DEPARTMENT OF HEALTH AND HUMAN SERVICES

Centers for Disease Control and Prevention

Notice Regarding Requirement for Annual Submission of 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 establishes a uniform protocol for the analysis of nicotine, total moisture, and pH in smokeless tobacco products. This protocol was designed to implement the requirement of the Comprehensive Smokeless Tobacco Health Education Act (CSTHEA) of 1986 (15 U.S.C. 4401 *et seq.*, Pub. L. 99–252), which requires 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, 1999, with subsequent submissions due by March 31 of each year.

ADDRESSES: The information shall be submitted to: Michael P. Eriksen, Sc.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: Michael P. Eriksen, Sc.D., Director, Office on Smoking and Health, telephone: (770) 488–5701.

SUPPLEMENTARY INFORMATION: According to a 1986 report of an Advisory Committee to the Surgeon General, smokeless tobacco represents a significant health risk, is not a safe substitute for cigarette smoking, can cause cancer and a number of noncancerous oral conditions, and can lead to nicotine addiction.

The Centers for Disease Control and Prevention's (CDC) Office on Smoking and Health (OSH) has primary responsibility for the Department of Health and Human Services' (HHS) tobacco and health program. The overall goal of this program is to reduce death and disability resulting from cigarette smoking and other forms of tobacco use through programs of information, education, and research.

HHS uses the information collected to exercise its authority under CSTHEA to conduct research on the addictive nature of nicotine and general health effects of using smokeless tobacco. Nicotine data will provide a more complete picture of the addictive nature of smokeless tobacco products. Also, as authorized in the statute, HHS may report to the Congress information regarding its current and proposed research relative to nicotine levels in smokeless tobacco products. CSTHEA further requires that individuals who manufacture, package, or import smokeless tobacco products report to HHS the list of ingredients added to tobacco in the manufacture of such products, and this requirement has been implemented by a previous notice (59 FR 4714,).

In 1989 the smokeless tobacco industry submitted a business review letter to the Department of Justice (DOJ), in accordance with 28 CFR 50.6. This letter requested approval of a collaborative industry effort to develop a uniform analytical protocol for determining the nicotine and moisture content of smokeless tobacco products.

In January 1993, DOJ extended permission to the smokeless tobacco industry to develop a uniform analytical protocol for this purpose. A work group representing the 10 major domestic manufacturers of smokeless tobacco was convened on July 7, 1993. The workgroup developed and submitted to CDC for approval the ''Protocol for Analysis of Nicotine in Smokeless Tobacco Products.'' The protocol was revised by CDC based on individual comments from peer reviewers and the National Center for Environmental Health, CDC. The revised protocol, ''Protocol for Analysis of Nicotine, Total Moisture, and pH in Smokeless Tobacco Products,'' is hereafter referred to as the ''protocol.''

On May 2, 1997, a notice (62 FR 24115) was published in accordance with the Paperwork Reduction Act to solicit public comment on the proposed collection of data. A notice was also published (62 FR 24116, May 2, 1997) to solicit public comment on the protocol. The protocol consists of standard laboratory methods to measure nicotine, moisture, and pH in smokeless tobacco products, and an equation (Henderson-Hasselbalch) to calculate un-ionized nicotine. Nicotine is the major alkaloid in tobacco and the drug in tobacco that causes addiction. In the protocol, moisture is referred to as total moisture because the method measures the amount of water and tobacco constituents in a smokeless tobacco product that are volatile at temperatures of 99 degrees centigrade. pH is defined as the negative logarithm of the molar concentration of hydrogen ions in an aqueous solution and is a quantitative measure of acidity or alkalinity. The degree of nicotine ionization is calculated from the Henderson-Hasselbalch equation. Un-ionized nicotine is known to be the form of nicotine absorbed most easily in the mouth. This protocol will provide CDC with information on levels of nicotine found in smokeless tobacco products manufactured, packaged, or imported during the previous calendar year. The schedule for reporting this information to CDC corresponds to the reporting of ingredients added to tobacco in the manufacture of smokeless tobacco products November 8, 1994 (59 FR 55670,).

Following public request, on June 2, 1997, a notice (62 FR 29729) was published extending the comment period on the proposed protocol by an additional 30 days to July 2, 1997. A summary of the comments received and CDC's response follows.

One respondent, on behalf of several smokeless tobacco manufacturers, had several comments regarding the collection of data. The respondent asserted that the protocols exceeded statutory authorization by collecting pH and free base (hereinafter referred to as ''un-ionized'', as reflected in the revised protocol) nicotine. Furthermore, the respondent felt that the legislative history of CSTHEA contemplates the reporting of nicotine content alone.

