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Date: December 31, 1999
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1 SCOPE OF APPLICATIONS

1.1 This method describes the separation and identification of eugenol in the ethanol extract of whole tobacco and clove cigarettes without extensive sample prep. The extract is filtered and analyzed using reversed phase/isocratic High Performance Liquid Chromatography (HPLC) with ultra violet (UV) detection. This method has several advantages. It is both precise and rapid and can be used in routine analysis. Also, analysis can be achieved at ambient temperature thereby avoiding the risk of thermal rearrangement and or decomposition that may be associated with gas chromatography.

1.2 The method is designed to be used as a routine analysis without the need for derivitization. This is applicable to processed cigarettes (Kreteks), tobacco and finecut tobacco.

1.3 This method is to only be used to determine the amount of eugenol added to tobacco as a flavourant.

1.4 This method does not distinguish between the amount of eugenol added and the amount of naturally occurring eugenol (if any) found in whole tobacco.

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

3 METHOD SUMMARY

3.1 Two grams of conditioned whole tobacco is accurately weighed into a 75 mL culture tube with screw cap and 50 mL of ethanol is added.

3.2 The tube is sealed and the tobacco extracted for two hours at 50 ^{o}C.

3.3 An aliquot is syringe filtered into a 1.5 mL amber autosampler vial and analyzed by HPLC with UV detection.

3.4 Eugenol in whole tobacco is quantified by external standard calibration procedures where the relative response of the samples is compared against a six-point calibration.

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 Robot Coupe RS1 2V Batch Processor or equivalent.
4.2 Sonicator.
4.3 Bottletop Dispensor 10 - 50 mL.
4.4 Balance capable of reading to four decimal places.
4.5 Centrifuge.
4.6 Vortex.
4.7 100 mL Class A amber volumetric flasks.
4.8 10 mL Class A amber volumetric flasks.
4.9 50 mL glass graduated measuring cylinder.
4.10 200 X 25 mm screw-top culture/centrifuge tubes.
4.11 white polypropylene caps without liners.
4.12 Micropipettes 10 µL, 100 µL, 500 µL, 1000 µL 2500 µL and 5000 µL for the preparation of analytical standards.
4.13 Pipettes, Class A, 2 mL, 5 mL, 50 mL.
4.14 Syringe Filter, 0.45 µm PVDF.
4.15 Disposable 5 cc syringes.
4.16 Disposable Glass Pasteur Pipettes.
4.17 Rubber Bulbs.
4.18 Glass Filtering Funnel.
4.19 Parafilm® or equivalent.
4.20 Autosampler vials, screw caps and Teflon lined septa.
4.21 Shaker Bath.
4.22 PC controlled High Pressure Liquid Chromatography System consisting of:

- **4.22.1** Solvent Delivery System - ternary gradient pump.
- **4.22.2** Refrigerated Autosampler with partial fill sampling loop.
- **4.22.3** UV Detector.
- **4.22.4** Work Station.
- **4.22.5** RP 18e Column.
- **4.22.6** Disposable Guard Column.

5 REAGENTS AND SUPPLIES

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

5.1 Eugenol –purity > 99 %.
5.2 Ethanol – HPLC Grade.
5.3 Type I water (as per ASTM D1193).
5.4 Methanol.
5.5 Isopropanol.
5.6 Helium (UHP).

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 STANDARDS

7.1 Primary eugenol stock solution (2.0 mg/ml) is prepared by accurately weighing 200 mg of pure eugenol into a 100 ml volumetric flask and diluting to the mark with ethanol.

7.2 Six working standards in the range (2, to 1000 μg/ml) are prepared from the primary eugenol stock solution by dilution (0.01 to 5000 μL) to 10 mL with ethanol. (see **Appendix 1)**.

7.3 Transfer to 1.5 mL amber autosampler vials. Rinse vial first and then fill to minimize head space.

7.4 Place vials in a vial file and store at 4 ^{o}C, protected from light until analyzed.

7.5 Eugenol calibration standards are prepared fresh every five working days.

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 Extraction of Whole Tobacco

9.1.1 The whole tobacco required for analysis must be removed from its original package and inspected for extraneous material.

