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1 SCOPE OF APPLICATIONS

1.1 Applicable to the quantitation of Benzo[a]pyrene (B[a]P) content in whole tobacco and snuff by high performance liquid chromatography (HPLC)/ultra-violet (UV) detection.

2 NORMATIVE REFERENCES

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

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

2.3 Health Canada Test Method T-402 – Preparation of Cigarettes, Cigarette Tobacco, Cigars, Kreteks, Bidis, Packaged Leaf, Pipe and Smokeless Tobaccos for Testing, 1999-12-31.

3 METHOD SUMMARY

3.1 This method is a modification to AOAC Method 973.30, Polycyclic Aromatic Hydrocarbons and Benzo[a]pyrene in Food - Spectrophotometric Method. This consists of the extraction of the sample and the initial purification stages of partitioning and florisil treatment prior to HPLC analysis. Sample size (and subsequent reagent volumes) are adjusted to meet the analytical requirements of the sample as well as the availability of the sample and equipment.

3.2 Polycyclic aromatic hydrocarbons are extracted from a sample of tobacco after a saponification with an alcoholic KOH solution and partitioning into iso-octane. The solvent is then completely evaporated and the sample is reconstituted in 2 mL of iso-octane. This reconstituted solution is passed through a 1 g (6 mL) florisil cartridge and washed with 15 mL of iso-octane. The B[a]P is eluted using 15 mL benzene, the solvent is evaporated to dryness, and the sample is reconstituted in 2 mL volume of acetonitrile. The sample is subjected to reversed phase liquid chromatography and quantitated *via* fluorescence detection.

3.3 Some matrices may be too complex to achieve accurate quantitation using this solid phase extraction clean-up procedure. These types of samples may require an HPLC column clean-up using normal phase chromatography on a silica column where the B[a]P fraction may be collected. This fraction may then be concentrated and subject to reversed phase HPLC.

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.

4 APPARATUS AND EQUIPMENT

4.1 Supelco Visi-Prep Solid Phase Extraction (SPE) unit (24 cartridge unit) or equivalent.
4.2 Condensor - Friedrich-type.
4.3 Argon cylinder with manifold for purging flasks during saponification.
4.4 Glass Pipettes (2, 20, 40, 50 mL).
4.5 Micro-pipette (1000 mL).
4.6 16 X 125 mm culture tubes (20 mL).
4.7 250 mL round bottom flasks.
4.8 1000 mL separatory funnels.
4.9 Zymark TurboVap Concentrator or equivalent.
4.10 Buchi Rotovap or equivalent.
4.11 Balance, capable of measuring to four decimal places.
4.12 Pasteur Pipettes.
4.13 0.45 µm nylon filter.
4.14 1 g florisil cartridges (6 mL capacity).
4.15 2 mL screw-cap autosampler vials with Teflon-lined caps.
4.16 High Performance Liquid Chromatograph consisting of:

4.16.1 Autosampler.
4.16.2 Tertiary pump.
4.16.3 Fluorescence Detector.
4.16.4 Data collection system.
4.16.5 Merck 250 X 4 mm, RP-18e, 5 µm packing, HPLC column.
4.16.6 Lichrocart 4-4 Lichrosphere 100 RP-18 endcapped, 5 µm guard column.

5 REAGENTS AND SUPPLIES

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

5.1 Benzo[a]pyrene (B[a]P).
5.2 2,2,4-trimethylpentane.
5.3 Benzene.
5.4 Acetonitrile.
5.5 Potassium Hyroxide (KOH) – 50 % (w/v).
5.6 Anhydrous sodium sulphate.
5.7 Argon – UHP.
5.8 Reagent Alcohol.
5.9 Type I water (as per ASTM specifications).
5.10 Sodium Chloride (NaCl) – saturated solution.
5.11 Isopropanol (IPA).
5.12 Tetrahydrofuran (THF).

