

CONFIDENTIAL

UK SMOKE CONSTITUENTS STUDY

Part 10: Determination of Mono and Dihydroxy Phenols Yields in Cigarette Smoke

Annex A - method

Commissioned by:
Tobacco Manufacturers Association
55 Tufton Street
London SW1P 3QL

March 2003

DEFINITIONS

The acronyms used in this procedure are listed and defined below.

(v/v)	Volume/Volume
1R4F	Industry Reference Cigarette Produced by the University of Kentucky
AA	Acetic Acid
Cal	Calibration
Cigt.	Cigarette
Conc	Concentration
D	Difference
FTC	Federal Trade Commission
HPLC	High Performance Liquid Chromatograph(y)
ID	Identification
LOD	Limit of Detection
LOQ	Limit of Quantitation
LRB	Laboratory Record Book
MeOH	Methanol
MS	Mainstream
MSDS	Material Safety Data Sheet
N	Number of Replicates
Phenolics	Hydroquinone, resorcinol, catechol, phenol, <i>m+p</i> cresol, and <i>o</i>-cresol
PTFE	Polytetrafluoroethylene (Teflon)
QAU	Quality Assurance Unit
QC	Quality Control
RE	Relative Error
RSD	Relative Standard Deviation
SD	Standard Deviation
Soln	Solution
SOP	Standard Operating Procedure

DETERMINATION OF PHENOLIC COMPOUNDS IN MAINSTREAM SMOKE

I. PURPOSE/SCOPE

Phenolic compounds are known components of mainstream cigarette smoke. This procedure describes the quantification of phenolic compounds from sampling media using high performance liquid chromatography (HPLC) with fluorescence detection.

II. PRINCIPLE OF METHOD

The phenolics are collected from mainstream smoke from five cigarettes smoked under ISO conditions (ISO). All mainstream smoke is collected on a Cambridge filter pad and then extracted for analysis.

All data in this SOP was collected under ISO conditions unless explicitly specified. ISO smoking refers to a 35cc puff, one puff every 60 seconds, 2-second puff duration with none of the ventilation holes blocked.

An HPLC equipped with a reversed-phase column and a fluorescence detector is used for the analysis. This system utilizes an increasing methanol fraction in a methanol:water gradient to elute potentially interfering compounds, and the analytes from the column, at different times. All phenolics are well separated except m-cresol and p-cresol, which co-elute. Therefore, only one value is reported for m, p-cresol. The eluted compounds are detected with a fluorescence detector.

Increased sensitivity is generated with the use of a constant amount of acetic acid in the mobile phase (~1% by volume). The acid functions to decrease the mobile phase pH, ultimately resulting in the formation of the protonated aromatic hydroxyl forms of the phenolic compounds. The quantum yield of the protonated form of an aromatic hydroxyl compound is greater than for the corresponding conjugate base, and therefore, the method detection limit is decreased. The fluorescence responses are calibrated from standard solutions of known concentration.

The concentrations of the phenolic compounds are reported in units of mass-to-volume (i.e., µg/mL). The measured phenolics concentration, the number of cigarettes smoked, and the sample solution volume(s) are also used to calculate the total mass of phenolics on a per cigarette basis.

III. APPARATUS, CHEMICALS, AND LABORATORY SUPPLIES

A. Preparation of Mobile Phase, Extraction Solution and Standard Preparatory Solution

See appendix A

B. Apparatus

Equipment	Use
Analytical Balance	Weighing materials
Mechanical Dispenser	Transferring solution for filter pad extraction
Wrist Action Shaker	Filter extraction
Water System	Provide purified, deionized water
Weight Set	Calibrate balance

C. Chemicals

See appendix B

D. Laboratory Supplies

- Aluminum Foil
- Automated liquid sampler (ALS) vials, with PTFE-lined lids.
- Amber volumetric flasks, calibrated “to contain” specified volumes, Class A.
- Disposable pasteur pipets
- Volumetric glass pipets, calibrated “to deliver” specified volumes, Class A.
- 44-mm Cambridge filter
- Erlenmeyer flasks, 50-mL
- Glass syringe with a Luer-Lok tip, Model 2440, Becton-Dickinson (Rutherford, NJ) or equivalent plastic syringe.
- Syringe filter, 13 mm PTFE, part number 187-1345, Nalgene Nunc (Rochester, NY) or equivalent plastic syringe.