9.1.2 Before testing, several grams of the whole tobacco samples are finely chopped using a batch processor, ground to pass through a 20 mesh screen.

9.1.3 The sample is then conditioned as specified in T-115.

9.1.4 Accurately weigh 2 g of finely chopped tobacco into a 200 X 25 mm screw cap glass culture tube.

9.1.5 Add 50 mL of ethanol to the sample. Screw the cap on and seal the cap with a little Parafilm.

9.1.6 Extract in a shaker bath for two hours at 50 ^{o}C.

9.1.7 It may be necessary to centrifuge at about 1200 rpm for 10 minutes to compress whole tobacco.

9.1.8 Allow the sample to cool to room temperature, then filter an aliquot into an amber autosampler vial (in duplicate) using a disposable syringe filter attached to a disposable syringe. Cap and store at 4 ^{o}C.

10 REVERSED PHASE HPLC ANALYSIS

10.1 Chromatographic Conditions (Reversed Phase Analysis)

10.1.1 Column Temperature: 30 °C.

10.1.2 Mobile Phase: Reagents.

10.1.2.1 Solvent A: Methanol : Type I water (80:20) filter and degas. (UHP Helium sparged).

10.1.3 Sample Wash: Solvent A.

10.1.4 Mobile Phase: Gradient.

Flowrate	0.7 mL/minute		
Time (minutes)	Composition		
0.0	100 % A	0 % B	0 % C
20.0	100 % A	0 % B	0 % C
Method End Action (Equilibrate 10 minutes).	100% A	0 % B	0 % C

10.2 Sample Analysis

10.2.1 Sample vials are loaded onto the autosampler such that every 10th vial is a standard solution.

10.2.2 Twenty (20) µL of each sample vial is injected onto the HPLC column and analyzed. Elution pattern should be similar to **Figure 1a, 1b**

11 CALCULATIONS

11.1 Construct a Calibration Curve:

11.1.1 Twenty (20) µL of each calibration standard is injected onto the HPLC and analyzed as per the chromatographic conditions under "System Control". Do in duplicate. Elution pattern should be similar to **Figure 2.**

11.1.2 A calibration curve is prepared by plotting the concentration of eugenol vs. the peak area. Determine the response factor from the calibration curve.

11.2 Sample Quantification

11.2.1 The amount of eugenol in whole tobacco samples is quantified by the external standard method.

11.2.2 The identification of peaks is by comparison of retention times with standards, and the spiking of whole tobacco samples.

11.3 Determination of Eugenol Deliveries in µg/g

11.3.1 $$\text{Eugenol [µg/g]} = \frac{\text{Peak Area}}{\text{Resp. Factor}} \times \frac{\text{mL of Solution}}{\text{Wt(g) of Tobacco}}.$$

11.4 By entering the correct multiplier (overall volume of the original sample in mL) and divisor (the original sample weight in (g)), the concentration of eugenol is automatically calculated in µg/g.

11.5 To convert this concentration to a percentage (%), the µg/g result must be divided by 10000.

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

12 QUALITY CONTROL

12.1 Recoveries and Levels of Contamination

12.1.1 Each analytical run of whole tobacco should also include:

12.1.1.1 A Laboratory Reagent Blank (LRB) to evaluate the extent of any interference due to the glassware, trapping reagents, solvent and analyzer effects.

12.1.1.2 A Laboratory Fortified Blank (LFB) to evaluate the extent of potential analyte loss

12.1.1.3 A Laboratory Fortified Matrix (LFM) to assess matrix interference. This is accomplished by spiking a true sample with a known concentration and determining a per cent recovery.

12.2 Method Detection Limit (MDL) and Limit of Quantitation

12.2.1 Method Detection Limit (MDL)

12.2.1.1 The method detection limit is determined by analyzing the lowest level standard at least 10 times as an unknown over several days. The MDL is then calculated as three times the standard deviation of these determinations.

12.2.2 Limit of Quantitation (LOQ)

12.2.2.1 The limit of quantification is determined by analyzing the lowest level standard at least 10 times as an unknown over several days. The LOQ is then calculated as 10 times the standard deviation of these determinations.

