

DEPARTMENT OF THE TREASURY

Alcohol and Tobacco Tax and Trade Bureau

[Notice No. 66]

Total Reducing Sugars Analytical Method

AGENCY: Alcohol and Tobacco Tax and Trade Bureau, Treasury.

ACTION: General notice.

SUMMARY: The Alcohol and Tobacco Tax and Trade Bureau issues this notice to describe the analytical method that it uses to determine the total reducing sugars content in the cigars and cigarettes that it analyzes. We reference this method in our notice of proposed rulemaking entitled "Tax Classification of Cigars and Cigarettes," which is published elsewhere in this issue of the **Federal Register**. We are giving notice of this method so that interested members of the public may use the same method to evaluate the total reducing sugars of tobacco products for the purpose of commenting on the proposed rule.

FOR FURTHER INFORMATION CONTACT: Linda Wade Chapman, Regulations and Rulings Division, Alcohol and Tobacco Tax and Trade Bureau, 1310 G Street NW., Suite 200-E, Washington, DC 20220; telephone 202-927-8210.

SUPPLEMENTARY INFORMATION:

Background

The Alcohol and Tobacco Tax and Trade Bureau (TTB) is responsible for the administration of the Federal excise tax and related provisions that apply to tobacco products under Chapter 52 of the Internal Revenue Code of 1986, as amended. Cigars and cigarettes are among the tobacco products covered by those provisions.

Elsewhere in this issue of the **Federal Register**, we are publishing a notice of proposed rulemaking, "Tax Classification of Cigars and Cigarettes," which sets forth proposed new regulatory standards for the classification of cigars and cigarettes for tax purposes. The proposed regulatory standards include a specification regarding the total reducing sugar content of cigar and cigarette filler tobacco. This specification is based on the results of a study TTB performed on cigars and cigarettes using the Astoria 2 + 2 Flow Analyzer, which is the apparatus that TTB currently uses for tobacco product analysis. The purpose of this notice is to advise the public of the apparatus and method we employ to determine the total reducing sugars found in tobacco products, thereby

allowing interested industry members and others to evaluate the total reducing sugar content of tobacco products in the same way for the purpose of commenting on the proposed new regulatory standards.

TTB uses the Astoria 2 + 2 Flow Analyzer and the Astoria-Pacific International Total Reducing Sugars A250 method with one modification—we prepare the extraction solution without methanol, thereby increasing the extraction time to 30 minutes, instead of 15 minutes, per operating note 10 in method A250.

The method is set forth below.

Laboratory Method

Total Reducing Sugars—A250

(Astoria-Pacific International, Rev. D 8/2003)

A. Scope and Application

This method is used for the determination of total reducing sugar in acetic acid extracts of tobacco. The applicable range of this method is 20 to 400 mg/L. The range can be extended by changing the detector sensitivity or by sample dilution.

B. Summary of Method

Samples (containing sucrose) are first hydrolyzed by invertase to form reducing monosaccharides.¹ Reducing sugars react with p-hydroxybenzoic acid hydrazide (PAHBAH) in an alkaline media to form a yellow color measured at 410 nm.² Calcium is used to enhance the color development.³

C. Interferences

No interferences are known for this methodology.

D. Sample Handling and Preservation

Casing and flavoring products should be refrigerated until analyzed. Samples should be stored in airtight containers prior to analysis. Extracted samples are stable for 48 hours when refrigerated.

E. Raw Materials Required

Note: Chemicals should be of ACS grade or equivalent.

- Acetic Acid, Glacial CH₃COOH (FW 60.05);
- Brij®-35, 30% w/v (API p/n 90-0710-04);
- Calcium Chloride CaCl₂·2H₂O (FW 147.02) (API p/n 80-7040-72);
- Citric Acid HOCCOOH (CH₂COOH)₂·H₂O (FW 210.14) (API p/n 80-7035-61);
- Deionized Water (ASTM Type I or II);
- Fructose C₆H₁₂O₆ (FW 180.16) (API p/n 80-7062-72);

- Glucose C₆H₁₂O₆ (FW 180.16) (API p/n 80-7061-72);
- Hydrochloric Acid HCl (FW 36.46);
- Invertase (See Operating Note #2) (API p/n 80-7050-61K);
- p-Hydroxybenzoic Acid Hydrazide (PAHBAH) C₇H₈N₂O₂ (FW 152.20) (API p/n 80-7030-60);
- Methanol CH₃OH (FW 32.04);
- Sodium Hydroxide NaOH (FW 40.00); and
- Sucrose C₁₂H₂₂O₁₁ (FW 342.30) (API p/n 80-7063-72).