IV. PREPARATION OF ANALYTICAL SOLUTIONS AND CALIBRATION CHECK STANDARDS

A. Preparation of Stock and Working Calibration Solutions

Prepare all solutions in Class A, “to contain” amber glassware. All pipetting will be performed with Class A, “to deliver” pipettes unless otherwise stated. Transfer all solutions to Teflon-capped amber vials for storage at $\leq 10^{\circ}\text{C}$.

All solution concentration values are to be calculated to the number of significant figures given with the approximate solution concentrations.

The phenolic compounds are hygroscopic (they absorb moisture from the air and will gain mass as they sit on the balance). Therefore, the time the standard materials are exposed to air on the balance must be minimized. If the technician is unable to weight out the material required for one standard in <10 minutes, the lead chemist will be contacted for advice on technique.

All stock, working and calibration solutions expire 6 days after the preparation date, when stored in sealed amber bottles at $\leq 10^{\circ}\text{C}$.

To prepare standard stock I, accurately weigh 0.125 ± 0.0125 mg of each analyte into an amber 25-mL volumetric flask. Slowly add ~5-10 mL of the Standard Preparatory solution and mix well to ensure the solids have dissolved. Dilute to volume with 1% Acetic Acid in 100 Mm Ascorbic Acid and mix the solution well. Combined stock II is prepared in 100 mL flasks from the volumes of stock I listed below. Stock II is only prepared for resorcinol, m-cresol, p-cresol, and o-cresol.

TABLE IV.1 Preparation of Calibration Standards (Stock I)

Stock I					
Analyte	Target Wt.	Wt (g)	Purity	Stock I Volume mL	Stock I Conc mg/mL
hydroquinone	0.1250	0.125	0.99	25	4.950
catechol	0.1250	0.125	0.99	25	4.950
phenol	0.1250	0.125	0.99	25	4.950
resorcinol	0.1250	0.125	0.99	25	4.950
m-cresol	0.1250	0.125	0.99	25	4.950
p-cresol	0.1250	0.125	0.99	25	4.950
o-cresol	0.1250	0.125	0.99	25	4.950

TABLE IV.2. Preparation of Calibration Standards (Combined Stock II)

Combined Stock II			
Analyte	Volt Stock 1 mL	Stock II Conc [mg/mL]	Dilute to Volt mL
hydroquinone	n/a	n/a	n/a
catechol	n/a	n/a	n/a
phenol	n/a	n/a	n/a
resorcinol	2.5	0.1238	100
m-cresol	10	0.4950	100
p-cresol	5	0.2475	100
o-cresol	5	0.2475	100

To prepare the combined **Stock III Solution**, 1 mL of hydroquinone, catechol, and phenol are pipetted from **Stock Solution 1** into a 50 mL flask. Into the same 50-mL flask, 4 mL of resorcinol, m-cresol, p-cresol, and o-cresol are pipetted from combined **Stock Solution II**. Dilute to volume with 1 % Ascorbic Acid in 100 mM Ascorbic Acid and mix well. Transfer all solutions into 40 mL amber bottles for storage if possible.

TABLE IV.3. Preparation of Calibration Standards (Combined Stock III)

Combined Stock III				
Analyte	vol stock I (ml)	vol stock 2 (ml)	Dilute to vol (ml)	Main Stock [µg/ml]
hydroquinone	1	-	50	99.00
catechol	1	-	50	99.00
phenol	1	-	50	99.00
resorcinol	-	4	50	9.900
m-cresol	-	4	50	39.60
p-cresol	-	4	50	19.80
o-cresol	-	4	50	19.80

In amber, 50-mL volumetric flasks, add the following amounts of standard solutions as indicated in Table A.3-3. Dilute all standards with 1 % Acetic Acid in 100 Mm Ascorbic Acid.