It is CDC's belief that the collection of pH of smokeless tobacco products and the un-ionized nicotine content of each is authorized by section 4 of CSTHEA. There is ample scientific evidence that mere quantity of nicotine alone is insufficient in determining its effect on a user; knowledge of pH and un-ionized nicotine content of the overall nicotine quantity is essential in determining the rate of nicotine absorption. pH and un-ionized nicotine content are essential factors affecting nicotine bioavailability. Furthermore, Congress has never defined exactly what it meant by ''quantity of nicotine'' in section 4(b) of CSTHEA. In light of ample scientific evidence indicating the importance of pH and un-ionized nicotine content in assessing the overall quantity of nicotine, CDC's interpretation of its statutory authority is clearly permissible.

It is CDC's belief that the legislative history provides support for requiring the reporting of moisture, pH, and un-ionized nicotine content. The Senate

Report accompanying and establishing Congress' views on CSTHEA repeatedly emphasized that ''it is essential that we inform the public of the health effects of smokeless tobacco use and continue research on such health effects as expeditiously as possible'' (Senate Report 99–209, Dec. 4, 1985, p.13). Since scientific evidence has established that knowledge of pH and un-ionized nicotine content is essential in determining the health effects of smokeless tobacco, the reporting of these elements is supported by the legislative history.

The respondent also commented that CDC failed to comply with procedural obligations in violation of 5 USC § 551 *et seq.*, the Administrative Procedure Act (APA). Specifically, the respondent claimed that CDC failed to provide opportunity for advance review and comment and failed to inform the public of the true nature of the proposed protocol.

CDC feels that the notice given to the tobacco manufacturers was clearly adequate under the APA. CDC's purpose in publishing the protocol in the **Federal Register** was to solicit comments from the public. This is the appropriate time for the manufacturers to review the protocol and relay their comments to CDC, not before publication. As a matter of courtesy, CDC has provided the manufacturers with a copy of draft protocols before publication. CDC did that here as well, for the tobacco companies were given an advance copy of the protocol before formal publication. Therefore, CDC did provide the tobacco companies with advance knowledge of the protocol, even though such notice was not required.

Furthermore, CDC has not failed to inform the public of the true nature of its proposed protocol. CDC sufficiently apprised the public of the agency's legal authority to issue the proposed protocol, for it explicitly states that it is operating under the authority of CSTHEA. Thus, the public is on sufficient notice of the legal authority under which CDC issued the proposed protocol, and has had full opportunity to comment.

CDC is also not required to lay out potential criticisms of its scientific positions in its notice and request for public comment. The purpose of the notice and comment period is to provide interested parties with the opportunity to conduct their own analysis of the merits of the protocol, and to provide scientific or other criticisms, if desired. CDC has clearly stated the terms and substance of its proposed protocol as to provide the public with sufficient opportunity to comment.

This respondent also commented that CDC failed to meet the requirements of the Paperwork Reduction Act by failing to provide the public with an accurate estimate of the burden of compliance. CDC disagrees. CDC based its original estimate on the figures that were submitted by the manufacturers themselves. Moreover, the 60-day notice and comment period is designed to solicit comment on the accuracy of the agency's estimate of the burden of the proposed collection of information. 44 U.S.C. 3506(c)(2)(A)(ii). The tobacco manufacturers felt that the estimate of burden had changed and commented as such at that time. CDC then sought revised estimates from the manufacturers. Only one manufacturer responded to the request for this information. Based on this response, CDC conducted new analyses and revised its estimate of burden accordingly. Thus, CDC clearly complied with the Paperwork Reduction Act's requirements.

CDC also received comments regarding the protocol, which raised technical and scientific concerns regarding collection and calculation of the requested information. Several respondents supported the protocol requirement of reporting not only moisture and nicotine content but also pH and unionized nicotine levels. Support for this aspect of the protocol was based on scientific evidence that the nicotine-specific effects of a given amount of smokeless tobacco depend as much on the pH of the product as on the nicotine content itself. CDC agrees with this scientific observation regarding the utility of determining smokeless tobacco pH and calculating unionized nicotine levels in smokeless tobacco products.

