

- Updates on cruise ship outbreaks; and
- Inspections for WHO Ship Sanitation Certificates.

The official record of this meeting will remain open for 15 days (through April 30, 2008) so that additional materials or comments may be submitted and made part of the record.

Advanced registration is encouraged. To do so, please provide your name, title, company name, mailing address, telephone number, facsimile number, and e-mail address to Stephanie Lawrence at telephone: (770) 488-3141, fax: (770) 488-4127, or e-mail: [slawrence@cdc.gov](mailto:slawrence@cdc.gov). You may also contact her if you need any additional information.

Dated: March 7, 2008.

**James D. Seligman,**

Chief Information Officer, Centers for Disease Control and Prevention.

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**BILLING CODE 4163-18-P**

**DEPARTMENT OF HEALTH AND HUMAN SERVICES**

**Centers for Disease Control and Prevention**

**Notice Regarding Revisions to the Laboratory Protocol To Measure the Quantity of Nicotine Contained in Smokeless Tobacco Products Manufactured, Imported, or Packaged in the United States**

**AGENCY:** Centers for Disease Control and Prevention (CDC), Department of Health and Human Services.

**ACTION:** Notice.

**SUMMARY:** This notice amends the uniform protocol for the analysis of nicotine, total moisture, and pH in smokeless tobacco products (the "protocol"). The protocol, originally published in 1999 [Federal Register, Vol. 64, No. 55, "Annual Submission of the Quantity of Nicotine Contained in Smokeless tobacco products manufactured, imported, or packaged in the United States Requirement; Notice," 14085-14096 (FR Doc. 99-7022)], implements the requirement of the Comprehensive Smokeless Tobacco Health Education Act (CSTHEA) of 1986 (15 U.S.C. 4401 *et seq.*, Pub. L. 99-252) that each entity manufacturing,

packaging, or importing smokeless tobacco products shall annually provide the Secretary of Health and Human Services (HHS) with a specification of the quantity of nicotine contained in each smokeless tobacco product.

**DATES:** The first report of information is due June 30, 2008, with subsequent submissions due by March 31 of each year.

**ADDRESSES:** The information shall be submitted to: Matthew McKenna, M.D., Director, Office on Smoking and Health, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention (CDC), 4770 Buford Highway, NE., Atlanta, GA 30341-3724.

**FOR FURTHER INFORMATION CONTACT:** Matthew McKenna, M.D., Director, Office on Smoking and Health, telephone: (770) 488-5701.

**SUPPLEMENTARY INFORMATION:** Several smokeless tobacco product categories have entered the U.S. smokeless tobacco market since the implementation of the protocol in 1999 including snus, low moisture snuff sold in portion pouches, and smokeless tobacco sold in a compressed, pellet form. Some of the new smokeless tobacco product categories differ physically from previous smokeless tobacco categories.

**TABLE 1.—SUMMARY OF PH LEVELS FOUND IN SEVEN TYPES OF SMOKELESS TOBACCO PRODUCTS: PLUG; LOOSE LEAF OR SCRAP; TWIST; DRY SNUFF-LOW MOISTURE/NO POUCH; DRY SNUFF-LOW MOISTURE/POUCH; SNU; AND MOIST SNUFF**

Category	Smokeless tobacco product	pH <sup>a</sup>						pH change	Percent change
		Condition A 10 mL <sup>b</sup>			Condition B 20 mL <sup>b</sup>				
		Mean <sup>c</sup>	SD <sup>d</sup>	Mean <sup>c</sup>	SD <sup>d</sup>	Mean <sup>c</sup>	SD <sup>d</sup>		
Plug .....	Days O Work Chew .....	5.06	± 0.02	5.11	± 0.03	0.048	0.95		
	Conwood Company's Sun Cured .....	5.12	± 0.02	5.19	± 0.02	0.067	1.30		
	Levi Garrett Plug Chew .....	5.83	± 0.02	5.91	± 0.03	0.074	1.26		
	Taylor's Pride Plug Chew .....	5.92	± 0.03	5.97	± 0.03	0.052	0.89		
Loose Leaf .....	Beech-Nut Chew .....	5.56	± 0.01	5.62	± 0.01	0.062	1.11		
	Redman Chew .....	5.93	± 0.01	5.99	± 0.04	0.067	1.12		
Twist .....	Cumberland .....	5.68	± 0.01	5.79	± 0.02	0.107	1.88		
Dry Snuff/No Pouch ..	Tube Rose Sweet Scotch Snuff .....	5.64	± 0.00	5.69	± 0.02	0.051	0.90		
	RailRoad Mills Sweet Scotch Snuff .....	5.91	± 0.02	6.02	± 0.00	0.115	1.95		
Dry Snuff/Pouch .....	Taboka .....	6.44	± 0.01	6.52	± 0.00	0.081	1.26		
	Skoal Dry Cinnamon .....	6.78	± 0.00	6.83	± 0.01	0.056	0.83		
Snus .....	Camel Snus Original .....	7.43	± 0.00	7.44	± 0.00	0.010	0.13		
Moist Snuff .....	Renegades Wintergreen .....	6.45	± 0.03	6.53	± 0.03	0.079	1.22		
	Copenhagen Regular .....	7.61	± 0.02	7.52	± 0.01	-0.090	-1.18		
	Kodiak Ice Long Cut Regular .....	8.13	± 0.04	8.13	± 0.01	0.001	0.01		