(Reagents may be purchased in a pre-packaged kit, API p/n 80-7000-13K).

F. Reagent Preparation

1. Sodium Hydroxide Solution, 1 N (1 L)

Caution: The dissolution of sodium hydroxide in water releases a great amount of heat.

Sodium Hydroxide 40.0 g
NaOH (FW 40.00)
Deionized Water

Dissolve 40.0 g of sodium hydroxide in 800 ml of deionized water contained in a 1 L volumetric flask. Dilute to the mark. Mix well. Filter to 0.45 μm with a membrane filter. Keep tightly closed; stable for one month at room temperature.

2. Hydrochloric Acid Solution, 0.75 N (1 L)

Hydrochloric Acid, Concentrated 63 ml
(36-37%).
HCl (FW 36.46)
Deionized Water

Cautiously add 63 ml of hydrochloric acid to 800 ml of deionized water contained in a 1 L volumetric flask. Dilute to 1 L with deionized water and mix well. Filtering is not necessary. Keep tightly closed; stable for 60 days at room temperature.

3. p-Hydroxybenzoic Acid Solution (PAHBAH) (500 ml)

p-Hydroxybenzoic Acid 1 vial or 30.0 g
Hydrazide, 80-7030-60.

C₇H₈N₂O₂ (FW 152.20)

Citric Acid, 80-7035-61. 1 vial or 10.5 g

HOCCOOH
(CH₂COOH)₂·H₂O
(FW 210.14)

Hydrochloric Acid Solution, 0.75N

Dissolve one vial or 30.0 g of p-hydroxybenzoic acid hydrazide and one vial or 10.5 g of citric acid in 400 ml of 0.75 N hydrochloric acid solution contained in a 500 ml volumetric flask. Dilute to volume with hydrochloric acid solution and mix well using a magnetic stirring bar. Filter to 0.45 μm with a

membrane filter. Store at room temperature; stable for 60 days.

4. Calcium Chloride Solution (500 ml)

Calcium Chloride, 80–7040–72. 1 vial or 0.34 g

CaCl₂•2H₂O (FW 147.02)

Deionized Water

Dissolve one vial or 0.34 g of calcium chloride in 400 ml of deionized water contained in a 500 ml volumetric flask. Dilute to volume with deionized water and mix well. Filter to 0.45 µm with a membrane filter. Store at room temperature; stable for 60 days.

5. Extraction Solution, 1% acetic acid, 2% methanol (v/v) (20 L)

Note: If analyzing Nitrate/Nitrite simultaneously, see Operating Note 10.

Acetic Acid, Glacial 170 ml
CH₃COOH (FW 60.05)

Methanol 340 ml
CH₃OH (FW 32.04)

Deionized Water

Add 170 ml of glacial acetic acid and 340 ml of methanol to 16.49 L of deionized water contained in a 20 L polyethylene carboy. Do not dilute to volume. Mix well. Store at room temperature. Stable for 30 days.

Note: Extraction Solution can be prepared in smaller quantities. This solution will be used for extraction, calibrants and sampler wash.

6. Invertase Solution (500 ml)

Invertase, 80–7050–61K 1 vial or 0.20 g
(See operating note #2)

Extraction Solution

Dissolve one vial or 0.20 g invertase into 300 ml extraction solution contained in a 500 ml volumetric flask. Dilute the solution to volume with Extraction Solution and mix well. Filter to 0.45 µm with a membrane filter. Store refrigerated; stable for 10 days.

7. Sampler Wash Solution (1 L)

Extraction Solution 1 L
Brij-35, 30% solution 1 ml

Add 1 ml of Brij-35, 30% solution to 1 L Extraction Solution and mix well.

8. Startup and Shutdown Solution (1 L)

Deionized Water 1 L
Brij-35, 30% solution 1 to 2 ml

Add 1 to 2 ml of Brij-35, 30% solution to 1 L deionized water and mix well.

G. Calibrants

Specific Stock and Working Calibrant preparation instructions can be found on the back of the flow diagram. Be sure to use the flow diagram which covers

the concentration range you wish to analyze.

Working calibrants may be prepared to cover alternate ranges by adding the appropriate volumes of stock or intermediate calibrant to 100 ml volumetric flasks that contain approximately 80 ml of extraction solution. Dilute the solution to 100 ml with extraction solution and mix well.