One respondent, on behalf of several smokeless tobacco manufacturers, stated that the protocols for nicotine analysis and total moisture determination are scientifically flawed. Specifically, the respondent stated that the Standards Addition Assay is flawed and unnecessary, that the protocol specifies an unavailable vegetable-based matrix, that triplicate determinations are unnecessary, and that the protocol requires smokeless tobacco manufacturers to use a protocol specific to cigarettes.

With respect to the Standards Addition Assay, CDC reaffirms the function of the Standards Addition Assay and disputes the inadequacies of the extraction testing offered by the respondent as a rationale for eliminating the Standards Addition Assay. CDC revised the protocol to facilitate preparation of a standard curve for the Standards Addition Assay that encompasses the range of values expected from adding known concentrations of nicotine to the smokeless tobacco product. Also, CDC revised the protocol to specify when the Standards Addition Assay is to be conducted by the testing facility.

Regarding use of a vegetable-based matrix, CDC acknowledges that a nicotine-free tobacco surrogate is not readily available to serve as a vegetable-based matrix; that is why the protocol thus specifies adding known concentrations of nicotine to the smokeless tobacco product when performing the Standards Addition Assay. CDC revised the text in Endnote 1 of the protocol to eliminate the phrase ''routine testing of random blind samples.''

Regarding triplicate determinations, CDC asserts that the potential sources of smokeless tobacco product variability necessitate triplicate determinations for evaluation of precision. In response to the comment that CDC was attempting to ''bind the manufacturers'' to a cigarette testing protocol in the smokeless tobacco testing protocol, CDC clarifies that the disputed protocol is a sampling protocol, not a testing protocol. Therefore, testing facilities should make use of the document as reference material. However, for clarification, Endnote 11 of the protocol (Endnote 10 of the public comment version of the protocol) was revised to read—''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.''

This respondent also commented that the protocol for pH measurement is scientifically flawed. Specifically, the respondent states that the procedure is based on a nonvalidated protocol, that an inappropriate volume of liquid is specified, that proper calibration of instruments has not been incorporated in the protocol, that temperature is not considered in the protocol, and that multiple pH measurements are unnecessary.

CDC disagrees that the protocol for pH measurement is scientifically flawed. The protocol to determine smokeless tobacco pH is based on the validated protocol published by Henningfield et

al. (1995), which also provides the rationale for the quantity of smokeless tobacco and the volume of liquid. Parameters that can be standardized (sample size, sample preparation, quantity and purity of standard and reagents, instrumentation, measurement time and conditions, etc.) are specified in the protocol. Of note, the Henningfield et al. (1995) reference was provided in the version of the protocol that the respondent received for comment.

CDC agrees that careful calibration across the range of unknown values to be measured is needed. The protocol was revised to read—''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.''

CDC also agrees that conditions such as sample preparation, sample size, extraction time, volume and purity of the water used, and temperature must be controlled during determination of the pH of a smokeless tobacco product. The protocol was revised to specify room temperature for nicotine extraction and pH determination.

As described above, CDC recognizes that there are several potential sources of smokeless tobacco product variability that necessitate triplicate determinations for evaluation of precision. With respect to pH determination, CDC recognizes the need for multiple measurements to determine if pH values for the smokeless tobacco product vary systematically with time. For edification, the protocol was revised to read—''The first time pH values are determined for each lot of 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 for the lot 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.''

This respondent further asserted that the ''smokeless tobacco pH'' theory has been discredited. In summary, the respondent states that calculation of un-ionized nicotine is based on a discredited scientific theory and that the ''smokeless tobacco pH'' theory ignores the chemical, biological, and behavioral factors that govern absorption of smokeless tobacco.

Un-ionized nicotine is known to be the form of nicotine most easily absorbed in the mouth. pH determination is a component of the protocol to allow calculation of un-ionized nicotine. The degree of nicotine ionization is calculated from the Henderson-Hasselbalch equation. In a document written at the request of the United States Tobacco Company, Dr. Jeffrey R. Idle recognizes pH as one of the ''chemical factors which determine the absorption of a substance dissolved in water across a biological barrier with which the solution is in intimate contact.'' In the same document Dr. Idle concludes that ''The concept of a pH for snuff depends upon a standardized and validated pH assay for aqueous tobacco suspensions' and that ''The concept of pH for a moist solid such as tobacco can only apply to a solution derived from a stirred suspension of a standardized amount of tobacco in a standardized volume of water.''