<sup>a</sup> The standard protocol published in the FEDERAL REGISTER to measure pH in smokeless tobacco products is as follows: 10 mL of deionized distilled water is added to 2.00 grams of smokeless tobacco product measuring pH at 5, 15, 30 and 60 minute intervals. Recently introduced low moisture dry snuff smokeless tobacco products packed in pouches had a thick paste-like consistency when prepared in 10 mL of deionized distilled water. When 2.00 grams<sup>e</sup> of low moisture dry snuff smokeless tobacco products packed in pouches were prepared in 20 mL of deionized distilled water, the sample remains suspended in liquid and is well mixed.

<sup>b</sup> n = 1.

<sup>c</sup> Average pH from four measured intervals.

<sup>d</sup> Standard Deviation.

<sup>e</sup> Accurately weighed: 2.000 ± 0.0005 grams.

After evaluating information that has recently come to the attention of the Centers for Disease Control and Prevention's Office on Smoking and Health (OSH) regarding low moisture smokeless tobacco products packaged in portion pouches, OSH conducted an independent comparison of pH measurements in a variety of low and high moisture smokeless tobacco products. The results of this comparison, presented in Table 1, indicate that there is an acceptable (less than 2%) level of change in pH values when measurements are taken with 20 mL deionized, distilled water compared to 10 mL of deionized, distilled water. Increasing the volume of water in the mixture ensures that the matrix is sufficiently fluid to facilitate ease of measure.

OSH has determined that certain revisions will improve the applicability of the protocol to all currently marketed categories of smokeless tobacco and will allow manufacturers to test smokeless tobacco products less frequently than specified in the original version of the protocol. Briefly, the revised protocol requires the Standards Addition Assay to be performed and reported the first time each smokeless tobacco brand name is tested and whenever a change is made to the product formulation (including a change to the tobacco blend or cultivar); allows test materials to be analyzed in duplicate, rather than triplicate; provides a formula for testing frequency based on the manufacturing duration of each brand; and increases the volume of liquid in the pH determination from 10 milliliters (mL) to 20 mL. The change in the volume of liquid in the pH determination facilitates the ease of measure of smokeless tobacco pH for all currently marketed smokeless tobacco categories (i.e., plug, twist, moist snuff, dry snuff, snus, loose leaf, chew, moist snuff in portion pouches, smokeless tobacco compressed into a pellet, and dry snuff in portion pouches).

#### Collection of Information

This proposed amendment does not call for any new collection of information under the Paperwork Reduction Act of 1995 (44 U.S.C. 3501–3520).

Dated: March 7, 2008.

**James D. Seligman,**

*Chief Information Officer, Centers for Disease Control and Prevention.*

### Revised Protocol for Analysis of Nicotine, Total Moisture, and pH in Smokeless Tobacco Products

#### I. Requirements<sup>1,2</sup>

##### A. Reagents<sup>3</sup>

1. Sodium hydroxide (NaOH), 2N.
2. Methyl t-butyl ether (MTBE).
3. (-)-Nicotine (Fluka 72290) >99% purity.<sup>4,5</sup>
4. Quinoline (Aldrich).
5. Standard pH buffers; 4.01, 7.00, and 10.00.
6. Deionized distilled water.

##### B. Glassware and Supplies

1. Volumetric flasks, class A.
2. Culture tubes, 25 mm × 200 mm, with Teflon-lined screw caps.
3. Pasteur pipettes.
4. Repipettors (10 mL and 50 mL).
5. Linear shaker (configured to hold tubes in horizontal position).<sup>6,7</sup>
6. Weighing dishes, aluminum.
7. Teflon-coated magnetic stirring bars.
8. Polypropylene containers, 50 mL.

##### C. Instrumentation

1. Robot Coupe Model RSI 2V Scientific Batch Processor.
2. Capillary gas chromatograph, Hewlett Packard, Model 6890, with split/splitless injector capability, flame ionization detector, and a capillary column (Hewlett Packard HP-5, Crosslinked 5% PH ME Siloxane, 30 m length × 0.32 mm ID, film thickness 0.25 or 0.52 μm).
3. Orion Model EA 940 pH meter equipped with Orion 8103 Ross combination pH electrode.

