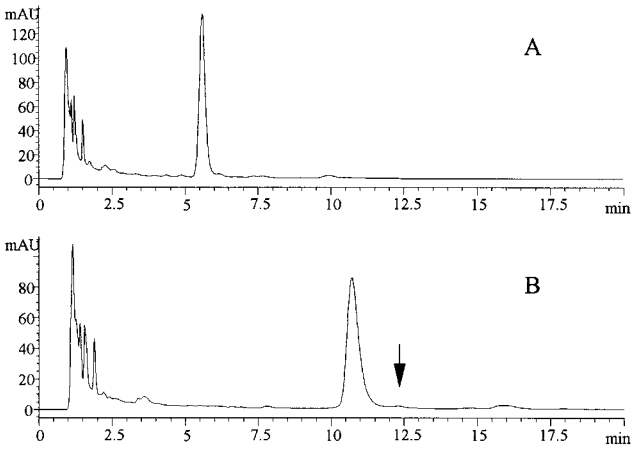

Figure 1. Chromatograms of a commercial moist snuff product obtained using optimized reversed phase ion-pairing conditions and a variation: (A) 65:35 buffer/methanol, buffer pH at 3.0; (B) 70:30 buffer/methanol, buffer pH at 3.2. The variation provided a significant increase in the resolution between nicotine and a closely eluting minor component, indicated by the arrow in (B).

For eight of the nine moist snuff composites, a mobile phase of 65:35 buffer/methanol was used. For composite 3, it was necessary to adjust the mobile phase conditions to 70:30 buffer/methanol to resolve a coeluting component. This increased the retention time of nicotine to ∼11 min and extended the run time for composite 3 to 20 min. The coeluting component was detected by monitoring the spectral purity of the nicotine peaks. A further variation of the conditions comprising 70:30 buffer/methanol, with the buffer pH at 3.2, was developed after work done with commercial cigarettes (Ciolino et al., 1999b). This variation was also applied to the analysis of moist snuff and was shown to be suitable (Figure 1B, arrow indicates the resolved minor component).

Linear calibration curves were obtained by all four analysts over the specified concentration range (0−100 μg/mL) with correlation coefficients (r^2) all >0.9999 for the 10 calibration curves generated during this portion of the study. The average nicotine contents, standard deviations, and %RSDs obtained by the four analysts for the nine moist snuff composites are given in Table 4. %RSDs ranged from 0.5 to 3.8% across analysts and composites. The overall method precision defined as the overall average %RSD was 1.3%.

Each of the eight commercial composites (composites 1−8) was analyzed by two analysts, and the reference composite (composite 9) was analyzed by all four ana-

Table 4. Results of Nicotine Determinations Using Ion-Pair LC Method (Three Extractions): Percent by Weight, As Is Basis

composite no.	analyst no. 1 av	1 SD	1 %RSD	2 av	2 SD	2 %RSD	3 av	3 SD	3 %RSD	4 av	4 SD	4 %RSD	analyst to analyst precision[a] (%)
1	0.69	0.03	3.8							0.70	0.00	0.6	1.4
2							1.26	0.01	0.7	1.30	0.01	0.7	3.1
3	1.48	0.05	3.2				1.43	0.02	1.1				3.4
4				1.42	0.01	0.9				1.41	0.01	0.9	0.7
5	1.25	0.03	2.3	1.26	0.02	1.7							0.8
6	1.03	0.01	1.3							1.05	0.01	0.8	1.9
7				0.95	0.00	0.5	0.92	0.01	1.1				3.2
8				0.46	0.00	0.9	0.44	0.01	1.2				4.4
9	1.23	0.02	1.9	1.25	0.01	0.6	1.19	0.01	1.1	1.24	0.01	0.8	4.9

[a] See text for details of calculation of analyst to analyst precision.