The following formula can be used to calculate the amount of stock (or intermediate) calibrant to be used.

$$C_1 V_1 = C_2 V_2$$

Where:

C₁ = desired concentration (in mg/L) of working calibrant to be prepared;

V₁ = final volume (in ml) of working calibrant to be prepared (generally 100 ml);

C₂ = concentration (in mg/L) of stock (or intermediate) calibrant; and

V₂ = volume (in ml) of stock (or intermediate) calibrant to be used.

Rearranging the equation to solve for V₂ yields:

$$V_2 = \frac{C_1 V_1}{C_2}$$

For example, to prepare a 1.0 mg/L working calibrant from a 1000 mg/L stock calibrant, use 0.1 ml (100 µl) of the stock calibrant in 100 ml final volume.

$$V_2 = \frac{(1.0 \text{ mg/L})(100 \text{ ml})}{1000 \text{ mg/L}}$$

$$V_2 = 0.1 \text{ ml}$$

Add this amount of stock calibrant to the volumetric flask and then dilute to volume with the extraction solution.

H. Operation Procedure

1. Set up the cartridge as shown in the flow diagram [The flow diagram is shown at the end of this document.¹ Check all tubing and connections. Replace if necessary.

2. Place reagent lines in startup solution.

3. Turn on the power to all units including heat bath and latch pump platens to begin liquid flow.

4. Verify that the bubble sizes and spacing are consistent throughout the cartridge. If bubbles are splitting up as they enter or exit a coil or heat bath, check and replace fittings if necessary. The bubbles should flow smoothly without dragging. If dragging occurs,

add more Brij-35 to the startup and sampler wash solutions.

5. Check all reagent containers on the instrument for particulate matter. Reagents should be filtered prior to use. Be sure all containers are properly labeled and filled before pumping reagents.

6. After the heat bath has reached the desired temperature and a stable baseline has been verified on the startup solution, place reagent lines in reagent bottles.

7. If using data collection software, set up the appropriate sample table.

8. Allow reagents to run for 5 to 10 minutes and verify a stable baseline.

9. Load the sampler tray with calibrants, blanks, samples, and QC or monitor samples.

10. Select the appropriate parameters for the detector and sampler. (See Flow Diagram at the end of methodology.)

11. Begin analysis.

12. At the end of analysis place all reagent lines in startup/shutdown solution and turn off the heat bath. Pump for 20 to 30 minutes to flush all of the reagents out of the cartridge and to allow the heat bath to cool.

13. Turn off the power to all units and release pump platens.

I. Operating Notes

1. If this cartridge is run as a single cartridge on the system, a sample "helper" line may be necessary. Problems such as poor precision, erratic washout between peaks, irregular peak shape, or inconsistent ISAC artifacts could be an indication that a "helper" line is needed. (See Section 9 of the Astoria® Analyzer Operation Manual.) Pecking may also be turned ON.

2. Invertase, grade VII from Bakers Yeast, available from Sigma Chemical Company, St. Louis, MO is recommended.

3. To check hydrolysis efficiency, prepare a check calibrant of the same nominal concentration from sucrose. If hydrolysis efficiency is poor, replace the invertase reagent.

4. This cartridge has a 0.015" ID pulse suppressor installed at the NaOH reagent addition tee. Insure that no air bubbles are present in this suppressor before analyzing samples. Air bubbles or debris in the suppressor will produce an unstable baseline and loss of sensitivity.

5. Another common cause of low sensitivity and noise in the baseline is debris in the flowcell. Particulate matter from the reagents and samples become lodged in the flowcell restricting the amount of light that is passed through the flowcell. Flushing the flowcell with approximately 10 ml of sampler wash solution with a syringe will dislodge

¹ **Note:** Some proprietary information of Astoria-Pacific International was removed from this diagram.

any debris. Following proper filtration procedures for the reagents and samples will reduce the frequency of clogging.

6. Avoid using carbon in the filtration of samples. It will effect the color development.

7. Adding 1 ml/L of Brij-35 to the reagents just before use rather than when they are prepared will decrease the possibility of bacteria or algae contamination.

8. If bubbles are sticking in a debubbler, cleaning the debubbler will allow bubbles to escape smoothly out the bubble line. Bubbles sticking in the debubbler can cause a loss in the overall precision of the peak height. To clean, soak the debubbler for 2–3 hours in a mixture of 20–30% Contrad®NF (API p/n 80–0007–04) and hot tap water. Rinse thoroughly.

