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**Date:** December 31, 1999
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## 1 SCOPE OF APPLICATIONS

**1.1** This method is to be used to determine the amount of nicotine, nornicotine, anabasine, myosmine, and anatabine (alkaloids) in whole tobacco. The method is designed to quantitate nicotine directly against a nicotine calibration that is also used to determine the amounts of each of the other alkaloids assuming a similar response factor.

**1.2** Nornicotine, anabasine, myosmine and anatabine (if desired) may also be quantitated against calibrations generated from commercially available standards. Anatabine must be quantitated using the response factor generated by the anabasine standard if pure anatabine is not available.

**1.3** Total alkaloids are determined as the sum of nicotine, nornicotine, anabasine, myosmine, and anatabine.

## 2 NORMATIVE REFERENCES

**2.1** American Society for Testing and Materials (ASTM) D1193-77 – Standard Specifications for Reagent Water, Version 1977.

**2.2** Health Canada Test Method T-115 – Determination of Tar, Water, Nicotine and Carbon Monoxide in Mainstream Tobacco Smoke, 1999-12-31.

**2.3** Health Canada Test Method T-402 – Preparation of cigarettes, cigarette tobacco, cigars, kreteks, bidis, packaged leaf tobacco, pipe tobacco, and smokeless tobacco for testing, 1999-12-31.

## 3 DEFINITIONS

**3.1** Refer to T-115 for definitions of terms used in this document.

## 4 METHOD SUMMARY

**4.1** This method is a gas chromatographic (GC) method using a fused silica capillary column and a thermionic specific detector (TSD). 25 mg of freeze-dried and ground tobacco is extracted with 1.0 mL of a methanolic KOH solution (containing 2,4-dipyridyl as internal standard) in an ultrasonic bath for three hours. The mixture is then centrifuged at low speed to separate the solid tobacco from the solution. The supernatant is transferred to an autosampler vial where it is analyzed by GC.

**4.2** The alkaloids are analyzed on a CAM fused silica capillary column which has a polyethylene glycol (PEG) stationary phase that has been specifically base deactivated for volatile amine analysis. Quantitation is achieved using an internal standard calibration by comparing the TSD response of the analytes in the samples against a five-point calibration of nicotine in the standards.

*Note:* The testing and evaluation of certain products against this test method may require the use of materials and or equipment that could potentially be

hazardous and this document does not purport to address all the safety aspects associated with its use. Anyone using this test method has the responsibility to consult with the appropriate authorities and to establish health and safety practices in conjunction with any existing applicable regulatory requirements prior to its use.

## 5 APPARATUS AND EQUIPMENT

**5.1** Lyophilizer.
**5.2** Culture tubes, disposable borosilicate – 10 mm X 125 mm.
**5.3** Volumetric flasks – 10 mL, 1000 mL, 2000 mL.
**5.4** Glass Pasteur transfer pipettes.
**5.5** Micropipettes or gas-tight syringe for the preparation of working standards.
**5.6** Dispensor (1 – 5 mL) and bottle.
**5.7** Parafilm (for closure of the extraction tubes).
**5.8** 20 mL scintillation vial with aluminum lined cap.
**5.9** Bench grinder with # 40 screen.
**5.10** Cotton swabs -for cleaning grinder.
**5.11** Varian 3400 / 3600 Gas Chromatograph or equivalent.
**5.12** Varian 8100 / 8200CX Autosampler or equivalent.
**5.13** Autosampler vials/caps/Teflon faced septa.
**5.14** CAM fused silica capillary column 30 m X 0.25 mm X 0.25 µm.
**5.15** Ultrasonic bath.
**5.16** Analytical balance capable of measuring to four decimal places.
**5.17** Centrifuge.
**5.18** Vortex mixer.

## 6 REAGENTS AND SUPPLIES

*Note:* All reagents shall be, at the least, recognized as analytical reagent grade in quality.

**6.1** Methanol Distilled in Glass (DIG).
**6.2** Potassium Hydroxide.
**6.3** Type I water (meets ASTM D 1193 specification).
**6.4** 2,4-dipyridyl - used as an internal standard (ISTD).
**6.5** (-)-Nicotine*.
**6.6** If quantitation of minor alkaloids is specifically requested:

(+/-)-Anabasine - Neonicotine*
(+/-)-Nornicotine - 2-[3-pyridyl]-pyrrolidine*
Myosmine - 3-[1-Pyrrolin-2-yl]pyrroline*
Anatabine*

***Note:* All neat compounds must be of known purity and be as close to 100% as possible

## 7 PREPARATION OF GLASSWARE

**7.1** Glassware should be cleaned and dried in such a manner that ensures that contamination from glassware does not occur.