TABLE IV.4. Preparation of Working Calibration Standards

Label Vol (mL) Stock III	s-1 0.5	s-2 1	s-3 2	s-4 5	s-5 9
hydroquinone	0.9900	1.980	3.960	9.900	17.82
catechol	0.9900	1.980	3.960	9.900	17.82
phenol	0.9900	1.980	3.960	9.900	17.82
resorcinol	0.09900	0.1980	0.3960	0.9900	1.782
o-cresol	0.1980	0.3960	0.7920	1.980	3.564
m+p cresol	0.5940	1.188	2.376	5.940	10.69

Phenolic Quality Control Standards (QC)

To prepare **QC Standard Stock I**, accurately weigh 0.125 ± 0.0125 mg of each analyte into an amber 25-mL volumetric flask. Slowly add ~5-10 mL of the 1 % Acetic Acid in 100 mM Ascorbic Acid and mix well to ensure the solids have dissolved. Dilute to volume with 1% AA/ 5% MeOH in H₂O and mix the solution well. Combined Stock II is prepared in 100 mL flasks from the volumes of stock I listed below. **Combined Stock II** is only prepared for resorcinol, m-cresol, p- cresol, and o-cresol.

TABLE IV.5. Preparation of Calibration Check Standards

Analyte	Target Wt.	Wt (g)	Purity	Stock I Volume mL	Stock I Conc mg/mL
hydroquinone	0.1250	0.125	0.99	25	4.950
Catechol	0.1250	0.125	0.99	25	4.950
Phenol	0.1250	0.125	0.99	25	4.950
resorcinol	0.1250	0.125	0.99	25	4.950
m-cresol	0.1250	0.125	0.99	25	4.950
p-cresol	0.1250	0.125	0.99	25	4.950
o-cresol	0.1250	0.125	0.99	25	4.950

TABLE IV.6. Preparation of Calibration Standards (Combined Stock II)

Combined Stock II			
Analyte	Vol Stock 1 mL	Stock II Conc [mg/mL]	Dilute to Vol mL
hydroquinone	n/a	n/a	n/a
catechol	n/a	n/a	n/a
phenol	n/a	n/a	n/a
resorcinol	2.5	0.1238	100
m-cresol	10	0.4950	100
p-cresol	5	0.2475	100
o-cresol	5	0.2475	100

To prepare the **Combined QC Stock solution III**, 1 mL of hydroquinone, catechol, and phenol are pipetted from **Stock Solution 1** into a 50-mL flask, into the same 50-mL flask 4 mL

of resorcinol, m-cresol, p-cresol, and o-cresol are pipetted from **QC Stock Solution II**. Dilute to volume with Standard Preparatory Solution and mix well. Transfer all solutions into 40 mL amber bottles for storage if possible.

TABLE IV.7. Preparation of Calibration Check Standards (Combined Stock III)

Combined Stock III				
Analyte	vol stock I (ml)	vol stock 2 (ml)	Dilute to vol (ml)	Main Stock [µg/ml]
hydroquinone	1	-	50	99.00
catechol	1	-	50	99.00
phenol	1	-	50	99.00
resorcinol	-	4	50	9.900
m-cresol	-	4	50	39.60
p-cresol	-	4	50	19.80
o-cresol	-	4	50	19.80

In amber, **50-mL volumetric flasks**, add 1 mL of the **QC Check Standard Combined Stock Solution III** as indicated in Table A.4-3. Dilute to volume with 1% Acetic Acid in 100 mM Ascorbic Acid.

TABLE IV.8. Preparation of Working Check Standard

QC stock III	
Label	s-2
Vol (mL)	1
hydroquinone	1.980
catechol	1.980
phenol	1.980
resorcinol	0.1980
o-cresol	0.3960
m+p cresol	1.188

V. SAMPLE COLLECTION AND WORKUP

A. Sample Collection

Authors Comment – conditions for smoke generation and collection are described elsewhere – a summary is reproduced below.

