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WITHDRAWAL

October 2016

Standards in series Tobacco

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Octobre 2016

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CAN/CGSB-176.1-92

Preparation of cigarettes from cigarette tobacco for testing (ICS 65.160)

CAN/CGSB-176.2 No.1-96

Methods of sampling and testing tobacco
Determination of Benzo(α)pyrene in total particulate matter of tobacco smoke (ICS 65.160)

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CAN/CGSB-176.1-92

Préparation des cigarettes à l'aide de tabac aux fins d'essais (ICS 65.160)

CAN/CGSB-176.2 N° 1-96

Méthodes d'échantillonnage et d'essai du tabac
Détermination du benzo(α)-pyrène dans la matière particulaire totale de la fumée de tabac (ICS 65.160)

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CAN/CGSB-176.2

No. 1-96

Methods of Sampling and Testing Tobacco

**Determination of Benzo[*a*]pyrene in Total Particulate Matter of
Tobacco Smoke**

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National Standard of Canada

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**METHODS OF SAMPLING
AND TESTING TOBACCO**

**DETERMINATION OF BENZO[a]PYRENE IN
TOTAL PARTICULATE MATTER OF TOBACCO SMOKE**

Prepared by the
Canadian General Standards Board 

Approved by the
Standards Council of Canada 

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Acknowledgment is made for the translation of this National Standard of Canada by the Translation Bureau of the Secretary of State.

Preface to the National Standard of Canada

This National Standard of Canada has been developed by the Canadian General Standards Board (CGSB) Committee on Tobacco Test Methods on behalf of Health Canada.

Withdrawn/Retirée

 Ottawa Canada K1A 1G6	METHODS OF SAMPLING AND TESTING TOBACCO	CAN/CGSB-176.2
	Determination of Benzo[a]pyrene in Total Particulate Matter of Tobacco Smoke	No. 1-96

1. SCOPE

- 1.1 This method is used to isolate and to determine the benzo[a]pyrene (B[a]P) content in total particulate matter (TPM) of tobacco smoke.
- 1.2 The testing and evaluation of a product against this method may require the use of materials and/or equipment that could be hazardous. This document does not purport to address all the safety aspects associated with its use. Anyone using this method has the responsibility to consult the appropriate authorities and to establish appropriate health and safety practices in conjunction with any existing applicable regulatory requirements prior to its use.

2. PRINCIPLE

- 2.1 The total particulate matter is collected on a tared Cambridge filter pad spiked with ^{14}C -B[a]P and then extracted with acetone by successive sonication and centrifuging. The crude extract is dried, taken up in tetrahydrofuran and hexane and then filtered through a silica gel cartridge. The solution from the cartridge is dried, taken up in 1 mL of hexane, and a 250 μL aliquot is fractionated using preparatory normal phase liquid chromatography. Radioactive fractions are collected, pooled and dried under nitrogen.
- 2.2 The residue is then dissolved in acetonitrile and subjected to reverse phase liquid chromatography. Quantification of the B[a]P is based on the detection of fluorescence and the recovered radioactivity.

3. REFERENCED PUBLICATIONS

- 3.1 The following publications are referenced in this method:
- 3.1.1 American Society for Testing and Materials (ASTM)
D 1193 — Standard Specification for Reagent Water.
- 3.1.2 International Organization for Standardization (ISO)
ISO 2602 — Statistical interpretation of test results — Estimation of the mean confidence interval
ISO 3308:1986 — Cigarettes — Routine analytical cigarette — Smoking machine definitions and standard conditions
ISO 3402 — Tobacco and tobacco products — Atmospheres for conditioning and testing
ISO 3534 — Statistics — Vocabulary and symbols
ISO 4387 — Cigarettes — Determination of total and dry particulate matter using a routine analytical cigarette-smoking machine — Glass fibre filter smoke trap method
ISO 8243 — Cigarettes — Sampling.
- 3.2 A reference to a regulation is always to the latest issue. A dated reference is to the issue specified. An undated reference is to the latest issue, unless otherwise specified by the authority applying this method. The sources are given in the Notes section.