9. Cover all reagents and other solutions to avoid interference due to dust and other particulates.

10. Methanol in the extraction buffer has been found to cause a 0.2–0.3% increase in Nitrate results. If running nitrate simultaneously with total reducing sugars, removing the methanol from the extraction solution will take care of the bias. However, you will need to increase the extraction time to 30 minutes.

J. Sample Preparation Procedure

Note: This procedure is applicable for all tobacco products including leaf, stem, scrap, and cut filler. The total sugar results achieved from this procedure will be on a dry weight basis.

1. Place approximately 3 to 5 grams of product into a screen wire basket.

2. Place baskets into a 90°C convection oven for one hour.

3. Remove baskets from oven.

4. Grind samples to 20 mesh using a Wiley Mill Grinder or equivalent.

5. Place ground samples into air tight containers.

6. Using an analytical balance, accurately weigh approximately 100 mg of the dried and ground tobacco sample.

7. Transfer the weighed sample to a 125 ml Erlenmeyer flask.

8. Accurately dispense 100 ml of Extraction Solution into the 125 ml flasks.

9. Stopper the flasks and place them on a wrist action or orbital shaker for 15 minutes.

10. Filter the samples through Schleicher and Schuell #560 pleated filter paper or equivalent.

11. Contain the filtered solution in plastic vials with hinged caps until the appropriate time for analysis.

12. If refrigerated, samples will be stable for 48 hours.

K. References

1. *Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC)*, Thirteenth Edition 31.134, 1980, Washington, DC.

2. Ferraro, J.J., Caccavo, F.A., Saifer, A., p-Hydroxybenzoic Acid Hydrazide Procedure for Serum Glucose Adapted to the Technicon “SMA 12/60”, and Compared with Other Glucose Methods. *Clin Chem* 22/2, 263–266 (1976).

3. Davis, R. E., A Combined Automated Procedure for the Determination of Reducing Sugars and Nicotine Alkaloids in Tobacco Products Using a New Reducing Sugar Method. *Tobacco Science*, XX: pp. 146–151 (1976).

Acknowledgements

- Astoria® and FASPac™ are trademarks of Astoria-Pacific, Inc., Clackamas, Oregon.
- Brij®-35 is a registered trademark of ICI Americas, Wilmington, Delaware.
- Contrad® is a registered trademark of Decon Laboratories, Bryn Mawr, PA.

Calibrant Preparation

Total Reducing Sugars in Extracts of Tobacco

200–A250–A00
20–400 mg/L Sugars
(Astoria-Pacific International, Rev. A 11/2003)

4,000 mg/L Sugar Stock Standard (500 ml)

(1) Dissolve into 300 ml extraction solution:

- 1.0 g (one bottle) Fructose (API p/n 80–7062–72), C₆H₁₂O₆ (FW 180.16), dried at 110°C, and
- 1.0 g (one bottle) Glucose (API p/n 80–7061–72), C₆H₁₂O₆ (FW 180.16), dried at 110°C.

(2) Dilute to 500 ml with extraction solution.

(3) Add 2 drops Chloroform, CHCl₃ (FW 119.38) and mix well.

(4) Store @ 2–8°C. Stable for 60 days.

Working Standard (100 ml)

Use adjustable, microliter pipettes to add the designated volumes of stock, intermediate or working standard to 100 ml volumetric flasks containing approximately 80 ml of extraction solution. Dilute each solution to the mark with the extraction solution and mix well. Make standards covering the range being run.

Range	Working standard concentration	Standard to pipet (Stock, Intermediate or Working)	Volume to pipet (μL)–(ml)
20–400 mg/L	400 mg/L	4,000 mg/L	10,000 C6 10.0
20–400 mg/L	300 mg/L	4,000 mg/L	7,500 C5 7.5
20–400 mg/L	200 mg/L	4,000 mg/L	5,000 C4 5.0
20–400 mg/L	100 mg/L	4,000 mg/L	2,500 C3 2.5
20–400 mg/L	40 mg/L	4,000 mg/L	1,000 C2 1.0
20–400 mg/L	20 mg/L	4,000 mg/L	500 C1 0.5

Note: To prepare alternate calibrant concentrations consult the methodology.

2,000 mg/L Sucrose Stock for Liquid Control (500 ml)

(1) Dissolve into 300 ml extraction solution:

• 1.0 g (one bottle) Sucrose (API p/n 80–7063–72), C₁₂H₂₂O₁₁ (FW 180.16), dried at 110°C.

(2) Dilute to 500 ml with extraction solution.