- Cigarettes are conditioned¹ at a temperature of $22 \pm 1^\circ\text{C}$ and $60 \pm 3\%$ relative humidity for a minimum of 48 hours but not exceeding 10 days.
- Butt marking will be ISO butt length specifications². Filtered cigarettes will be smoked to a measured butt length equal to either the tipping paper + 3 mm or filter length + 8 mm whichever is longer. The minimum butt length will be 23 mm and this will also be used for non filter brands. All smoking shall be conducted in an environment of temperature $22 \pm 2^\circ\text{C}$ and $60 \pm 5\%$ relative humidity¹.
- ISO conditions³ for smoking cigarettes will apply. The smoking machine puffing parameters will be $35 \pm 0.2\text{ cm}^3$ puff volume with 2.0 ± 0.05 second puff duration once every 60.0 ± 0.5 seconds.

¹ ISO 3402: 2000 - Tobacco and tobacco products – atmosphere for conditioning and testing

² ISO :4387: 2000 - Methods for chemical analysis of tobacco and tobacco products – Determination of total and nicotine- free dry particulate matter using a routine analytical smoking machine

³ ISO 3308:2000 – Routine analytical cigarette smoking machine – 1: Definitions and standard conditions

- As a check that cigarettes have been smoked in accordance with ISO standard conditions, TPM yields were determined and compared with that normally achieved. Results for cigarettes that give significantly low or high TPM yields ($\pm 3 \times$ standard deviation) will be discarded.
- A minimum of five determinations will be performed for each brand. The smoking of the cigarette brands is randomised so that samples from the same brand are smoked on different days.
- With each batch of samples a 2R4F cigarette is smoked.

For mainstream smoke collection, the sampling apparatus will consist of a 44-mm Cambridge filter pad in a holder, connected directly behind the cigarettes to an analytical smoking machine.

Each mainstream sample will consist of the smoke collected from five cigarettes smoked under ISO conditions.

VI. PROCEDURE

A. Mainstream Sample Extraction

Cambridge Filter Sample Extraction (for Mainstream 44-mm Cambridge filter samples):

With tweezers, carefully remove the Cambridge filter from the holder. Fold the filter to expose the "clean" side, and wipe the inside of the holder with the filter. Place the filter into an aluminum foil-wrapped, 50-mL Erlenmeyer flask, and unfold it on the bottom of the flask.

Using a repeating pipette, add 20.0 mL of 1% Acetic Acid/ 5% MeOH in H₂O and stopper the flask with a ground glass stopper. Next, agitate the samples for 30 minutes on a wrist action shaker.

Filter the solution by removing the plunger from a syringe and attach a 0.45- μ m filter to the syringe barrel. With a Pasteur pipette, pipette ~1.5 mL of the solution into the barrel of the syringe. Replace the plunger, and "push" the solution directly into a labeled ALS vial. Cap the vial with a Teflon-lined cap.

For the Phenolics Sample Analysis Procedure See Appendix C

VII. INSTRUMENT ANALYSIS

A. HPLC Apparatus and Operation Parameters

TABLE VII.1 HPLC Parameters

Equipment/Parameter	Make	Model	Serial Number
HPLC System Detector	Waters W474 Fluorescence Detector Waters 2690 Separation Unit	474 2690	
Data Acquisition:	Water Millennium ³²	Version 3.2	
Analytical Columns:	Metachem Carbasorb 3 μ Carbasorb column	Part Number 0220-250X030	
	Phenomenex Columbus 5 μ m C18, guard column	Part Nos. 00G-4108-E0	
Mobile Phase	Gradient See Table		
Analytical Run Time	~35 min with a five minute post time		
Injection Program	5 μ L sample		
Lamp	Xenon-arc		
Response Time	3.0 seconds		
Slit Width: Entrance/Exit	18nm / 18nm		
Analytical Wavelengths:	(Time Table)		
Time	Excitation (nm)	Emission (nm)	Gain
0.0	304	338	
8.0	274	298	10
Expected Retention Times	Hydroquinone: ~5.7 minutes resorcinol: ~9.5 minutes Catechol: ~12.8 minutes Phenol: ~20.2 minutes m+p-cresol: ~25.5 minutes o-cresol: ~26.0 minutes		