It is the intent of the protocol to provide smokeless tobacco manufacturers with a ''standardized measurement'' of pH. Parameters that can be standardized for pH, moisture, and nicotine determination (sample size, sample preparation, quantity and purity of standards and reagents, instrumentation, measurement time and conditions, etc.)—not random conditions or circumstances unique to each smokeless tobacco user such as ''residues of beverages'' in the mouth of the smokeless tobacco user (chemical factors), ''surface area of the absorptive tissues'' (biological factors), and ''expectoration'' or ''swallowing'' (behavioral factors)—are specified in the protocol. In addition, the protocol's methodology is supported by the conclusions presented in a recent review article that ''pH is a major determinant of nicotine absorption across mucosal tissues'' in the mouth and that other ''behavioral and biological'' factors probably have ''little effect on the rate of nicotine absorption'' (Tomar and Henningfield, Tobacco Control 1997, 6:219–225).

Information Collection Provisions

This notice contains information collections which have been approved by the Office of Management and Budget (OMB) under the Paperwork Reduction Act of 1980 and assigned OMB Action Notice number 0920–0444 (expiration 01/31/2002). The title description, and respondent information are shown below with an estimate of the annualized costs and burden hours.

Title: Quantity of Nicotine Contained in Smokeless Tobacco Products Manufactured, Imported, or Packaged in the United States.

Description: The Comprehensive Smokeless Tobacco Health Education Act (CSTHEA) of 1986 requires HHS to collect this information. HHS is authorized under CSTHEA to conduct research on the addictive nature of nicotine and general health effects of using smokeless tobacco.

Description of Respondents: Businesses or other For-Profit Organizations.

Estimates of Annualized Burden Hours and Costs: The average annualized total cost to industry is $23,419. This is based on an annualized estimated cost for 11 companies at $2,129 per company. Some companies may choose to contract with an independent laboratory while others may elect to complete the determinations in-house. For those companies choosing to conduct the testing in-house, approximately US$60,000 would be required to purchase the necessary equipment, assuming none of the equipment was previously owned.

The annual response burden to the industry is estimated at 170 hours per smokeless tobacco company. Thus, for the 11 respondents, the hour-burden is 1,870 hours.

Respondents	Number of re-spond-ents	Avg. number of responses per respond-ent	Avg. hours per respondent	Estimated total hours	Avg. cost per respondent	Estimated total cost
Tob. Mfrs.	11	1	170	1,870	$2,129	$23,419

Procedures to maintain confidentiality of nicotine, pH, and moisture data: As provided by CSTHEA, HHS is required to treat the nicotine, pH, and moisture information as a trade secret or confidential in accordance

with 5 U.S.C. 552(b)(4)and 18 U.S.C. 1905. CSTHEA also requires HHS to establish written procedures to assure the confidentiality of the information provided. Consistent with these statutory provisions, HHS has developed strict procedures for treating and protecting relevant documents, including secured file storage and strictly-limited access to the information. The procedures that are applicable to the nicotine content of smokeless tobacco products comport with those already in place for protecting the confidential lists of ingredients in cigarettes and smokeless tobacco products. These procedures have proven workable, effective, and acceptable to the companies required to report the confidential information. The procedures, Guidelines for Maintaining and Releasing Privileged Information Obtained in Accordance With Sec. 4(b)(2)(a) of Public Law 99–252 (15 U.S.C. 4403), were previously published in the February 1, 1994, **Federal Register** (59 FR 4714), and are available from CDC's Office on Smoking and Health upon request.

Dated: March 17, 1999.

Martha Katz,

Acting Director, Centers for Disease Control and Prevention.

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

I. Requirements [1, 2]

A. Reagents [3]

1. Sodium hydroxide (NaOH), 2N
2. Methyl t-butyl ether (MTBE)
3. (-) -Nicotine (Fluka 72290) >99% purity [4, 5]
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 x 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) [6, 7]
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 x 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 [8, 9]

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 [10]
4. Injection volume: 2 μl
5. Column conditions: 110–185°C at 10°C min $^{-1}$; 185–240°C at 6°C min $^{-1}$, hold at final temperature for 10 min.

F. Sample Preparation [11]

There exist six different categories of commercial smokeless tobacco products:

1. Dry snuff;
2. Moist (wet) snuff;
3. Moist (wet) snuff portion packs;
4. Plug;
5. Twist; and
6. Loose leaf.

Because of their physical characteristics, samples of three of the six product categories must be ground 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 moist (wet) snuff 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.

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.

II. Nicotine Analysis [12]

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:
$Recovery = Nicotine_{calculated}/Nicotine_{actual}$

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 lot of a smokeless tobacco product is evaluated, 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 ± 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 ± 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.[13]

4. Pipette 50 mL of extraction solution (II.D.1) into each tube. Cap each tube and tighten.[14]

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:

$Recovery = Nicotine_{calculated}/Nicotine_{actual}$

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 lot.