4. TERMINOLOGY

- 4.1 The following definition applies in this method:

Total Particulate Matter (TPM): that fraction of tobacco smoke that is retained by a Cambridge glass fibre filter that is a least 99.9% efficient for particles $>0.1\ \mu\text{m}$.

5. SAMPLE PREPARATION AND CHARACTERIZATION

5.1 Cigarettes

- 5.1.1 When precise results are essential, the criteria for the rejection of cigarettes must be clearly defined, and records must be kept of the proportion rejected. It is always useful to determine the mass of the cigarettes before the smoking process is initiated. The relationship between smoke yield and cigarette mass is not straightforward, but if large variations in the mass are found, these can sometimes be corrected by special statistical analysis. Since the purpose of this method is to estimate the B[a]P content in mainstream smoke of commercially available cigarettes, selection based on the mass is not considered critical. The resulting increased variability in yields of B[a]P better reflects the true characteristics of the product.
- 5.1.2 The sorting of cigarettes by mass can often produce a sample that is not a representative of the population. For example, cigarettes are made to a mass specification, but a particular batch will often have a mean mass that differs enough from the specification to affect the test results. To sort a sample by mass on the basis of a bulk mass determination, rather than of a mass specification, can therefore give a test sample that is not representative of the original population.

5.2 Smoking

- 5.2.1 For all comparative testing, the conditioning of the cigarettes before testing shall be carried out at $60 \pm 2\%$ RH and $21 \pm 1^\circ\text{C}$ for at least 48 h, in accordance with ISO 3402. The cigarettes shall be exposed to the conditioning environment in a single layer.
- 5.2.2 The smoking preparation, the Cambridge filter assembly and the smoking machine operation shall be carried out in accordance with ISO 3308:1986, noting that:
- Most smoking machines have a number of smoking ports, and however carefully the machine is set up, there will be differences between these ports. There are also differences in results from one smoking run to another, usually much larger than those between ports in one smoking run.
 - It is simpler to smoke a different type of cigarette in each port of the machine, randomly changing the position from one run to the next so that each type of cigarette is tested more or less equally on each port. The standard deviation will then include port to port and run to run variations, and valid confidence limits can be calculated.
 - It is important to ensure that 70 to 100 mg of TPM have been deposited on the Cambridge filter pad before proceeding with the analysis. This can readily be accomplished if the TPM yield of the cigarette has previously been determined. If this is not the case, then five cigarettes should be smoked per Cambridge pad for most cigarettes. Low-yield cigarettes may require the smoking of ten or more cigarettes.

5.3 Total Particulate Matter

- 5.3.1 The TPM shall be determined in accordance with ISO 4387. Data from the TPM determination shall be used to characterize the samples and to monitor the smoking process.

6. TESTING

6.1 Glassware Preparation

- 6.1.1 Soak all glassware overnight in a 5% solution of Decon® cleaning solution and deionized water. After soaking, rinse all glassware three times with tap water, three times with deionized water, and twice with methanol (DIG). Dry in an oven at 160°C .

6.2 Extraction of TPM

6.2.1 Reagents

B[a]P Tracer (Note¹) — 50 μCi of Carbon-14-labelled [17,10-¹⁴C] B[a]P is diluted to 50 mL with acetone. This stock solution is diluted 5-fold in acetone to obtain a spiking solution of 0.2 $\mu\text{Ci/mL}$. At an activity of 205 $\mu\text{Ci/mg}$,

¹ A 30 to 50 $\mu\text{Ci/mmol}$ B[a]P Tracer is available from Amersham Corp.

the B[a]P concentration is 0.9756 µg/mL. The solution is stored in a series of 2 mL autosampler vials with teflon-lined caps, and the vials are stored in a vial box at 4°C. A fresh vial is used for each set of samples.

Acetone — Distilled-in-glass (DIG) grade or equivalent.

Tetrahydrofuran, (THF) — Distilled-in-glass (DIG) grade or equivalent.

Hexane — Distilled-in-glass (DIG) grade or equivalent.

6.2.2 Apparatus and Materials

Centrifuge — Capable of operating at a relative centrifugal force of 210 G.

Glass centrifuge tubes — Capacity of 20 mL, with teflon-lined caps.