Mobile Phase Gradient

Time	% (1% AA in H ₂ O)	% (1% AA/1% IPA in ACN)	Flow Rate (mL/min)
0.0	98	2	0.500
1.0	98	2	0.500
6.0	96	4	0.500
10.0	93	7	0.500
20.0	70	30	0.500
34.0	40	60	0.500
35.0	98	2	0.500

Equilibrate the column with the initial mobile phase conditions for 15-30 minutes or until obtaining a stable baseline before use.

Example chromatograms are depicted in Figures VII.1.

B. Sample Analysis Order for the Assay of Phenolics

Sample analysis is to begin with the blank extraction solution 1% AA / 5%MeOH in H₂O. The calibration standards, arranged in increasing concentration levels, are next in the analysis scheme.

One check standard will be injected before the first sample. A check standard will be injected before and after each sample set.

An example sampling analysis order for 10 smoke samples (including calibration blank, 1R4F, and test cigarette smoke samples) follows:

BLK, Cal Stds (low to high), CS, Samples 1 through 5, CS (medium), Samples 6 through 10, CS (BLK = Calibration Blanks; Cal Stds = Calibration Standards; CS = Check Standards)

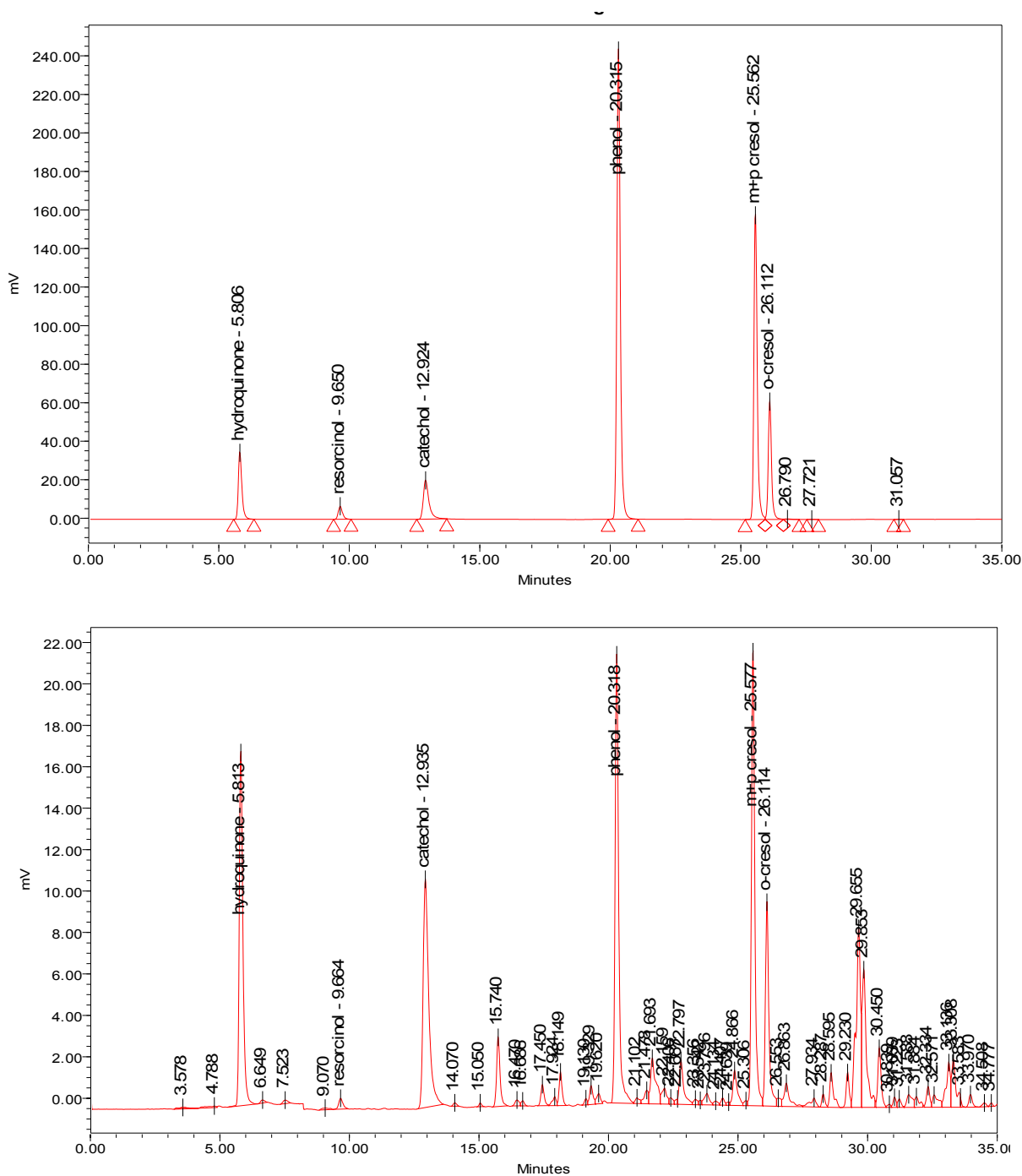


FIGURE VII.1. Example Chromatograms. Top: High Standard. Bottom: 1R4F Mainstream Smoke Filter Extract.

VIII. DATA REDUCTION AND EXAMPLE CALCULATIONS

This section describes the calculations for the calibration of the HPLC and the method used for determining the amount of phenolic compound per cigarette.

A. Instrument Calibration Calculations

The analysis is carried out using the calibration capabilities of the Waters Millennium³² software and assumes the operator is already familiar with the procedure for setting up a

calibration on the Waters Millennium³² software. For the initial run of calibration standards, clear calibration is selected.

B. Data Reduction of Raw Data from HPLC

The analyte concentration is determined by the external calibration method using the regression equation derived from the calibration curve. Calculation of each specific analyte is obtained by using the calculation capabilities of the Waters Millennium³² software and assumes the operator is already familiar with the procedure for setting the Waters Millennium³² software. The operator must supply the correct values for sample dilution and number of cigt. per sample. The Waters Millennium³² software will also calculate the percent relative error (%RE) of the calibration check standards.