6 PREPARATION OF GLASSWARE

6.1 Glassware should be cleaned and dried in such a manner to ensure that contamination from glassware does not occur.

7 PREPARATION OF SOLUTIONS AND STANDARDS

7.1 Preparation of Working Standards and Spike Solutions

7.1.1 Primary (1°) B[a]P Stock: Dissolve 10 mg B[a]P to 50 mL Acetonitrile.

7.1.2 Secondary(2°) Stock: Pipette 100 µL of 1° Stock to 50 mL Acetonitrile.

7.1.3 Working Standards:

Standard #	Vol.of 2° Standard (µL)	Final Volume (mL)	Concentration [ng/mL]
1	40	25	0.6400
2	175	25	2.800
3	350	25	5.600
4	600	25	9.600
5	900	25	14.40
6	2mL of Std 1	10	0.1280
7	4mL of Std 1	10	0.2560

Note: All weights, volumes, and purity must be recorded and used to accurately calculate the standard concentrations. These concentrations are only representations of standards used in a calibration curve**.**

8 SAMPLING

8.1 The sampling of tobacco products for the purpose of testing shall be as specified in T-115.

9 SAMPLE PREPARATION

9.1 Whole tobacco shall be prepared for testing as specified in T-402.

10 SAMPLE PROCESSING

10.1 Extraction - Base Saponification

10.1.1 Accurately weigh 2 g of prepared tobacco sample into a 250 mL round bottom flask.
10.1.2 Add 60 mL of reagent alcohol.
10.1.3 Add 4.5 mL of KOH solution.
10.1.4 Purge the round bottom flask with argon.
10.1.5 Set up refluxing apparatus such that the apparatus is continually purged with argon at a slow rate.
10.1.6 Reflux for two hours at a rapid rate.

Note: To prevent foaming, gradually increase the heat after refluxing at a relatively slow rate for five to 10 minutes.

10.1.7 Let cool down to room temperature while continuing to purge with argon.
10.1.8 Stopper the flask and store in the dark until ready for partitioning.

10.2 Extraction - Partitioning

10.2.1 Transfer the content of the round bottom flask into a 1000 mL separatory funnel (funnel I).

Note: Some solid is allowed to get into the separatory funnel, however, this can make it very difficult to draw off the bottom layer due to clogging of the stopcock.

10.2.2 Wash the flask with 2 X 20 mL portions of type I water and transfer into the separatory funnel (I).

10.2.3 Wash the flask with 2 X 15 mL portions of reagent alcohol and transfer into the separatory funnel (I).

10.2.4 Wash the flask with a 25 mL portion of iso-octane and transfer into the separatory funnel (I).

10.2.5 Shake the separatory funnel for three minutes and allow the layers to separate.

10.2.6 Carefully drain the lower layer into a second separatory funnel (II).

10.2.7 Repeat the above extraction by adding 20 mL of iso-octane to the round bottom flask and transfer into the separatory funnel (II).

10.2.8 Shake the separatory funnel for three minutes and allow the layers to separate (II).

10.2.9 Carefully drain the lower layer into a third separatory funnel (III).

10.2.10 Repeat the above extraction by adding 20 mL of iso-octane to the round bottom flask and transfer into the separatory funnel (III).

10.2.11 Shake the separatory funnel (III) for three minutes and allow the layers to separate.

10.2.12 Carefully drain the lower layer to waste and discard.

10.2.13 Wash each iso-octane layer (separatory funnels I, II, III) three times with a solution containing 50 mL of warm Type I water and 500 µL saturated NaCl.

Note: Use a gentle swirling motion for washing. Vigorous shaking will result in a “soapy” aqueous phase that will not separate properly, resulting in low recoveries.

10.2.14 Discard the aqueous layer (lower layer) after each washing.

10.2.15 After washing is complete, drain each of the iso-octane layers through a funnel containing anhydrous sodium sulphate into a 250 mL round bottom flask (drain in the order I, II, then III) combining the iso-octane fractions in the same flask.

10.2.16 Rinse the third separatory funnel (III) with 25 mL of fresh iso-octane, and shake.

10.2.17 Transfer the iso-octane into the second separatory funnel (II), and shake.

10.2.18 Transfer the iso-octane into the first separatory funnel (I), and shake.

10.2.19 Transfer the iso-octane of the first separatory funnel (I), combining with the previous iso-octane fractions in the same flask.