Sonicator — Branson Model 3200 or equivalent.

Round-bottomed flasks — With capacities of 500 mL and 250 mL.

Rotary evaporator — Büchi type rotary evaporator or equivalent.

Silica Sep-Paks (Note²) — 1 g packing.

Disposable syringes — Capacity 5 mL.

Pasteur pipettes.

Rubber bulbs.

"Autosampler" vials — 20 × 10 mm, screw-capped type, with teflon-lined caps.

6.2.3 Procedure

- 6.2.3.1 After the smoking run is completed (Note³), determine the mass of the Cambridge filter pads for use in the TPM determination. Quarter the pads and place in a 20 mL centrifuge tube using a clean pair of tweezers.
- 6.2.3.2 Add 100 µL of B[a]P tracer solution to each centrifuge tube. The two control samples are not spiked.
- 6.2.3.3 Add exactly 16 mL of acetone to the tube.
- 6.2.3.4 Cap the tube. Sonicate for 30 min in a 22°C water bath; then centrifuge for 5 min at 210 G.
- 6.2.3.5 At this stage, and if required, a 20 µL aliquot of the acetone extract can be taken for nicotine analysis (Note⁴). Transfer the remaining acetone solution to a 500 mL round-bottomed flask using a pasteur pipette.
- 6.2.3.6 Add approximately 12 mL of acetone to the tube. Shake by hand for 1 min, then centrifuge 5 min at 210 G. Transfer to the round-bottomed flask using the same pasteur pipette.
- 6.2.3.7 Repeat par. 6.2.3.6 four more times.
- 6.2.3.8 Remove the acetone from the flask by rotary-evaporation in a water bath maintained at 40°C.
- 6.2.3.9 Prepare the Sep-Pak by attaching a 5 mL syringe to one end and forcing 5 mL of hexane through it into a beaker, ensuring that air is not pushed through the Sep-Pak. Pull back 5 mL of hexane into the syringe. Continue this step until the silica is well saturated, with no evidence of air pockets. Mount the syringe and cartridge above a 250 mL round-bottomed flask.
- 6.2.3.10 Add 2 mL of THF to the 500 mL round-bottomed flask (par. 6.2.3.8) and dissolve the tar residue.
- 6.2.3.11 Swirl the 500 mL flask while adding 30 mL of hexane. A sticky, tar-like precipitate will form and coat the side of the flask.

² Available from Waters Chromatography, Division of Millipore.

³ One smoking machine run consists of 18 samples and 2 controls. A working group consists of 5 samples.

⁴ ISO 10315 — Cigarettes — Determination of Nicotine in Smoke Condensates — Gas Chromatographic Procedure.

- 6.2.3.12 Using a pasteur pipette, transfer approximately 5 mL of hexane from this flask to the 5 mL syringe. Allow the hexane to filter by gravity into the 250 mL round-bottomed flask. The level of the hexane solution in the syringe is maintained at about 5 mL until all the hexane has been transferred.
- 6.2.3.13 When the hexane transfer is completed, rinse the 500 mL flask twice with 5 mL of hexane, which is transferred to the syringe.
- 6.2.3.14 After all of the hexane has passed through the Sep-Pak into the 250 mL round-bottomed flask, remove the hexane by rotary evaporation in a water bath maintained at 40°C.
- 6.2.3.15 Dissolve the residue in approximately 2 mL of hexane and transfer to a 2 mL "autosampler" vial.
- 6.2.3.16 Reduce the content (volume) of the vial under a gentle stream of nitrogen in a heating block maintained at 40°C.
- 6.2.3.17 As the hexane in the vial evaporates, rinse the 250 mL flask twice with 2 mL of hexane which is transferred to the vial.
- 6.2.3.18 Continue evaporating the hexane from the vial until approximately 1 mL remains. Cap the vial, store at 4°C and protect from light until ready for isolation by HPLC.

6.3 Preparatory Liquid Chromatography (Normal Phase)

6.3.1 Reagents

Hexane — Distilled-in-glass (DIG) grade or equivalent.

Dichloromethane (DCM) — Distilled-in-glass (DIG) grade or equivalent.