12.3 Stability of Reagents and Supplies

12.3.1 All primary stock eugenol standards are prepared fresh weekly.

12.3.2 All work standards, and extraction solvents are prepared fresh weekly.

12.3.3 All samples are analyzed within 24 hours.

13 REFERENCES

13.1 Myint, S., Daud, W.R.W., Mohmand, A.B., and Kadhum, A.A.H., 1995. Separation and Identification of Eugenol in Ethanol Extract of Cloves by Reversed-Phase High Performance Liquid Chromatography, *Journal of American Oil Chemist Society* 72, p.1231-1233.

13.2 Smith, R.M., and Beck, S., 1984. High Performance Liquid Chromatographic Analysis of Eugenol in Pimento using Ultraviolet and Electrochemical Detection, *Journal of Chromatography* 291 p. 424-427.

13.3 Fischer, I.U., and Dengler, H.J., 1990. Sensitive High Performance Liquid Chromatographic Assay for the Determination of Eugenol in Body Fluids, *Journal of Chromatography* 525, p. 369-377.

APPENDICES

Appendix 1: Eugenol Calibration Standards

Standard ID	**Vol (ml) 1° Eugenol**	**Final Volume in mL**	**Eugenol [µg/mL]**
Std 1	5.0	10	1000
Std 2	2.5	10	500
Std 3	1.0	10	200
Std 4	0.500	10	100
Std 5	0.100	10	20.0
Std 6	0.010	10	2.0

Note 1: The concentration of Eugenol will vary depending on the exact concentration of primary stock prepared.

Figure 1a: Overlay Chromatogram of whole tobacco sample sample (Kretek type cigarette tobacco) and calibration standard. (Eugenol peak is at RT 4.672 minutes)

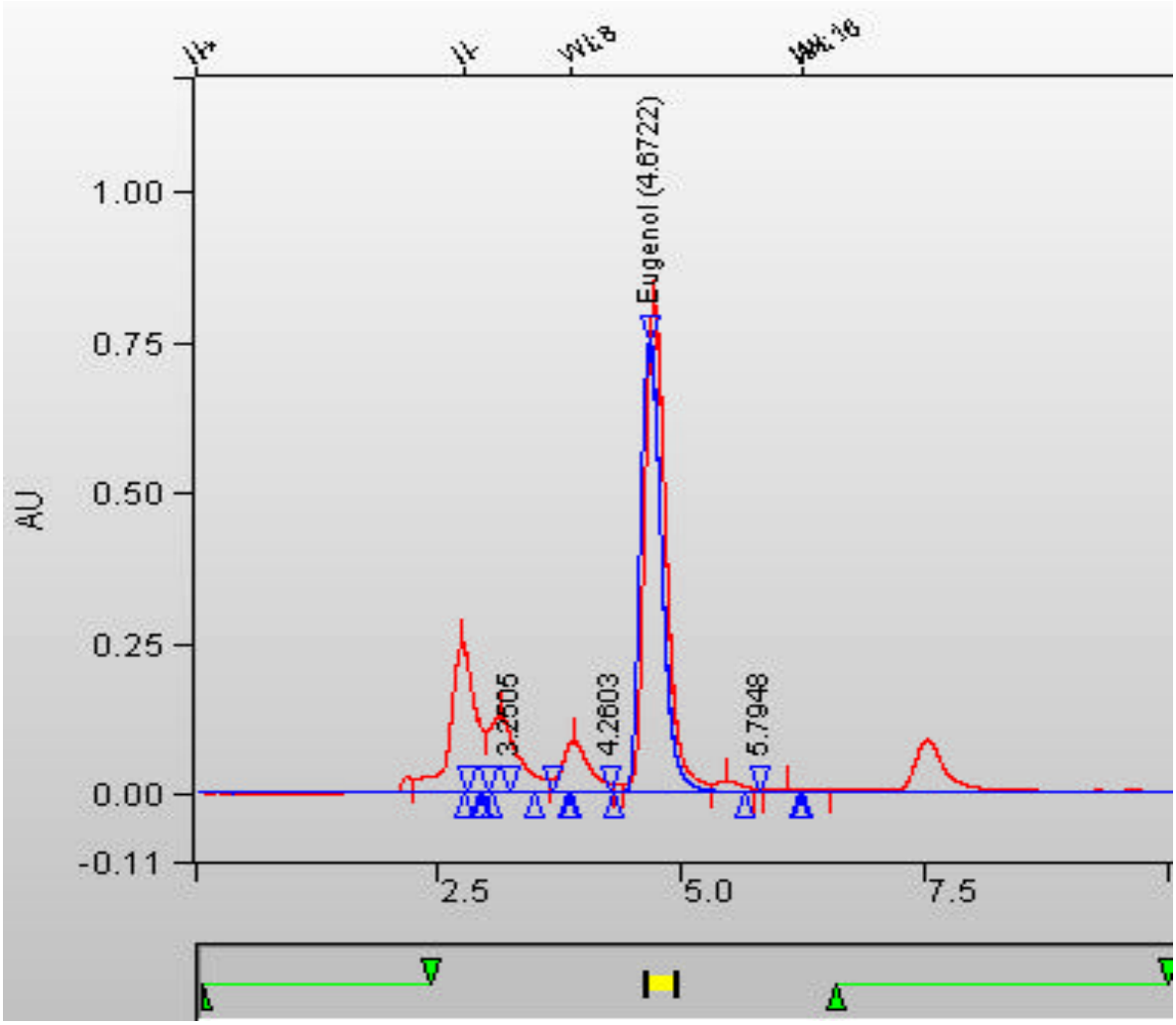
Eugenol (4.6722)
3.2505
4.2603
5.7948
AU
1.00
0.75
0.50
0.25
0.00
-0.11
2.5
5.0
7.5