##### D. Additional Equipment

Forced-air oven, Fisher Isotemp®, regulated to 99 ± 1.0 °C. Suggested dimensions: 18 x 18 x 20.”

##### E. Chromatographic Conditions<sup>8,9</sup>

1. Detector temperature: 250 °C.
2. Injector temperature: 250 °C.
3. Flow rate at 100 °C—1.7 mL/min; with split ratio of 40:1.<sup>10</sup>
4. Injection volume: 2 μL.
5. Column conditions: 110–185 °C at 10 °C min<sup>-1</sup>; 185–240 °C at 6 °C min<sup>-1</sup>, hold at final temperature for 10 min.

##### F. Sample Preparation<sup>11</sup>

There are ten different categories of commercial smokeless tobacco products:

1. Dry snuff;
2. Moist (wet) snuff;

3. Moist (wet) snuff portion packs;
4. Plug;
5. Twist;
6. Loose leaf;
7. Dry snuff portion packs;
8. Snus;
9. Snus portion packs; and
10. Pellet or Compressed.

Because of their physical characteristics, some of the ten product categories must be ground (whole or in part) before nicotine, total moisture, and pH analyses can be conducted. The objective of grinding the samples is to obtain a homogeneous sample with particles measuring approximately 4 mm. Grinding to achieve this particle size should take no more than 3 minutes. To ensure proper grinding and an adequate amount of the ground sample for analysis, the minimum sample size of all commercial products to be ground should not be less than 100 grams.

To ensure precision of analyses for nicotine, total moisture, and pH, the samples that require grinding should be ground using a Robot Coupe Model RSI 2V Scientific Batch Processor or its equivalent. This is a variable speed (0 to 3000 RPM) processor. The variable speed motor is required to ensure proper grinding of the tobacco tissues (and in the case of pH determination, the portion pack). Elevated temperatures can result in moisture loss and an underestimated value for moisture content. Hence, care must be taken during grinding to avoid elevated temperatures. The bowl should be cleaned after each grinding to obtain accurate results. Freeze- or cryo-grinding is also an acceptable grinding method.

1. *Dry snuff*: Dry snuff samples do not need to be ground since the product is a powder. The sample must be thoroughly mixed before weighing for nicotine, total moisture, and pH analysis.

2. *Moist (wet) snuff*: Moist (wet) snuff samples do not need to be ground. The sample must be thoroughly mixed before weighing for nicotine, total moisture, and pH analysis.

3. *Moist (wet) snuff portion packs*: The tobacco contents of the moist (wet) snuff portion packs do not need to be ground for nicotine, total moisture, or pH analysis. The tobacco packaging material (the “pouch”) should be separated from the tobacco and ground to obtain particles measuring approximately 4 mm for pH analysis. The tobacco of the moist (wet) snuff portion pack and the ground pouch are combined and thoroughly mixed before pH analysis.

4. *Plug tobacco*: Break or cut apart plugs and add in portions to grinder at 2000 RPM. Reduce RPM or stop grinding if sample bowl becomes warm. Pulse the Robot Coupe, when needed, to complete grinding. Grind samples until approximately 4 mm in size. The total grinding time should be no more than 3 minutes.

5. *Twist tobacco*: Separate twists, add to grinder and grind at 2000 RPM. Reduce RPM or stop grinding if sample bowl becomes warm. Continue grinding until sample particles are approximately 4 mm in size. The total time for grinding should be no more than 3 minutes.

6. *Loose leaf*: Grind in the same manner as described in 4 and 5 to obtain product with particle size of approximately 4 mm.

7. *Dry snuff portion packs*: The tobacco contents of the dry snuff portion packs do not need to be ground for nicotine, total moisture, or pH analysis. The tobacco packaging material (the "pouch") should be separated from the tobacco and ground to obtain particles measuring approximately 4 mm for pH analysis. The tobacco of the dry snuff portion pack and the ground pouch are combined and thoroughly mixed before pH analysis.

8. *Snus*: Snus samples do not need to be ground since the product is a powder. The sample must be thoroughly mixed before weighing for nicotine, total moisture, and pH analysis.

9. *Snus portion packs*: The tobacco contents of the snus portion packs do not need to be ground for nicotine, total moisture, or pH analysis. The tobacco packaging material (the "pouch") should be separated from the tobacco and ground to obtain particles measuring approximately 4 mm for pH analysis. The tobacco of the snus portion pack and the ground pouch are combined and thoroughly mixed before pH analysis.