Acetonitrile — Distilled-in-glass (DIG) grade or equivalent.

6.3.2 Apparatus and Materials

High-performance liquid chromatography system (HPLC) — Varian Star High Pressure Liquid Chromatography system with MS Windows-based software capable of complete instrument control, data handling, integration, quantitation and custom reporting or equivalent.

Ternary gradient pump.

Autosampler — With 50 and 250 µL sample loops.

UV-VIS detector — Variable wavelength UV-VIS detector.

Fluorescence detector — JASCO programmable scanning fluorescence detector, using an excitation wavelength of 365 nm and an emission wavelength of 425 nm or equivalent.

Radioactivity monitor — Berthold radioactivity monitor with YG-600 U4 cell or equivalent.

Fraction collector — Foxy II programmable fraction collector or equivalent.

HPLC column — Merck 250 mm long × 10 mm inside diameter column, packed with 10 µm particle size LiChrospher Si 60 or equivalent.

6.3.3 Procedure

- 6.3.3.1 Gradient Elution Pattern (Note⁵) is as follows: 100% hexane (flow rate of 3.5 mL/min) to 90:10 hexane:DCM at 20 min, to 50:50 hexane:DCM at 30 min, to 100% DCM at 32 min, hold 5 min, to 100% acetonitrile (flow rate of 5.0 mL/min) at 40 min, hold 5 min, to 100% DCM at 50 min, to 100% hexane at 55 min, then equilibrate for 10 min.
- 6.3.3.2 Inject 250 µL of the final extract (par. 6.2.3.18) into the HPLC. One run of 20 samples is analyzed at a time.
- 6.3.3.3 Collect two minute fractions (7 mL) from 20 to 30 min. The B[a]P is isolated by observing the response on the radioactivity monitor. Fractions containing the labelled B[a]P (normally 3 fractions around 25 min) are pooled.

⁵ Because the types and condition of the column and equipment may vary, it is necessary to optimize the conditions to separate the B[a]P as well as possible from the large amount of early elution apolar compounds. The elution pattern should be similar to that given in Figure 1.

- 6.3.3.4 Using a pasteur pipette, transfer the fractions into a 250 mL round-bottomed flask. Rinse the tubes twice with approximately 2 mL of hexane. Transfer the rinses to the flask. Reduce the content of the flask to approximately 1 mL by rotatory evaporation in a water bath maintained at 40°C.
- 6.3.3.5 Using a pasteur pipette, transfer the content of the round-bottomed flask into a 2 mL autosampler vial. Place the vial in a heating block maintained at 40°C and under a gentle stream of nitrogen. Rinse the round-bottomed flask twice with 2 mL of hexane. Transfer the rinses to the vial. Add 25 µL of the tracer solution to the two vials containing the two previously unspiked control samples (par. 6.2.3.2). Take the content of the vial to dryness.
- 6.3.3.6 Add 250 µL of acetonitrile to the vials. Cap the vials with a teflon-lined cap and store at 4°C. Protect from the light, until ready for Analytical Liquid Chromatography (LC).
- 6.4 **Analytical Liquid Chromatography (Reverse Phase)**
- 6.4.1 **Reagents**
- Acetonitrile* — Distilled-in-glass (DIG) or equivalent.
- Reagent Water* — Conforming to Type I of ASTM Specification D 1193.
- Primary B[a]P Stock Solution* (200 µg/mL) — Dissolve 10 ± 0.01 mg of B[a]P to 50 mL of acetonitrile.
- Secondary B[a]P Stock Solution* (5 µg/mL) — Dilute 250 µL of the above primary stock solution to 10 mL of acetonitrile.
- B[a]P Calibration Standards* — From the above stock solution, prepare in duplicate, three dilutions in the range of approximately 0.02 to 0.1 µg/mL. Store calibration standards in 2 mL autosampler vials with teflon-lined caps at 4°C, until ready for use (Note⁶).
- 6.4.2 **Apparatus and Materials**
- High-performance liquid chromatography system (HPLC)* — See par. 6.3.2.
- HPLC cartridge* — Merck 250 mm long × 4 mm inside diameter column packed with 3 µm particle size Superspher 100 RP-18 with a guard column 4 mm long × 4 mm inside diameter packed with a 5 µm Superspher 100 RP-18, or equivalent.
- 6.4.3 **Procedure**
- 6.4.3.1 Gradient Elution Pattern (Note⁷) is as follows: 40:60 water:acetonitrile at a flow rate of 1.5 mL/min, increase to 25:75 water:acetonitrile in a linear gradient over a period of 23 min, then to 100% acetonitrile (no gradient) for 5 min. Equilibrate under the initial conditions (40:60 water:acetonitrile) for another 10 min.
- 6.4.3.2 Inject 50 µL of the final extract (par. 6.2.3.18) into the HPLC.
- 6.4.3.3 Monitor the fluorescence response using an excitation wavelength of 365 nm and an emission wavelength of 425 nm.
- 6.4.3.4 Determine the recovery of the radio-labelled isotope using the Berthold Radioactivity monitor.
- 6.5 **Calculations**
- 6.5.1 **Calibration Curve**
- 6.5.1.1 Inject 50 µL of each calibration standard (in duplicate) into the HPLC with each set of 20 samples (giving a run of 26 vials).
- 6.5.1.2 Record the B[a]P fluorescent peak area for each sample and calibration standard in the run. Plot the curve of the peak area of the calibration standards against their concentration.