Table 5. Results of Nicotine Determinations Using Ion-Pair LC Method (Single Extraction): Percent by Weight, As Is Basis

composite no.	analyst no. 1 av	1 SD	1 %RSD	2 av	2 SD	2 %RSD	3 av	3 SD	3 %RSD	4 av	4 SD	4 %RSD	analyst to analyst precision[a] (%)
1	0.65	0.01	1.1							0.67	0.01	1.4	3.0
2							1.26	0.03	2.5	1.25	0.01	0.6	0.8
3	1.37	0.02	1.3				1.33	0.01	0.5				3.0
4				1.39	0.02	1.3				1.34	0.01	0.5	3.7
5	1.14	0.02	1.4	1.17	0.01	0.9							2.6
6	0.97	0.01	1.1							1.00	0.01	1.1	3.1
7				0.89	0.00	0.3	0.89	0.02	2.0				0.6
8				0.41	0.01	3.7	0.43	0.01	2.6				4.8
9	1.10	0.01	0.7	1.15	0.01	1.2	1.16	0.04	3.5	1.14	0.03	2.3	5.3

[a] See text for details of calculation of analyst to analyst precision.

lysts. A simple comparison of the average nicotine content results from the two analysts for each composite reveals good analyst to analyst precision. The analyst to analyst precision can also be calculated as (|difference|/average) × 100%, where |difference| refers to the absolute value of the difference between the nicotine content results from the two analysts. The results of these calculations are given in Table 4 (last column) and range from 0.7 to 4.4% across composites (composites 1−8). In an analogous manner, the nicotine content results from all four analysts represented a range of 4.9% relative to the average for the reference moist snuff composite (composite 9).

Both the Zorbax and Supelco columns were suitable for use in the analysis. However, the Supelco columns were found to be much less rugged than the Zorbax columns, exhibiting degraded performance after ∼300 injections. In contrast, the Zorbax columns were still usable after ∼500 injections. Lesser retention of nicotine was also observed with the Supelco columns (retention time in the range 4.0−4.5 min) vs the Zorbax columns (retention time in the range 5.0−6.0 min) using 65:35 buffer/methanol.

Efficiency of a Single Extraction, Ion-Pair LC Method. The ion-pair LC method uses three extractions to obtain all of the extractable nicotine. However, on the basis of the extraction experiments, it was expected that the majority of the nicotine was obtained in the first extraction. The entire ion-pair LC portion of the study was repeated by modifying the method to use only a single 10 mL extraction. The nicotine standard calibration curve was extended to cover the concentration range 0−500 μg/mL to reflect the higher concentration of nicotine obtained in a single extraction. Again, linear calibration curves were obtained by all four analysts with correlation coefficients (r^2) > 0.9993 for the nine calibration curves generated during this portion of the study.

Table 6. Nicotine Recovery from Moist Snuff in a Single Extraction Using Citric Acid/IPR Buffer

composite no.	single extrn % nicotine, overall lab av	three extrn % nicotine, overall lab av	% recovery (single extrn) ratio one/three extrn	% recovery (single extrn) spike/recovery
1	0.66	0.70	95	93
2	1.26	1.28	98	92
3	1.35	1.46	93	nt[a]
4	1.37	1.42	96	94
5	1.16	1.26	92	97
6	0.99	1.04	95	95
7	0.89	0.94	95	94
8	0.42	0.45	93	95
9	1.14	1.23	93	95

[a] Not tested.

The average nicotine contents, standard deviations, and %RSDs obtained by the four analysts for the nine moist snuff composites using a single 10 mL extraction are given in Table 5. %RSDs obtained by the four analysts were similar to the results obtained with three extractions, ranging from 0.3 to 3.7%. The overall method precision as previously defined was 1.5%. Good analyst to analyst precision as previously defined was also obtained (Table 5, last column) for the commercial composites (0.6−4.8%) as well as the reference composite (5.3%).

Table 6 lists the average nicotine contents (average across analysts) obtained for the nine moist snuff composites using one versus three extractions. These results indicate that the citric acid/sodium octanesulfonate buffer extracts most of the nicotine content of the tobacco in a single extraction. The percentage of the nicotine removed in the first extraction can be

Table 7. Comparison of Nicotine Content Determination Results for Ion-Pair LC Method vs AOAC and GC Methods (Percent by Weight, As Is Basis)

composite no.[a]	ion-pair LC method (three extrn)	GC method[b]	AOAC 967.02
1	0.70	0.72	0.80
2	1.28	1.35	1.40
3	1.46	1.52	1.60
4	1.42	1.46	1.52
5	1.26	1.25	1.36
6	1.04	1.08	1.17
7	0.94	0.97	1.00
8	0.45	0.47	0.52
9	1.23	1.28	1.33

[a] Composites 1−8 were made from eight different brands of commercial moist snuff. Composite 9 was made from University of Kentucky Tobacco and Health Research Institute Moist 1S3 Snuff. [b] GC Method (Gottscho et al., 1988).

calculated from the ratio of the single extraction and three extraction results and ranged from 92 to 98% across composites, with an average of 94%.