10. *Pellet or Compressed*: Break apart compressed tobacco pellets and add in portions to grinder at 2000 RPM. Reduce RPM or stop grinding if sample bowl becomes warm. Pulse the Robot Coupe, when needed, to complete grinding. Grind samples until approximately 4 mm in size. The total grinding time should be no more than 3 minutes.

## II. Nicotine Analysis<sup>12</sup>

### A. Calibration Standards

#### 1. Internal Standard (IS)

Weigh 10.00 grams of quinoline, transfer to a 250 mL volumetric flask and dilute to volume with MTBE. This solution will be used for calibration of the instrument for the nicotine calibration curve (II.A.2), for the

standards addition assay (II.B), and for preparation of the extracting solution (II.D).

#### 2. Nicotine Calibration Curve

a. Weigh 1.0000 gram of nicotine into a clean, dry 100 mL volumetric flask and dilute to volume with MTBE. This gives a nicotine concentration of 10 mg/mL for the stock solution.

b. Accurately pipette 0.5 mL of IS from stock solution (II.A.1) to five clean, dry 50 mL volumetric flasks. To prepare a nicotine standard corresponding to a concentration of 0.8 mg/mL, pipette exactly 4.0 mL of the nicotine standard (II.A.2.a) to a 50 mL volumetric flask containing the internal standard and dilute to volume with MTBE. To obtain nicotine concentrations equivalent to 0.6, 0.4, 0.2, and 0.1 mg/mL, pipette precisely 3.0, 2.0, 1.0, and 0.5 mL, respectively, of the nicotine standard into the four remaining flasks and dilute to volume with MTBE.

c. Transfer aliquots of the five standards to auto sampler vials and determine the detector response for each standard using gas chromatographic conditions described in I.E.

d. Calculate least squares line for linear equation from these standards by obtaining the ratio of  $Area_{nicotine} / Area_{IS}$ . This ratio will be the Y value and the concentration of nicotine will be the X value for determining the linear equation of the line (Equation 1):

Equation 1:

$$Y = a + bX;$$

Where:

X = Concentration of nicotine in mg

Y =  $Area_{nicotine} / Area_{IS}$

a = intercept on the ordinate (y axis)

b = slope of the curve

The final result will be reported in the following units:

Concentration of nicotine = mg of nicotine/gram of tobacco sample.

e. Determine the recovery of nicotine by pipetting 10 mL of the 0.4 mg/mL nicotine standard to a screw capped tube containing 1.0 mL of 2 N NaOH. Cap the tube. Shake the contents vigorously and allow the phases to separate. Transfer an aliquot of the organic phase to an injection vial and inject. Calculate the concentration of nicotine using the equation of the line in II.A.2.d above. This should be repeated two more times to obtain an average of the three values. The recovery of nicotine can be obtained by using the following equation:

Equation 2:

$$\text{Recovery} = \frac{\text{Nicotine}_{\text{calculated}}}{\text{Nicotine}_{\text{actual}}}$$

### B. Standards Addition Assay

Prior to analyzing a smokeless tobacco product for nicotine content, the testing facility must validate the system to verify that matrix bias is not occurring during nicotine extraction. This is done by analyzing the nicotine calibration standards in the same vegetable matrix as the smokeless tobacco. The first time each smokeless tobacco product is tested and whenever a change is made to the product formulation (including a change to the tobacco blend or cultivar), the Standards Addition Assay will be performed, and documentation of its performance and of the nicotine concentrations selected for the standard curve (II.B.2) will be submitted to the Centers for Disease Control and Prevention.

1. Using an analytical balance, accurately weigh  $1.000 \pm 0.020$  gram of the homogeneous, prepared tobacco sample into a culture tube. Repeat this five times for a total of 6 culture tubes containing the smokeless tobacco product. Record the weight of each sample.

2. Prepare a five-point standard curve for the Standards Addition Assay. The standard curve must consist of nicotine concentrations that encompass the range of values expected from adding known concentrations of the nicotine standard (II.A.2.a) to a measured quantity of the smokeless tobacco product ( $1.000 \pm 0.020$  gram, described in II.B.1.). The sixth culture tube is not supplemented with nicotine and serves as an analytical blank. Allow the samples to equilibrate for 10 minutes.

3. Pipette 5 mL of 2 N NaOH into each tube. Cap each tube. Swirl to wet sample and allow to stand 15 minutes.<sup>13</sup>

4. Pipette 50 mL of extraction solution (II.D.1) into each tube. Cap each tube and tighten.<sup>14</sup>

5. Place tubes in rack(s), place racks in linear shaker in horizontal position and shake for two hours.

6. Remove rack(s) from shaker and place in vertical position to allow the phases to separate.

7. Allow the solvent and nicotine supplemented samples and the blank to separate (maximum 2 hours).

8. Transfer aliquots of the five standards and the blank from the extraction tubes to sample vials and determine the detector response for each using gas chromatographic conditions described in I.E.