⁶ Each vial is used only once.

⁷ The Elution Pattern should be similar to that given in Figure 2.

6.5.2 Recovery Determination

6.5.2.1 Determine the area response of the ^{14}C -labelled B[a]P and average the value for the two controls.

6.5.2.2 Calculate the recovery for each sample by dividing the area response of the ^{14}C -labelled B[a]P by the average calculated in par. 6.5.2.1.

6.5.3 Calculate the B[a]P delivery as follows:

$$D = \frac{(1000x - tz)}{yz}$$

where:

D = delivery (ng/cig)

x = B[a]P obtained from standard curve ($\mu\text{g/mL}$)

y = number of cigarettes smoked

z = recovery of labelled B[a]P from the Monitor cigarette

t = amount of B[a]P tracer added (ng)

7. METHOD VALIDATION

7.1 Recovery Study

7.1.1 Five Canadian tobacco industry control cigarettes (Monitor 4) were smoked in each of ten randomly selected ports of a 20 port smoking linear machine and the mainstream tar collected on a tared Cambridge filter pad.

7.1.2 During the cleanup, 100 μL of the tracer solution was added to six of the samples at the following stages before HPLC isolation:

a. par. 6.2.3.2

b. before par. 6.2.3.8

c. before par. 6.2.3.10

d. par. 6.2.3.12

e. before par. 6.2.3.15

f. par. 6.2.3.18

25 μL of the tracer solution was added to two of the samples at the following stages after HPLC isolation and before HPLC quantitation.

g. before par. 6.3.3.4

h. par. 6.3.3.5

To determine 100% recovery, two of the samples were left unspiked until just before injecting (par. 6.4.3.2) into the analytical column.

7.1.3 The observed recovery in this study was $100.3 \pm 8.2\%$.

7.2 Validation

7.2.1 Five Kentucky Reference 2R1 cigarettes were smoked in ten random ports of a 20 port smoking machine and the TPM was collected on tared Cambridge filter pads.

7.2.2 All the filter pads were spiked with 100 μL of the tracer solution. Five of the filter pads were further spiked with 107.6 ng of unlabelled B[a]P.

7.2.3 The workup then proceeded as in par. 6.2.3.1 through 6.4.3.4.

- 7.2.4 The observed delivery of 31.9 ± 3.7 ng/cig from the unspiked 2R1 cigarette was in good agreement with the literature values of 28.4 ± 3.4 (Note⁸) and 32 ng/cig (Note⁹).

Gas Chromatograph/Mass Spectrophotometer (GC/MS) Confirmation

7.3.1 Apparatus and Reagents

Gas Chromatograph/Mass Spectrophotometer (GC/MS) — Varian 3400 Gas Chromatograph with the Saturn Ion Trap Mass spectral detector (Note¹⁰) and SPI injector with high performance insert and 8100 autosampler or equivalent.