D. Sample Extraction Procedure[12]

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 ± 0.020 gram of prepared tobacco sample into culture tube and record weight.[15] The number of products sampled per lot should reflect an acceptable level of precision.[16] The test material is to be representative of the product that is sold to the public and therefore should consist of sealed, packaged samples from each lot of finished product that is ready for commercial distribution. Triplicate determinations will provide precision data.

3. Pipette 5 mL of 2 N NaOH into the tube. Cap the tube. Swirl to wet sample and allow to stand 15 minutes.[13]

4. Pipette 50 mL of extraction solution into tube, cap tube and tighten.[14]

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.[17]

BILLING CODE 4163–18–P

Equation 3:[18]

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

BILLING CODE 4163–18–C

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 lot and 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 per lot, 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 ± 1.0°C.

B. Accurately weigh 5.00 grams of the sample (ground to pass ≤ 4 mm screen)[19] into a weighed moisture dish and place uncovered dish in oven.[20] The number of products sampled per lot should reflect an acceptable level of precision.[16] The test material is to be representative of the product that is sold to the public and therefore should consist of sealed, packaged samples from each lot of finished product that is ready for commercial distribution. Triplicate determinations will provide precision data.

C. Do not exceed 1 sample/10 sq in. (650 sq cm) shelf space, and use only 1 shelf. Dry 3 hr at 99 ± 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 lot and 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 per lot, 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[12,21]

A. Test samples as soon as possible after they are received. The number of products sampled per lot should reflect an acceptable level of precision.[16] The test material is to be representative of the product that is sold to the public and therefore should consist of sealed, packaged samples from each lot of finished product that is ready for commercial distribution. Triplicate determinations will provide precision data.

B. Accurately weigh 2.00 grams of the sample. Place in a 50 mL polypropylene container with 10 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 each lot of 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 for the lot 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 lot and 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 per lot, 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-Hasselbalch equation (Equation 4), based on measured pH and nicotine content.

BILLING CODE 4163–18–P

Equation 4:

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

$$B + H^+ \rightleftharpoons BH^+$$

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

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

[B] = amount of un-ionized (free) nicotine

$[BH^+]$ = amount of ionized nicotine

BILLING CODE 4163–18–C

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 lot and 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 per lot, 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.).

Billing Code 4163–18–P

Sample calculation:

Mean total nicotine = 10.30 (mg/g)

Mean pH = 7.50

pKa = 8.02

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

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

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

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

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

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

% un-ionized (free) nicotine = 23.20

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

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

Total free nicotine (mg/g) = 2.39

BILLING CODE 4163–18–C

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 [18]

The coefficient of determination between $Area_{nicotine}/Area_{IS}$ 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 per lot (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 ± 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 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.

[20] 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.

[21] The method is based on a method published by Henningfield et al. (1995).

References

AOAC (Association of Official Analytical Chemists). Official Methods of Analysis. 966.02: Moisture in Tobacco. (1990) Fifth Edition. K. Helrich (ed). Association of Official Analytical Chemists, Inc. Suite 400, 2200 Wilson Boulevard, Arlington, Virginia 22201 USA.

CORESTA (Centre de Coopération pour les Recherches Scientifiques relatives au Tabac). Recommended Method No. 39: Determination of the purity of nicotine and nicotine salts by gravimetric analysis—Tungstosilic acid method November, 1994. 87–90.

CRC Handbook of Chemistry and Physics. R.C. Weast, D.R. Lide, M.J. Astle, and WH. Beyer (eds). 70th ed. Boca Raton, Florida: CRC Press (1989–1990) D–162.

Henningfield, J.E., Radzius A., Cone E.J. (1995). Estimation of available nicotine content of six smokeless tobacco products. Tobacco Control 4:57–61.

ISO (International Organization for Standardization). IOS 8243: Cigarettes—Sampling. (1991). Second Edition. Prepared by Technical Committee ISO/TC 126, Tobacco and tobacco products. International Organization for Standardization, Case Postale 56, CH–1211 Genève 20, Switzerland.

Westgard J.O., Barry P., Hunt M., and Groth T. (1981). A multi-rule Shewhart chart for quality control in clinical chemistry. Clinical Chemistry 27:493.

[FR Doc. 99–7022 Filed 3–22–99; 8:45 am]

BILLING CODE 4163–18–P