(3) Add 2 drops Chloroform, CHCl₃ (FW 119.38) and mix well.

(4) Store @ 2–8°C. Stable for 60 days.

150 mg/L Sucrose Liquid Control (100 ml)

(1) 7.5 ml of 2,000 mg/L Sucrose Stock.

(2) Dilute with Extraction Solution to 100 ml.

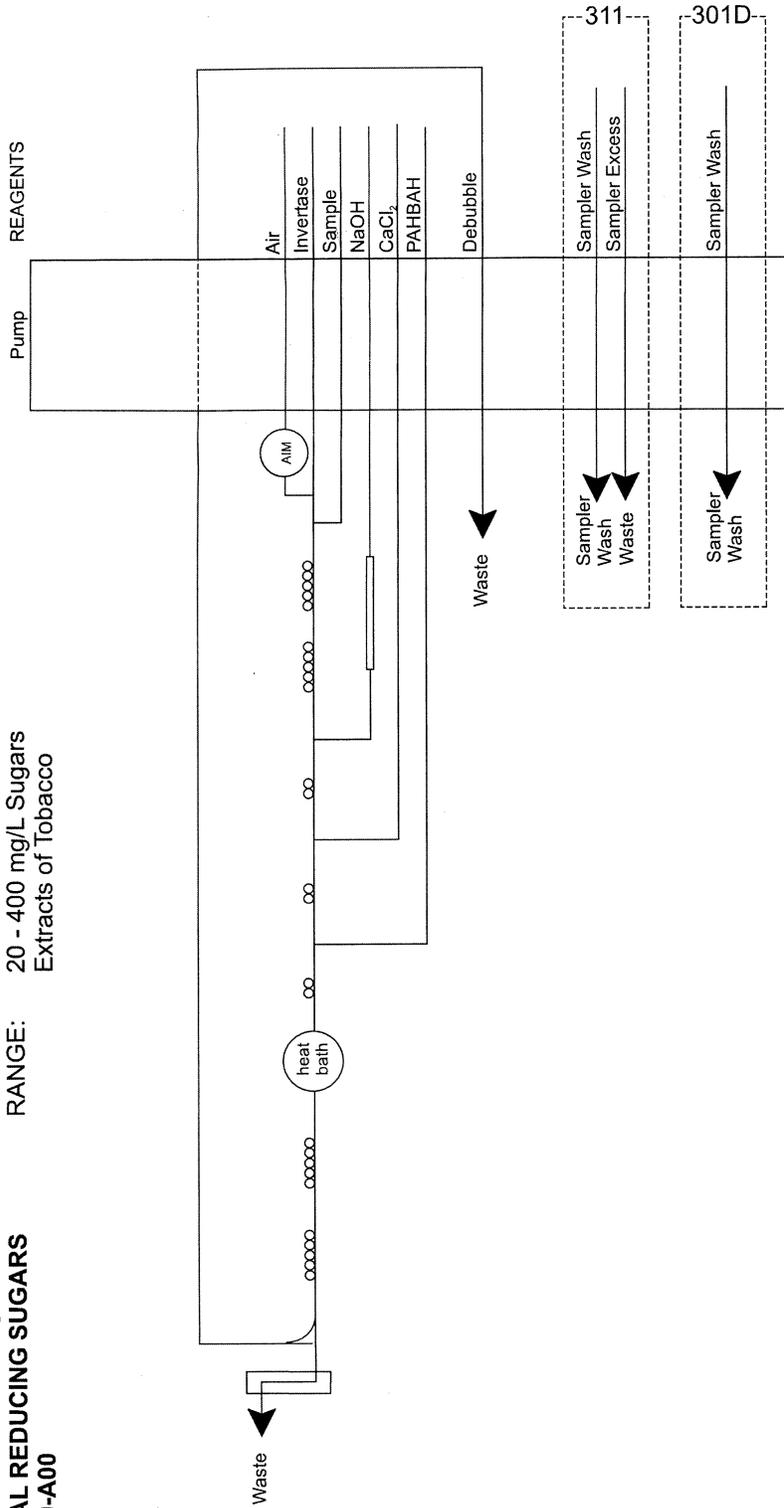
(3) Mix well.

BILLING CODE 4810–31–P

Astoria-Pacific International

**TOTAL REDUCING SUGARS
A250-A00**

**RANGE: 20 - 400 mg/L Sugars
Extracts of Tobacco**



BILLING CODE 4810-31-C

Signed: October 17, 2006.

John J. Manfreda,
Administrator.

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