Example Calculation (from a mainstream 1R4F sample):

Mainstream Cigarette Sample Concentration

µg/sample hydroquinone =

Example Calculation:

µg/mL (hydroquinone determined) * Volume / number of Cigts. in the sample

25.02 µg/mL hydroquinone determined, 10 mL and 5 cigarettes per sample

µg/sample hydroquinone determined = (25.02 µg/mL) * 10 mL = 250.2 µg

hydroquinone concentration (µg/cigt.) = (250.2 µg)/5 cigarettes = 50.04 µg/cigt.

C. Data Acceptance

In the event of poor chromatography (e.g., bad peak shape, no peaks, bad baseline, etc.), the lead chemist will be contacted. If the poor chromatography can be attributed to a single event, such as a bad injection, the standard or sample exhibiting the bad chromatography will be disregarded from any calculations, and a complete explanation will be included with the data.

D. Calibration and Quality Control Standard Acceptance Criteria

Agreement between the analyzed value and prepared value for calibrated standards must be achieved to prove conformance to the linear calibration model. Each calibration curve must have a correction of 0.990 or better. Calibrations that do not meet these requirements should be brought to the attention of the lead chemist immediately. All quality control standards must be within ±10% RE of their calculated values. All quality control standards that do not meet these requirements should be brought to the attention of the lead chemist immediately. If a quality control standard is not ± 10% RE of their calculated values the quality control standard may be replaced at the operators discretion with a different vial of the same standard and re-analyzed. If the new quality control standard is within ±10% RE of its calculated value then sample analysis may continue. If the new quality control standard is not ± 10% RE of the calculated value then sample analysis must stop, the problem corrected and the instrument recalibrated. Any samples that are bracketed with a failed quality control standard must be re-analyzed at the discretion of the lead chemist.