Column — J & W 30 mm long $\times$ 0.25 mm inside diameter column with a 1.25 μ m DB-5 film thickness or equivalent.

Isooctane — Distilled-in-glass (DIG) or equivalent.

Poly aromatic hydrocarbons (PAHs) — Supelco PAH standard mix.

7.3.2 Operating Conditions

Injector Program — 90°C for 12 s; then increase to 310°C at a rate of 180°C/min.

Oven program — 105°C for 4 min; then increase to 210°C at a rate of 8°C/min.

Manifold temperature — 240°C.

Transfer line temperature — 300°C.

Helium carrier gas — Pressure of 103.4 kPa (15 psi).

Acquisition — From 60 to 600 amu.

7.3.3 Procedure

- 7.3.3.1 During one set of HPLC analytical runs, 2 min fractions were collected from 8 to 18 min (B[a]P retention time was about 13 min). All of the sample fractions of the same retention time were pooled (i.e. all fractions from 8 to 10 min, etc.) into a round-bottomed flask, and the solvent was evaporated. The standard fractions were pooled separately for comparison with the sample fractions.

- 7.3.3.2 The flasks were rinsed with acetonitrile, and the contents were transferred to a 2 mL autosampler vial and blown to dryness under nitrogen. 200 μ L of isooctane was added to each vial and 1 μ L injected into the column. The PAH standard mix was diluted 1:50 with isooctane and 1 μ L injected into the GC/MS to establish spectra and retention times for the PAHs.

7.3.4 Observations

- 7.3.4.1 B[a]P was positively confirmed in the fourth of both the sample and standard fractions. No other PAHs were observed in these fractions. Numerous other peaks were observed, but these peaks were observed in every fraction.
- 7.3.4.2 It was concluded that the contaminants were either non-fluorescing compounds and/or artifacts from the post-analytical sample workup. Either way, these did not interfere with the B[a]P quantification.

8. NOTES

8.1 Related Publications

- 8.1.1 At the time of publication, the following documents were related publications. A related publication is not necessarily equivalent and does not take precedence over this standard.

⁸ Tomkins, B.A. et al. "Liquid Chromatographic Determination of Benzo[a]Pyrene in Total Particulate Matter of Cigarette Smoke," *Journal — Association of Official Analytical Chemists*, 68 (1985): 935-940.

⁹ Appel, B.R. et al. "Benzene, Benzo[a]Pyrene, and Lead in Smoke from Tobacco Products Other than Cigarettes," *American Journal of Public Health*, 80 (1990): 560-564.