9. Subtract the  $Area_{nicotine} / Area_{IS}$  of the blank from the  $Area_{nicotine} / Area_{IS}$  of each of the standards.

10. Calculate least squares line for linear equation from the corrected standards as described above (Equation

1) in II.A.2.d. The final corrected result will be reported in the following units: Concentration of nicotine = mg of nicotine/gram of tobacco sample.

11. Determine the recovery of nicotine by pipetting 10 mL of the 0.4 mg/mL nicotine standard to a screw capped tube containing 1.0 mL of 2 N NaOH and 10 mL of extraction solution (II.D.1). Cap the tube and tighten. Shake the contents vigorously and allow the phases to separate. Transfer an aliquot of the organic phase to an injection vial and inject. Calculate the concentration of nicotine using the equation of the line above in II.A.2.d. This should be repeated two more times to obtain an average of the three values. The recovery of nicotine can be obtained by using Equation 2: Recovery =  $\text{Nicotine}_{\text{calculated}} / \text{Nicotine}_{\text{actual}}$ .

12. Compare the results of steps II.A.2. and II.B. If they differ by a factor of 10% or more, the recovery of nicotine from the aqueous matrix is not equivalent to recovery from the vegetable matrix of the smokeless tobacco product. In this instance, the nicotine concentration of the smokeless tobacco product must be determined

from a nicotine calibration curve prepared from nicotine standards in a vegetable-based matrix.

#### C. Quality Control Pools

At least two quality control pools at the high and low ends of the expected nicotine values are recommended to be included in each analytical run. The pools should be analyzed in duplicate in every run. The quality control pools should be available in sufficient quantity to last for all analyses of a product.

#### D. Sample Extraction Procedure <sup>12</sup>

1. Extraction solution is prepared by pipetting 10 mL of the IS from the stock solution (II.A.1) to a 1000 mL volumetric flask and diluting to volume with MTBE.

2. Using an analytical balance, accurately weigh  $1.000 \pm 0.020$  gram of prepared tobacco sample into culture tube and record weight.<sup>15</sup> Sample each smokeless tobacco brand name according to the provided testing frequency schedule.<sup>19</sup> The number of products sampled should reflect an acceptable level of precision.<sup>16</sup> The test

material is to be representative of the product that is sold to the public and therefore should consist of sealed, packaged samples of finished product that is ready for commercial distribution. Samples are to be analyzed in duplicate.

3. Pipette 5 mL of 2 N NaOH into the tube. Cap the tube. Swirl to wet sample and allow to stand 15 minutes.<sup>13</sup>

4. Pipette 50 mL of extraction solution into tube, cap tube and tighten.<sup>14</sup>

5. Place tubes in rack(s), place racks in linear shaker in horizontal position and shake for two hours.

6. Remove rack(s) from shaker and place in vertical position to allow the phases to separate.

7. Allow the solvent and sample to separate (maximum 2 hours). Transfer an aliquot from the extraction tube to a sample vial and cap.

8. Analyze the extract using GC conditions as described above (I.E) and calculate the concentration of nicotine using the linear calibration equation. Correct percent nicotine values for both recovery and weight of sample by using Equation 3.<sup>17</sup>

Equation 3: <sup>18</sup>

$$\text{Nicotine (mg/g)} = \frac{(\text{Area}_{\text{nicotine}} / \text{Area}_{\text{IS}}) - a}{b \times \text{Sample Wt} \times \text{Recovery}}$$

9. Report the final nicotine determination as mg of nicotine per gram of the tobacco product (mg nicotine/gram), to an accuracy level of two decimal places for each brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.). All data should include the mean value with a 95% confidence interval, the range of values, the number of samples tested, the number of lots per brand name, and the estimated precision of the mean. Information will be reported for each manufacturer and variety (including brand families and brand variations) and brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.).

### III. Total Moisture Determination

A. This procedure is a modification of AOAC Method 966.02 (1990) and is referred to as "Total Moisture Determination" because it determines water and tobacco constituents that are volatile at temperatures of  $99 \pm 1.0$  °C.

B. Accurately weigh 5.00 grams of the sample (ground to pass  $\leq 4$  mm screen)<sup>20</sup> into a weighed moisture dish

and place uncovered dish in oven.<sup>21</sup> Sample each smokeless tobacco brand name according to the provided testing frequency schedule.<sup>19</sup> The number of products sampled should reflect an acceptable level of precision.<sup>16</sup> The test material is to be representative of the product that is sold to the public and therefore should consist of sealed, packaged samples of finished product that is ready for commercial distribution. Samples are to be analyzed in duplicate.