APPENDIX A:

PREPARATION OF MOBILE PHASE, EXTRACTION SOLUTION AND STANDARD PREPARATORY SOLUTION

Mobile phase solutions expire 3 months after the preparation date and are stored at ambient room temperature. Extraction solution expires 1 week after the preparation date and is stored at ambient room temperature.

Prepare all solutions in Class A, "to contain" glassware. All pipetting will be performed with Class A, "to deliver" pipettes unless otherwise stated. Transfer all solutions to sealed glass bottles for storage. Type 1 water should be used to prepare all solutions.

1% Acetic Acid in Water (Mobile Phase A)

Using a 20.0-mL volumetric pipette, pipette 20 mL of glacial acetic acid into a 2000-mL volumetric flask containing ~1400 mL (± 200 mL) of water (H_2O). Dilute to volume and mix the solution well.

1% Acetic Acid /1% IPA in ACN (Mobile phase B)

Using a 20.0-mL volumetric pipette, pipette 20.0 mL of glacial acetic acid into a 2000-mL volumetric flask containing approximately 500 mL of acetonitrile (ACN). Using a 20.0 mL volumetric pipette, pipette 20.0 mL of Isopropyl Alcohol. Dilute to volume with ACN and mix the solution well.

1% Acetic Acid /5% MeOH in Water (Extraction Solution)

Using a 20.0-mL volumetric pipette, pipette 20 mL of glacial acetic acid into a 2000-mL volumetric flask containing ~1000 mL (± 500 mL) of water (H_2O). Add 50.0 mL of MeOH using a 50.0 mL pipette. Dilute to volume with water and mix the solution well.

1% Acetic Acid in 100 mM Ascorbic Acid (Standard Preparatory Solution)

Weigh 35.2 ± 2.00 grams of Ascorbic Acid into a 2000-mL volumetric flask containing approximately 1000 mL of water. Make sure the ascorbic acid is in solution. Using a 20.0 mL volumetric pipette, pipette 20.0 mL of Acetic Acid to the mixture. Dilute to volume with water and mix well.

**APPENDIX B:
REQUIRED CHEMICALS**

Chemical	Supplier	Grade or Purity
Catechol	Aldrich (Milwaukee, WI) catalog #13,501-1 (or similar)	99+%
Hydroquinone	Aldrich (Milwaukee, WI) catalog #24,012-5 (or similar)	99+%
Resorcinol	Aldrich (Milwaukee, WI) catalog #39,804-7 (or similar)	99+%
Phenol	Aldrich (Milwaukee, WI) catalog #43,151-6 (or similar)	Reagent Plus™ 99.99%
<i>m</i> -cresol	Aldrich (Milwaukee, WI) catalog #C8,572-7 (or similar)	99+%
<i>p</i> -cresol	Aldrich (Milwaukee, WI) catalog #C8,575-1 (or similar)	99+%
<i>o</i> -cresol	Aldrich (Milwaukee, WI) catalog #C8,570-0 (or similar)	99+%
L-Ascorbic Acid	Aldrich (Milwaukee, WI) catalog #25,556-4 (or similar)	99+%
2-Propanol (Isopropyl Alcohol)	Fisher Scientific A520-4 (or similar)	Spectrophotometric/ Liquid chromatography grade
Methanol	EM Science MX04889-1 (or similar)	Spectrophotometric/ Liquid chromatography grade
Acetonitrile	EM Science AX0142-1	Spectrophotometric/ Liquid chromatography grade
Glacial Acetic Acid	Fisher Scientific (or similar)	99.5%
Reagent Water	Type 1 Water	HPLC Grade

**APPENDIX C:
PHENOLICS SAMPLE ANALYSIS PROCEDURE**