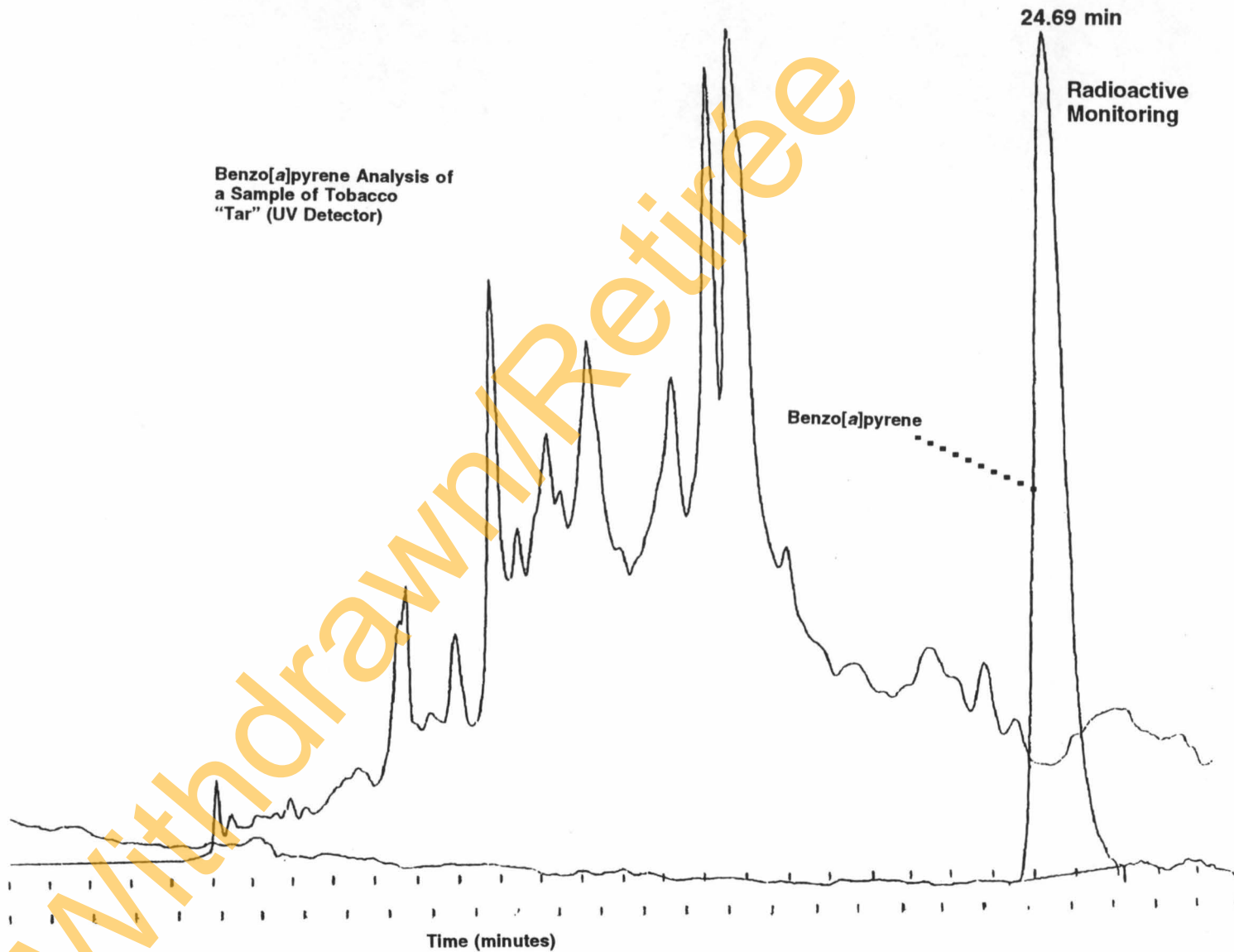
¹⁰ The Varian system is software controlled with a database capable of library spectral searches for positive identification, integration, analysis and custom reporting.

- 8.1.1.1 Kaiserman, M.J. and Rickert, W.S. "Carcinogens in Tobacco Smoke: Benzo[a]pyrene from Canadian Cigarettes and Cigarette Tobacco," *American Journal of Public Health*, 82 (1992): 1023-1026.
- 8.1.1.2 Risner, C.H. "The Determination of Benzo[a]pyrene and Benzo[a]anthracene in Mainstream and Sidestream of the Kentucky Reference Cigarette IR4F and a Cigarette which Heats but Does Not Burn Tobacco: A Comparison," *Beit, Zur Tabak*, 15 (1991): 11-17.
- 8.1.1.3 Risner, C.H. "The Determination of Benzo[a]pyrene in the Total Particulate Matter of Cigarette Smoke," *Journal of Chromatographic Science*, 26 (1988): 113-119.
- 8.1.1.4 Tomkins, B.A. et al. "Liquid Chromatographic Determination of Benzo[a]pyrene in Total Particulate Matter of Cigarette Smoke," *Journal — Association of Official Analytical Chemists*, 68 (1985): 935-940.

8.2 Sources of Referenced Publications

- 8.2.1 The publication referred to in par. 3.1.1 may be obtained from the American Society for Testing and Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428-2959, U.S.A., or from the Standards Council of Canada (par. 8.2.2).
- 8.2.2 The publications referred to in par. 3.1.2 may be obtained from the Standards Council of Canada, Standards Sales Branch, 45 O'Connor Street, Suite 1200, Ottawa, Canada K1P 6N7. Telephone (613) 238-3222. Fax (613) 995-4564.