## 8 PREPARATION OF SOLUTIONS

### 8.1 Extraction Solution - (0.25 mg/mL 2,4-dipyridyl in Methanolic-KOH)

**8.1.1** Weigh 5.6 g (+/- 0.05 g) of potassium hydroxide (KOH) into a 2000 mL volumetric flask and make to volume with methanol (MeOH).

*Note:* Sonication is necessary to dissolve the pellets.

**8.1.2** Label as "0.05N KOH in MeOH" with the preparation date.

**8.1.3** With an analytical balance, capable of measuring to four decimal places, accurately weigh 0.25g +/- 0.005g of 2,4-dipyridyl into a 1000 mL volumetric flask and make to volume with the 0.05N KOH in MeOH solution.

**8.1.4** Mix well and transfer the contents to 1250 mL Dispenser bottle and label as Alkaloids Extraction Solution with the date of preparation.

*Note:* When a new batch of extraction solution is prepared, a new series of working standards must be prepared to ensure a consistent amount of internal standard between samples and standards.

## 9 PREPARATION OF STANDARDS

### 9.1 Nicotine Primary Stock (concentration: approximately 5mg/mL)

**9.1.1** With an analytical balance, capable of measuring to four decimal places, accurately weigh 0.05 g (+/- 0.005 g) of Nicotine into a 10 mL volumetric flask and make to volume with extraction solution.

*Note 1*: Nicotine is very hygroscopic and weighing should be done carefully and quickly to prevent absorption of moisture from affecting the weight.

*Note 2*: Primary stocks of the minor alkaloids (if necessary) are to be made up similarly. Although their levels are considerably lower than that of nicotine, it is not recommended to weigh less of the standard, but to make a secondary stock for the preparation of working standards.

### 9.2 Working Standards

**9.2.1** All standards are made into volumetric flasks using the dilutions described in the following table:

| Nicotine Standard | Volume of Primary Std (µL) | Volume (mL) | Nicotine [µg/mL] | Nicotine: ISTD ratio |
|---|---|---|---|---|
| 1 | 2000 | 10 | 1000 | 4 |
| 2 | 1000 | 10 | 500 | 2 |
| 3 | 500 | 10 | 250 | 1 |
| 4 | 250 | 10 | 125 | 0.5 |
| 5 | 100 | 10 | 50 | 0.2 |

**9.2.2** Volumetric flasks are made to volume with extraction solution containing the internal standard.

*Note 1*: Analytical concentrations may vary depending on the concentration of primary stock and must be calculated in order to prepare an accurate calibration.

*Note 2*: In order to define a tighter calibration range for determining the concentration of the minor alkaloids, a standard of 1/10$^{th}$ that of the lowest standard of nicotine can be prepared. A separate response factor (or calibration curve) using the three lowest standards can be determined for calculating the minor alkaloids.

## 10 SAMPLE ANALYSIS

### 10.1 Determination of Moisture Content

**10.1.1** Accurately weigh and record the weight of an appropriately labeled, dry 20 mL scintillation vial with aluminum lined cap.

**10.1.2** Transfer the tobacco of three cigarettes (or 2 g) into the pre-weighed 20 mL scintillation vial with aluminum lined cap.

**10.1.3** Accurately weigh and record the total weight of the capped scintillation vial with tobacco.

**10.1.4** Place samples in the freezer for a minimum of one hour prior to placing them in the freeze-dryer.

**10.1.5** Place samples into the freeze dryer with the caps loosened to allow the moisture to be driven off from the tobacco.

**10.1.6** After a minimum of 48 hours, remove the vials and tighten the caps immediately to prevent re-absorption of moisture from the air.

**10.1.7** Accurately weigh and record the total weight of the capped scintillation vial with tobacco after freeze-drying and calculate the % Moisture Content.

### 10.2 Grinding of Samples

**10.2.1** Grind tobacco using the # 40 (40 sections/square inch) screen of the bench top grinder.

**10.2.2** After grinding, wipe off the remaining tobacco particles into the sample using a cotton swab, then vacuum the grinder to remove any tobacco dust.

**10.2.3** Disassemble components of grinder and clean with methanol.

**10.2.4** Reassemble the grinder and use the vacuum again in order to volatilize any residual methanol from the grinder.

**10.2.5** Repeat the cleaning process between samples.

### 10.3 Extraction of Sample

**10.3.1** Accurately weigh 25 mg (+/- 5 mg) of ground tobacco into a 10 mm X 125 mm disposable borosilicate culture tube.