C. Do not exceed 1 sample/10 sq in. (650 sq cm) shelf space, and use only 1 shelf. Dry 3 hr at  $99 \pm 1.0$  °C. Remove from oven, cover, and cool in desiccator to room temp. (about 30 min). Reweigh and calculate percent moisture.

D. Report the final moisture determination as a percentage (%), to an accuracy level of one decimal place for each brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.). All data should include the mean value with a 95% confidence interval, the range of values, the number of samples tested, the number of lots per brand name, and the estimated precision of the

mean. Information will be reported for each manufacturer and variety (including brand families and brand variations) and brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.).

### IV. pH Measurement <sup>12, 22</sup>

A. Test samples as soon as possible after they are received. Sample each smokeless tobacco brand name according to the provided testing frequency schedule.<sup>19</sup> The number of products sampled should reflect an acceptable level of precision.<sup>16</sup> The test material is to be representative of the product that is sold to the public and therefore should consist of sealed, packaged samples of finished product that is ready for commercial distribution. Samples are to be analyzed in duplicate.

B. Accurately weigh 2.00 grams of the sample. Place in a 50 mL polypropylene container with 20 mL deionized distilled water.

C. Place Teflon-coated magnetic stirring bar in container and stir mixture continuously throughout testing.

D. Measure pH of sample after a two-point calibration of the pH meter to an accuracy of two decimal places using standard pH buffers (4.01 and 7.00 or 7.00 and 10.00) that will encompass the expected pH value of the smokeless tobacco product.

E. The first time pH values are determined for a smokeless tobacco product, measure the pH of the smokeless tobacco product at 5, 15, and 30 minutes. If there is no systematic variation in pH values with time, all subsequent pH determinations are made at 5 minutes. If there is systematic

variation in pH values, continue to measure the pH of the smokeless tobacco product until the pH value is stable and does not vary more than 10% over 15 minutes. Report the final pH value.

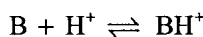
F. Report the final pH determination to an accuracy level of two decimal places for each brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.). All data should include the mean value with a 95% confidence interval, the range of values, the number of samples tested, the number of lots per

brand name, and the estimated precision of the mean. Information will be reported for each manufacturer and variety (including brand families and brand variations) and brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.).

G. Estimate the un-ionized (free) nicotine content with the Henderson-Hassel Balch equation (Equation 4), based on measured pH and nicotine content.

Equation 4:

$$\text{pH} = \text{pKa} + \log \frac{[\text{B}]}{[\text{BH}^+]}$$



$$\% \text{ un-ionized (free) nicotine} = \frac{\frac{[\text{B}]}{[\text{BH}^+]}}{\frac{[\text{B}]}{[\text{BH}^+]} + 1} \times 100$$

pKa = 8.02 (CRC Handbook of Chemistry and Physics, 1989–1990)

[B] = amount of un-ionized (free) nicotine  
[BH<sup>+</sup>] = amount of ionized nicotine

H. Report the final estimated un-ionized (free) nicotine as a percentage (%) of the total nicotine content, to an accuracy level of two decimal places and as mg of un-ionized (free) nicotine per gram of the tobacco product (mg un-ionized (free) nicotine/gram), to an accuracy level of two decimal places for

each brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.). All data should include the mean value with a 95% confidence interval, the range of values, the number of samples tested, the number of lots per brand name, and the estimated precision of the mean. Information will be reported for each manufacturer and variety (including brand families and brand variations) and brand name (e.g., Skoal

Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.).

Sample calculation:

Mean total nicotine = 10.30 (mg/g)

Mean pH = 7.50

pKa = 8.02

$$\text{pH} = \text{pKa} + \log \frac{[\text{B}]}{[\text{BH}^+]}$$

$$7.50 = 8.02 + \log \frac{[\text{un-ionized (free) nicotine}]}{[\text{ionized nicotine}]}$$

$$-0.52 = \log \frac{[\text{un-ionized (free) nicotine}]}{[\text{ionized nicotine}]} \quad 0.302 = \frac{[\text{un-ionized (free) nicotine}]}{[\text{ionized nicotine}]}$$

$$\% \text{ un-ionized (free) nicotine} = \frac{\frac{[\text{B}]}{[\text{BH}^+]}}{\frac{[\text{B}]}{[\text{BH}^+]} + 1} \times 100$$

$$\% \text{ un-ionized (free) nicotine} = \frac{0.302}{0.302 + 1} \times 100$$

$$\% \text{ un-ionized (free) nicotine} = 23.20$$

$$\text{Total free nicotine (mg/g)} = \text{total nicotine} \times \frac{\% \text{ un-ionized (free) nicotine}}{100}$$

$$\text{Total free nicotine (mg/g)} = 10.30 \times \frac{23.20}{100}$$

$$\text{Total free nicotine (mg/g)} = 2.39$$

## V. Assay Criteria for Quality Assurance

### A. Establishing Limits for Quality Control Parameters

All quality control parameters must be determined within the laboratory in which they are to be used. At least 10 within laboratory runs must be performed to establish temporary confidence intervals for the quality control parameters. Permanent limits should be established after 20 runs and should be reestablished after each additional 20 runs.