Figure 1b: Overlay Chromatogram of whole tobacco (1R4F cigarette type) and calibration standard. (Note the absence of eugenol peak in the sample at RT 4.672 minutes)

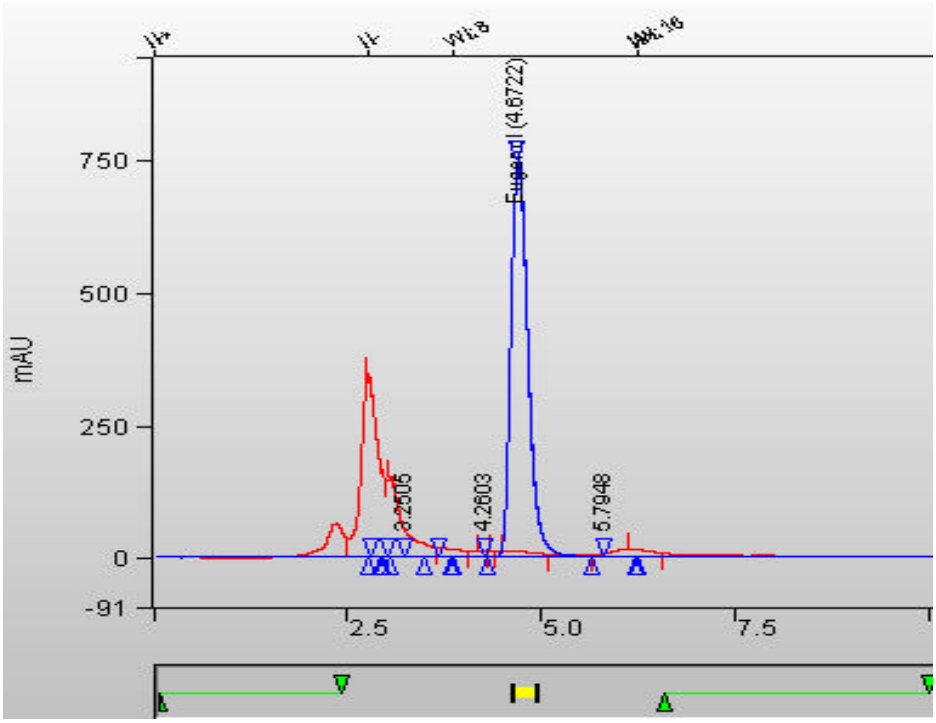

Figure 2: Chromatogram of Eugenol Calibration Standard. RT is 4.672 minutes