FIGURE 1
Preparatory Column Chromatograms



Benzo[a]pyrene Analysis of
a Sample of Tobacco
"Tar" (Fluorescence)

Benzo[a]pyrene
11.145 min

Authentic
Benzo[a]pyrene
(Radioactive Monitoring)

Time (minutes)

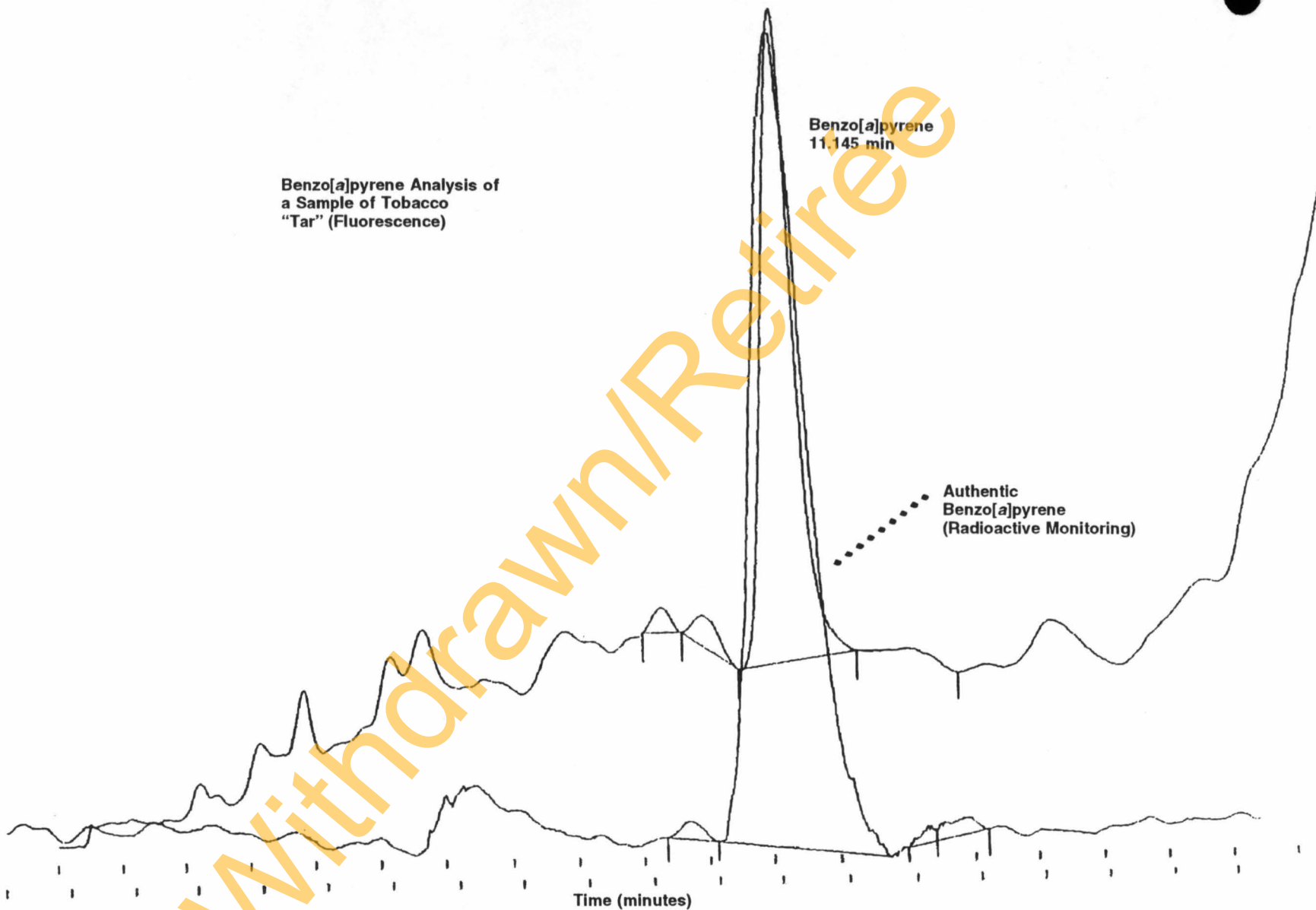


FIGURE 2

Analytical Column Chromatographs