10.2.20 Repeat this rinsing process with 25 mL of fresh iso-octane starting in the third separatory funnel (III) once again.

10.2.21 Wash the anhydrous sodium sulphate with an additional 2 X 5 mL portion of iso-octane.

10.2.22 Evaporate the iso-octane solution in the 250 mL round bottom flask on the rotary evaporator to dryness at approximately 55 ^{o}C.

10.2.23 Reconstitute the sample with 2 mL of iso-octane.

10.3 Sample Clean-up

10.3.1 Condition florisil cartridge by adding approximately 1g of anhydrous sodium sulphate to the cartridge and then by washing the column with 2 X 5 mL additions of iso-octane (allow to drip *via* gravity at the rate of approximately 1 drop/second).

10.3.2 Add the entire reconstituted sample (in 2 mL iso-octane) to the cartridge allowing the eluant to gravimetrically pass through the cartridge to waste.

10.3.3 Wash the flask with 3 X 5 mL additions of iso-octane transferring each fraction to the cartridge, allowing the eluant to gravimetrically pass through the cartridge to waste.

10.3.4 Place 20 mL disposable glass culture tubes beneath each of the cartridges.

10.3.5 Gravimetrically elute the B[a]P from the cartridges with 3 X 5 mL additions of benzene.

10.3.6 Add 1 mL of THF to each tube.

10.3.7 Place the tubes containing the 16 mL of collected eluant into the Zymark TurboVap.

Note: TurboVap conditions are to be set at 40 °C with a Nitrogen pressure of 7.5 psi.

10.3.8 Evaporate the samples to complete dryness.

*Note:*This will require an initial 30 minutes of evaporation in which the nitrogen pressure may be slowly increase to a maximum of 10.0 psi. in a manner as to prevent any loss of sample from splattering.

10.3.9 Pipette 1000 µL of acetonitrile into each of the dried tubes to dissolve the analyte and any residue that may be present.

10.3.10 Vortex the sample at high speed for approximately 15 seconds.

10.3.11 Using a glass transfer pipette, wash down the sides of the tube five times with the sample, and transfer to a 2 mL volumetric flask

10.3.12 Wash the tube with an additional 500 uL acetonitrile as previous and transfer to the 2 mL volumetric flask

10.3.13 Make to volume with acetonitrile

10.3.14 Transfer the sample to a 2 mL autosampler vial with a screw cap and Teflon-faced septa.

10.3.15 The samples are ready for HPLC analysis and may be stored at 4 °C until they are analyzed.

11 REVERSED PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS

11.1 Jasco Fluorescence Detector Conditions

Excitation Wavelength:	365 nm.
Emission Wavelength:	425 nm.
Gain:	X 1000.
Attenuation:	32.

Note: A different manufacturer's fluorescence detector may need to be programmed differently to maintain the full calibration range. A slight change in excitation and emission wavelength may be required dependent on manufacturer (i.e. 366 and 424 for the wavelengths).

11.2 Autosampler : Injection Volume

11.2.1 Analyze using a 50 µL sample loop and set the parameter for injection volume at 75 µL to ensure a thorough flushing of the sample loop with the sample.

11.3 Mobile Phase / Gradient Conditions (Tertiary Gradient System)

11.3.1 Solvent A: 55:45 Acetonitrile/1% IPA in Type I water (degassed and filtered using 0.45 µm nylon filter).
11.3.2 Solvent B: Methanol.
11.3.3 Solvent C: Acetonitrile.
11.3.4 Flow: 1.5 mL/minute.
11.3.5 Gradient: Adjustments to the gradient may be required depending on column conditions and resolution of analyte.

Time		Composition	
(minute)	% A	% B	% C
0.00	55	0	45
20.00	75	0	25

25.00	100	0	0
28.00	100	0	0
30.00	0	100	0
32.00	0	100	0
34.00	100	0	0
35.00	100	0	0
35.00	Method End Action:		Equilibrate

Equilibration Time: 8.00 minutes.