The efficiency of a single extraction was also measured in spike/recovery experiments. Nicotine was spiked onto the moist snuff at a level equal to its previously determined nicotine content so that the total nicotine content after spiking was twice the amount prior to spiking. Results are also given in Table 6 and show an average of 94% recovery across composites, consistent with the degree of extraction as calculated above. Therefore, all of the results based on a single extraction represent a reasonable estimate (within 10%) of the actual nicotine content. Use of a single extraction avoids the centrifugation step between extractions and may be a suitable approach for monitoring changes in nicotine content for a given product or for making product comparisons when one is working with larger numbers of samples.

Ion-Pair LC Method vs AOAC and GC Methods. The nicotine contents of the nine moist snuff composites were also determined using the AOAC steam distillation method (AOAC International, 1995a) and a GC method (Grottshco et al., 1988), which has been proposed as a replacement for the AOAC methods. A comparison of the average nicotine contents for the nine moist snuff composites as determined by the ion-pair LC method vs the AOAC and GC methods is given in Table 7.

As expected, both the ion-pair LC method and the GC method gave lower results than the AOAC method. This is expected because the AOAC method measures total alkaloids, whereas the chromatographic methods selectively determine nicotine content. The nicotine content as determined by the GC method averaged 94% of the total alkaloids as determined by the AOAC method, similar to the original comparison between these two methods conducted by the authors of the GC method (Gottshco et al., 1988).

Comparison of the results from the ion-pair LC method and the GC method shows good agreement with relative differences in the determined nicotine contents of 0−5% across composites. Inspection of the data reveals that the ion-pair LC method gave lower values than the GC method for eight of the nine composites. It is difficult to judge if either method provides more accurate results without a reference material certified for nicotine content. The nicotine content of the reference moist snuff (composite 9) was reported at 1.25 ± 0.08 wt % (THRI, 1986) at the time of its manufacture, although this material is not certified for nicotine content. The results from both the ion-pair LC method and the GC method are well within the reported range for the reference moist snuff.

General Evaluation of the Ion-Pair LC Method. The ion-pair LC method presented here offers a number of major advantages resulting in both time and cost savings for the determination of nicotine in commercial moist snuff. The extraction solvent is identical to the mobile phase buffer used in the LC procedure and is aqueous-based. This reduces the number of solutions that must be prepared, provides for minimal noise in the chromatographic baseline, and avoids the generation of halogenated or other organic solvent waste. Another advantage of the method is that no sample dilution is required after extraction. The extract is simply filtered into an LC sample vial.

A further advantage to the method is the use of the nicotine hydrogen tartrate salt rather than the free base as the standard. Unlike the free base, the nicotine salt is readily available at nominally 100% purity and is stable at room temperature. The free base form slowly degrades even when refrigerated and desiccated. The free base form also represents a more severe safety and handling hazard relative to the salt.

For the commercial moist snuff products we tested, the chromatographic conditions provided rapid separation of nicotine from the minor tobacco alkaloids, other endogenous tobacco components, and commercial additives. However, because of both the large number and variety of compounds that may be present in different commercial tobacco products and brands, there is a potential for interference as this method is applied more broadly. This issue can be readily addressed by using a diode array detector and monitoring the spectral purity of the nicotine peak. If coeluting interferences are encountered, the mobile phase conditions can be adjusted to resolve the interfering peaks. This was the case when we applied the method to commercial cigarettes, as we report in the subsequent paper (Ciolino et al., 1999b).

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Received for review January 19, 1999. Revised manuscript received June 18, 1999. Accepted July 9, 1999.

JF990049S