**10.3.2** Add 1 mL of the Extraction Solution to the sample.

**10.3.3** Seal the culture tube with four layers of Parafilm® and place into a metal rack suitable for placement into an ultrasonic bath.

**10.3.4** Sonicate samples in the ultrasonic bath for three hours.

*Note:* The sonication process will generate heat, and the bath should be monitored to not exceed 40 $^{o}$C.

**10.3.5** Remove samples from bath after one hour to vortex, swirling the tube to get all of the tobacco into the solvent, and return to the sonicator.

**10.3.6** After sonication is complete, centrifuge the tubes for five minutes at low speed to separate the tobacco from the solvent.

**10.3.7** Transfer the supernatant to an autosampler vial to be analyzed on the GC.

### 10.4 Gas Chromatograph Conditions

**Injector:** Split with a split flow of approximately 35 mL/min at 110 $^{o}$C.
**Column:** CAM –30 m X 0.25 mm X 0.25 µm.
**Detector:** Thermionic Specific (TSD), Channel A, 1 Volt Full Scale, Range:12, Atten:8.
**Autozero:** On, Bead Current: 3.175 Amps (specific to age and usage of Bead).
**Carrier:** He at 15.0 psi, linear velocity approximately 30 cm/second.
**Relays:** Initial relay state: all (-).

*Note 1*: Detector gas flows set as per manufacturer's specifications for $H_2$, X-Dry air, and N2 as make-up gas.

**Temperature Program**

**Injector:** 220 $^{o}$C.
**Detector:** 300 $^{o}$C.

**Start Temperature:** 110 $^{o}$C hold for one minute.
**Rate:** 5 $^{o}$C/min to 225 $^{o}$C hold for two minutes.

**Total Run Time:** 26.00 minutes .

**Autosampler Conditions: Injection volume:** 1.0 µL.

Load auto-sampler vials into rack starting with the highest standard to the lowest before any samples are to be run in order to create a new calibration.

*Note:* The first standard should be injected a minimum of three times initially to recondition the column.

Load samples into the rack, placing a standard in every 10th position to verify the calibration and the quality of the chromatography.

### 10.5 Calculations

**10.5.1** Generate an internal standard calibration curve from the area response of the standard solutions. These calibration factors are used to calculate the concentration of each analyte in each sample. By entering the correct multiplier (overall volume the original sample is diluted to in mL - one) and divisor (the original sample weight in g to fourth decimal place) the concentration of each of the alkaloids is automatically calculated in µg/g.

**Analytical Result (on a "dry matter" basis):**

Analyte [µg/g] = ($Area_{Analyte\ Sample}$ / $Area_{ISTD\ Sample}$) X $RF_{\mu g/mL}$ X (Multiplier $_{mL}$ / Divisor $_{g}$).

where the Response Factor (RF) is defined from the calibration.

**10.5.2** All results are expressed on a "dry matter" basis. These may be expressed on an "as received" basis using the appropriate moisture result.

**Conversion to an "as received" basis**

Analyte $[\mu g/g]_{\text{"as received"}}$ = Analyte $[\mu g/g]_{\text{"dry matter"}}$ X (1- (% Moisture / 100)).

where the % Moisture is determined by freeze drying in Preparation of Samples for Analysis - Determination of Moisture Content.

## 11 QUALITY CONTROL

### 11.1 Typical Chromatogram

**See Appendix 1- Figures 1 & 2.**

### 11.2 Recoveries and Levels of Contamination

**11.2.1** Each set for analysis should contain one laboratory reagent blank (LRB) per batch of up to 20 samples. LRBs may contain very low levels of nicotine but are often "not detected". Usually contamination can be minimized by cleaning glassware thoroughly and by using previously unopened solvent bottles for making up solutions.

*Note:* In addition to the LRB, a solvent blank should be injected after every $10^{th}$ sample to evaluate carry over. If carry over is increasing throughout the sample set, the injection liner and/or septum should be replaced.

### 11.3 Method Detection Limit (MDL)/Limit of Quantitation (LOQ)

**11.3.1** The MDL and LOQ for nicotine are not applicable (N/A) due to the very high levels this method is designed to analyze.

**11.3.2** The MDLs and LOQs for the minor alkaloids will be different depending on the detector and column conditions and should be evaluated any time minor alkaloids are required. A sample containing low levels of these alkaloids should be injected 10 times and the standard deviation of each alkaloid determined. The MDL is three times this standard deviation and the LOQ is 10 times this standard deviation.

**11.3.3** Alternately, the MDL can be determined as a peak which gives a signal to noise ratio (S/N) of 3:1. The LOQ can be determined as a peak which gives a S/N of 10:1.