12 CALCULATIONS

12.1 Determination of Response Factor

12.1.1 An initial calibration is performed by running prepared standards from high concentration to low concentration (injecting the very first standard a minimum of two times until the response and retention time are constant).

12.1.2 A calibration curve is prepared by plotting the concentration of B[a]P in the standard vs. the peak height response from the fluorescence detector.

12.1.3 The Response factor (RF) is the slope of the line as determined by linear regression (Height counts / unit concentration).

12.2 Determination of B[a]P Content [ng/g]

12.2.1 $$\text{B[a]P [ng/g]} = \frac{\text{Peak Height X Final Volume(mL)}}{\text{RF X Sample Weight (g)}}.$$

Note: Additional dilutions must be accounted for if they were used.

13 QUALITY CONTROL

13.1 Typical Chromatogram

13.1.1 Not available.

13.2 Typical Control Parameters

13.2.1 Each set analysis should contain at least one of each of the following per group of extractions:

13.2.1.1 Laboratory Reagent Blank (LRB): to determine background contamination from solutions, glassware, or materials used in the analysis process.

13.2.1.2 Laboratory Fortified Blank (LFB): to determine whether there is any loss of analyte as a result of the analysis process.

13.2.1.3 Laboratory Fortified Matrix (LFM) by spiking on of the control brand cigarettes: to determine whether there is any loss of analyte as a result of the analysis process and to determine potential matrix effects.

13.2.1.4 A Reference Sample (minimum of two per run of 20): to determine the inter-experimental reproducibility of the entire method of analysis

13.2.1.5 Duplicate Samples: to determine the reproducibility of the procedure within the same experiment or batch on analysis.

13.3 Recoveries and Levels of Contamination

13.3.1 Typical recoveries of Laboratory Fortified Blanks (LRB) and Laboratory Fortified Matrix (LFM) samples range from 75 - 95 % when a spiked solution (or sample) is carried out through the entire extraction process.

13.3.2 Recoveries lower than 65 % indicate either an insufficient elution of B[a]P from the solid phase extraction cartridges or a change in response factor (RF) of the fluorescence detector. A change in RF must first be investigated before re-processing of samples is initiated.

13.3.3 Typical Laboratory Reagent Blanks (LRB) range between 0-0.3 ng/g. Contamination of this type is usually associated with an inadequate cleaning of glassware.

13.4 Method Detection Limits (MDL) / Limit of Quantitation (LOQ)

13.4.1 The method detection limit (MDL) is determined by analyzing the lowest standard level a minimum of 10 times as an unknown over several days. The MDL is calculated as three times the standard deviation of these determinations.

13.4.2 The MDL (on a ng/g basis) can be manipulated by modifying the amount of tobacco extracted and the volumes used for extraction and clean-up in the procedure.

13.4.3 The practical limit of quantitation (LOQ) is determined by analyzing the lowest standard level a minimum of 10 times as an unknown over several days. The LOQ is calculated as 10 times the standard deviation of these determinations.

13.5 Stability of Reagents and Samples

13.5.1 Storage of analytical stocks and standard should be at –20 °C.

13.5.2 Stock standards and stock spike solutions remain stable for up to six months. Although there is no loss of analyte, evaporation (loss) of solvent may be an issue.

13.5.3 Working standards should be freshly prepared every two months.

13.5.4 Samples are stable at 4 °C for three weeks after extraction.

14 REFERENCES

14.1 AOAC Method 973.30, Polycyclic Aromatic Hydrocarbons and Benzo[a]pyrene in Food - Spectrophotometric Method, *AOAC 1995*, Volume II, 48.1.01. p. 1176-1178.

14.2 Tomkins, B.A.; Jenkins, R.A.; Griest, W.H.; Reagen, R.R. Liquid Chromatographic Determination of Benzo[a]pyrene in Total Particulate Matter of Cigarette Smoke. *J. Assoc. Off. Anal. Chem*., Vol. 68, 5, 1985. p. 935-940.