### B. Exclusion of Outliers From the Calibration Curve<sup>18</sup>

The coefficient of determination between  $\text{Area}_{\text{nicotine}} / \text{Area}_{\text{IS}}$  and nicotine concentration should be equal to 0.99 or higher. Any calibration standard having an estimated concentration computed from the regression equation (Equation 1) which is different from its actual concentration by a factor of 10% can be excluded from the calibration curve. Up to two concentrations may be excluded, but caution should be used in eliminating values, since bias may be increased in the calibration curve. If an outlier value is eliminated, its duplicate value must also be discarded to avoid producing a new bias. All unknowns must fall within the calibration curve; therefore, duplicate values excluded at either end of the calibration curve will restrict the useful range of the assay.

### C. Quality Control Pools and Run Rejection Rules

The mean estimated nicotine concentration in a pool should be compared with the established limits for that pool based on at least 20 consecutive runs. An analytical run should be accepted or rejected based upon the following set of rules adapted from Westgard *et al.* (1981).

1. When the mean of one QC pool exceeds the limit of  $x \pm 3$  standard deviations (SD), then the run is rejected

as out of control. Here,  $x$  and SD represent the overall mean and standard deviation of all estimated nicotine concentrations for a particular pool in the runs which were used to establish the control limits.

2. When the mean nicotine concentrations in two QC pools in the same run exceed the same direction, then the run must be rejected. The same direction is the condition in which both pools exceed either the  $x + 2$  SD or the  $x - 2$  SD limits.

3. When the mean nicotine concentrations in one or two QC pools exceed their  $x \pm 2$  SD limits in the same direction in two consecutive runs, then both runs must be rejected.

4. When the mean nicotine concentrations in two QC pools are different by more than a total of 4 SD, then the run must be rejected. This condition may occur, for example, when one QC pool is 2 SD greater than the mean, and another is 2 SD less than the mean.

### Endnotes

The comments and notes listed below can be described as Good Laboratory Practice guidelines; they are described in detail in this protocol to ensure minimal interlaboratory variability in the determination of nicotine, total moisture, and pH in smokeless tobacco.

1. This protocol assumes that the testing facility will implement and maintain a stringent Quality Assurance/Quality Control program to include, but not be limited to, regular interlaboratory comparisons, determination of the quality and purity of purchased products, and proper storage and handling of all reagents and samples.

2. When a specific product or instrument is listed, it is the product or instrument that was used in the development of this method. Equivalent products or instruments may also be used. Use of trade names is for identification only and does not

constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

3. All chemicals, solvents, and gases are to be of the highest purity.

4. Companies must ensure that the purity of the nicotine base is certified by the vendor and that the chemical is properly stored. However, nicotine base oxidizes with storage, as reflected by the liquid turning brown. If oxidation has occurred, the nicotine base should be distilled prior to use in making a standard solution.

5. A suggested method for the determination of nicotine purity is CORESTA Recommended Method No. 39.

6. Horizontal shaking will allow more intimate contact of this three phase extraction. There is a minimal dead volume in the tube due to the large sample size and extraction volume. This necessitates horizontal shaking.

7. If linear shaker is not available, a wrist action shaker using 250 mL stoppered Erlenmeyer flasks can be substituted. Values for nicotine are equivalent to those obtained from the linear shaker.

8. After installing a new column, condition the column by injecting a tobacco sample extract on the column, using the described column conditions. Injections should be repeated until areas of IS and nicotine are reproducible. This will require approximately four injections. Recondition column when instrument has been used infrequently and after replacing glass liner.

9. Glass liner and septum should be replaced after every 100 injections.

10. Most older instruments operate at constant pressure. To reduce confusion, it is suggested that the carrier gas flow through the column be measured at the initial column temperature.

11. The testing facility must ensure that samples are obtained through the use of a survey design protocol for sampling "at one point in time" at the

factory or warehouse. The survey design protocol must address short-, medium-, and long-term smokeless tobacco product variability (e.g., variability over time and from container to container of the tobacco product) in a manner equivalent to that described for cigarette sampling in Annex C of ISO Protocol 8243. Information accompanying results for each sample should include, but not be limited to:

1. For each product—manufacturer and variety (including brand families and brand variations) and brand name (e.g., Skoal Bandits, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.) information.