### 11.4 Stability of Reagents and Samples

**11.4.1** The CAM column has a polyethylene glycol stationary phase that has been base-deactivated for volatile amine analysis. Tailing, however, may affect quantitation of the minor alkaloids because of their smaller quantity, and therefore, smaller signal-to-noise ratio. These tailing effects are caused by two sources other than reactivity of the compounds:

1. solvent effect.
2. reactivity in the injector.

**11.4.2** Methanol is generally a poor solvent choice for injecting onto the GC because it causes an enormous amount of tailing. This effect can be minimized by using a large split ratio, a high linear velocity, a thick stationary phase, and de-activated glass injection liners.

**11.4.3** Reactivity in the injector is minimized by using de-activated glass inserts. It is necessary that the injection liner be changed between each set of samples (roughly 40 true samples) since the injected solution is quite dirty and creates active sites on the liner after repeated injections of sample.

**11.4.4** A Quality Control Standard (QCS) can be prepared and analyzed as a sample. This is a standard prepared from an independent stock and analyzed as an unknown to determine the accuracy of the calibration.

**11.4.5** The extraction solution can be used indefinitely as long as it does not become contaminated with nicotine.

**11.4.6** There is no indication of a problem with the stability of either samples or standards if kept at 4 °C.

**11.4.7** It is very important that the sonication process is closely monitored. The pieces of tobacco should be visibly vibrating and the solution should be homogenous in colour at the end of the sonication.

## 12 REFERENCES

**12.1** R.F. Severson, K.L., McDuffie, R.U. Arrendale, G.R. Gwynn, J.F. Chaplin, and A.W. Johnson. *J. Chromatog Rapid Method for the Analysis of Tobacco Nicotine Alkal*oids, 1981, p. 211, p.111-121.

**APPENDICES**

**Appendix 1 : Chromatographic Examples**

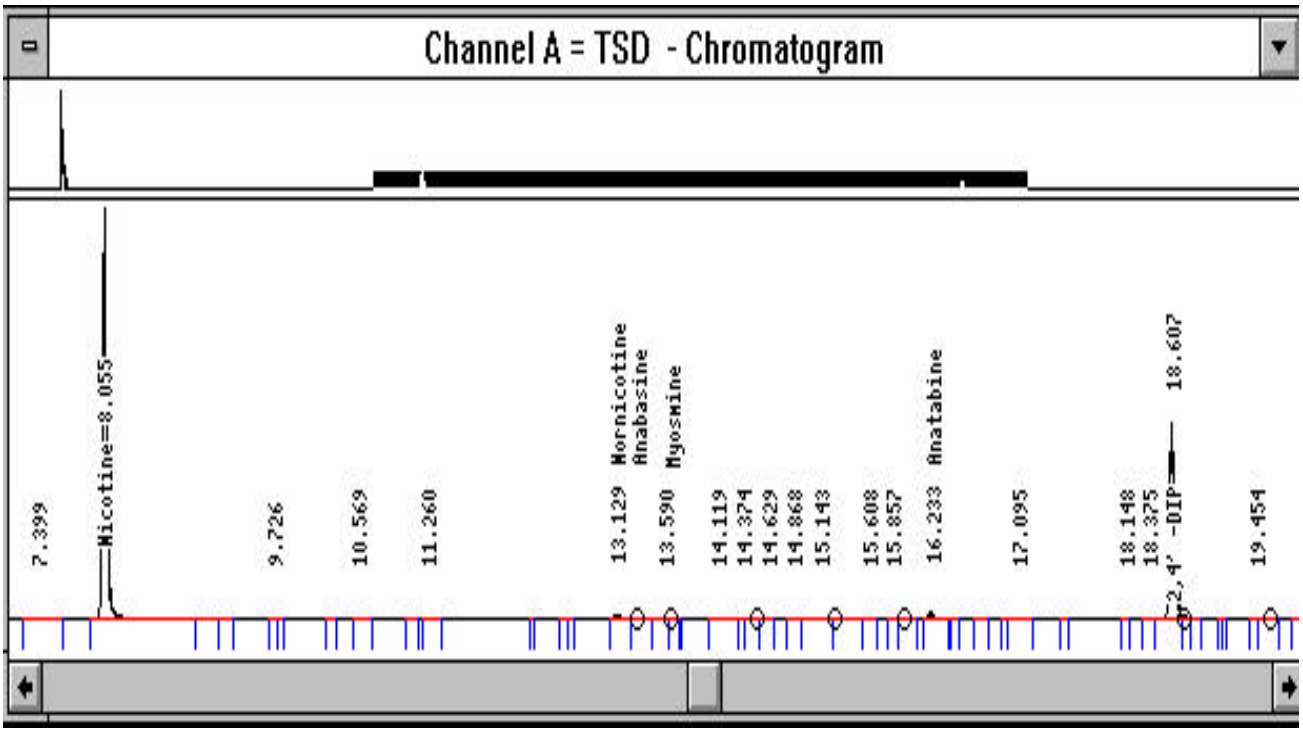


**Figure 1.** Example Chromatogram of a true tobacco sample showing the nicotine concentration elative to that of the minor alkaloids. Retention times are as follows:

Nicotine : 8.055 minutes.
Nornicotine : 13.129 minutes.
Anabasine: 13.307 minutes.
Myosmine : 13.622 minutes.
Anatabine : 16.233 minutes.
2,4-Dipyridyl (ISTD): 18.607.

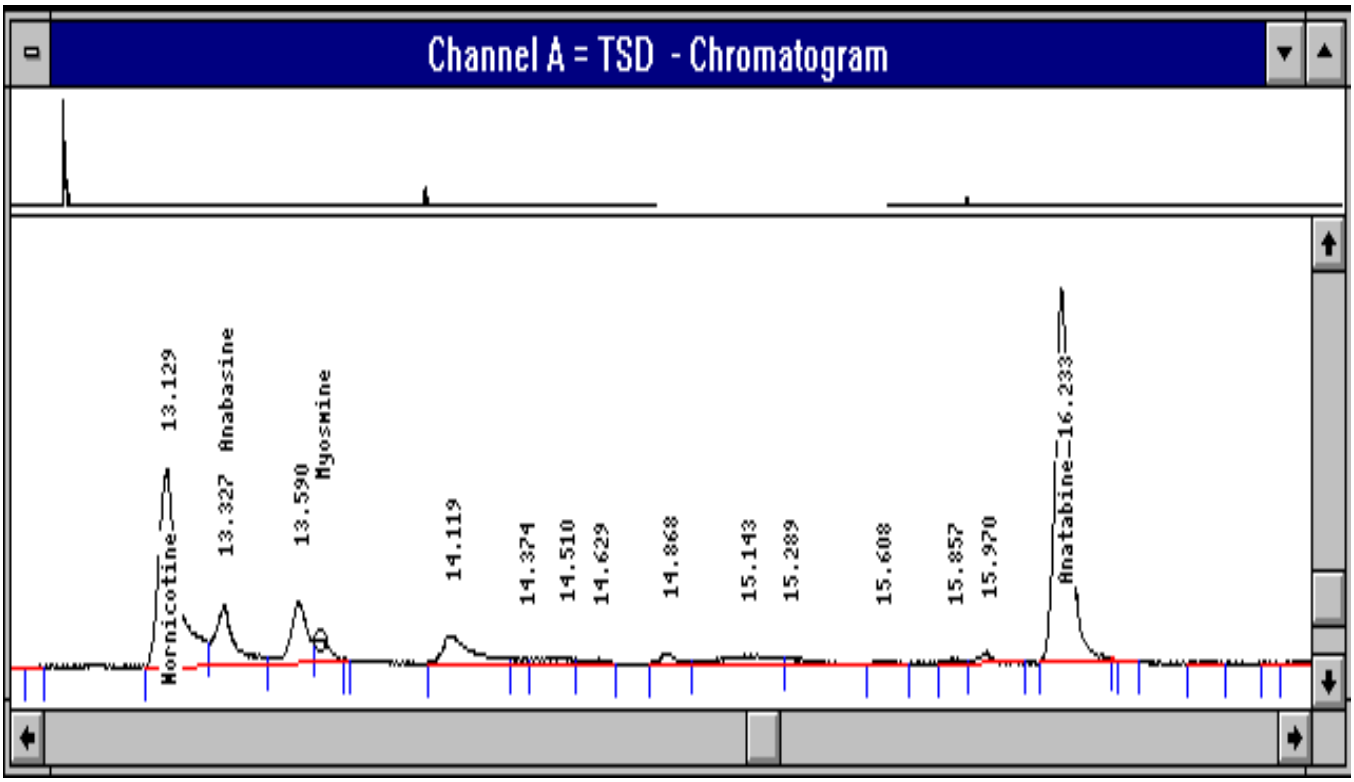


**Figure 2:** An expanded view of the minor alkaloid components of Figure 1.

*Note:* The amount of tailing present in Figure 2 is the maximum allowable. The primary indicators for requiring the analytical column to be cut (or replaced) is the resolution of the myosmine and the resolution of anabasine from nornicotine.