2. Product “category,” e.g., loose leaf, plug, twist, dry snuff, moist (wet) snuff, etc.

3. Lot number.

4. Lot size.

5. Number of randomly sampled, sealed, packaged (so as to be representative of the product that is sold to the public) smokeless tobacco products selected (sampling fraction) for nicotine, moisture, and pH determination.

6. Documentation of method used for random sample selection.

7. “Age” of product when received by testing facility and storage conditions prior to analysis.

12. Extraction of nicotine and pH determination must be performed with reagents and samples at a room temperature of 22–25 °C. Room temperature should not vary more than 1 °C during extraction of nicotine or pH determination.

13. Use non-glass 10 mL repipette for transferring NaOH solution.

14. Use 50 mL repipette for transferring MTBE.

15. For dry snuff, use  $0.500 \pm 0.010$  gram sample.

16. The testing facility is referred to ISO Procedure 8243 for a discussion of sample size and the effect of variability on the precision of the mean of the sample (ISO 8243, 1991).

17. When analyzing new smokeless tobacco products, extract product without IS to determine if any components co-elute with the IS or impurities in the IS. This interference could artificially lower calculated values for nicotine.

18. The calculated nicotine values for all samples must fall within the low and high nicotine values used for the calibration curve. If not, prepare a fresh nicotine standard solution and an appropriate series of standard nicotine dilutions. Determine the detector response for each standard using chromatographic conditions described in I.E.

19. The testing frequency for each smokeless tobacco brand name (e.g., Skoal Bandits Wintergreen, Skoal Long Cut Cherry, Skoal Long Cut Wintergreen, etc.) is based on the manufacturing duration (refer to table below). Each smokeless tobacco brand name will be sampled and tested for nicotine, total moisture, and pH no fewer than twice and no more than four times during a calendar year.

Manufacturing duration in weeks	Test frequency*
Up to and including 4 .....	2
Up to and including 28 .....	3
Up to and including 52 .....	4

\* Use a statistical program to determine random sampling dates based on the total manufacturing duration during a calendar year. Sampling dates should fall on actual manufacturing days for the product when test material that is representative of the product that is sold to the public (consisting of sealed, packaged samples) is available. If a statistically determined sampling date falls on a day that does not meet this criterion, sample the product on the next date that does meet the criteria.

For smokeless tobacco brand names with episodic production during a calendar year, the total number of sampling dates is determined by the sum of the individual test frequencies, not to exceed four. For the purpose of the Protocol, episodic production is defined as manufacturing intervals separated by periods of 30 or more days when the smokeless tobacco brand name is not manufactured.

*Example 1:* Within a single calendar year a smokeless tobacco brand name is manufactured from January 1 to March 31 and from September 1 to December 15. The testing frequency for the first manufacturing interval is 3 and for the second manufacturing interval is 3. The Protocol allows that each smokeless tobacco brand name be tested for nicotine, total moisture, and pH no more than four times during a calendar year. Therefore, 4 random sampling dates, as described in the footnote to the above table, are determined for the smokeless tobacco brand name. The values for nicotine, moisture, and pH determinations, and unionized (free) nicotine calculations and the mean of the 4 data points for that smokeless tobacco brand name are reported.

*Example 2:* Within a single calendar year a smokeless tobacco brand name is manufactured from April 5 to May 3 and from September 1 to December 15. The testing frequency for the first manufacturing interval is 2 and for the second manufacturing interval is 3. The values for nicotine, moisture, and pH determinations, and unionized (free) nicotine calculations and the mean of the 4 data points for that smokeless tobacco brand name are reported.

*Example 3:* Within a single calendar year a smokeless tobacco brand name is manufactured from January 1 to January 15 and from September 1 to September 22. The testing frequency for the first manufacturing interval is 2 and for the second manufacturing interval is 2. Four random sampling dates are selected to fall within the

6 weeks of manufacturing for the smokeless tobacco brand name. The values for nicotine, moisture, and pH determinations, and unionized (free) nicotine calculations and the mean of the 4 data points for that smokeless tobacco brand name are reported.

20. The method is a modification of AOAC Method 966.02 (1990) in that the ground tobacco passes through a 4 mm screen rather than a 1 mm screen.

21. When drying samples, do not dry different products (e.g., moist (wet) snuff, dry snuff, loose leaf) in the oven at the same time since this will produce errors in the moisture determinations.

22. The method is a modification of a method published by Henningfield *et al.* (1995).

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