

CONFIDENTIAL

UK SMOKE CONSTITUENTS STUDY

Part 9: Determination of Volatile Organic Compounds Yields in Cigarette Smoke

Annex A - method

Commissioned by:
Tobacco Manufacturers Association
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DEFINITIONS

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

µg	Microgram
µL	Microliter
1R4F	Industry Reference Cigarette Produced by the University of Kentucky
ALS	Automated Liquid Sampler
Cal	Calibration
Cigt.	Cigarette
Conc.	Concentration
D	Difference
FTC	Federal Trade Commission
GC/MSD	Gas Chromatograph/Mass-Selective Detector
ID	Identification
IPA	Isopropanol
ISO	International Organization for Standardization
ISTD	Internal Standard
LOD	Limit of Detection
LOQ	Limit of Quantitation
MeOH	Methanol
mL	Milliliter
MS	Mainstream Smoke
MSDS	Material Safety Data Sheet
N	Number of Replicates
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
Std	Standard
Volatiles	1,3-butadiene, isoprene, acrylonitrile, benzene, toluene and styrene, collectively

THE DETERMINATION OF VOLATILES IN MAINSTREAM SMOKE

I. PURPOSE/SCOPE

This method describes the procedure for the determination of 1,3-butadiene, Isoprene, Acrylonitrile, Benzene, Toluene and Styrene (referred to, collectively, as "Volatiles") in mainstream smoke.

II. PRINCIPLE OF METHOD

Mainstream (MS) smoke from test cigarettes is generated under conditions specified by the study protocol, passed through a 44-mm Cambridge filter and collected in one impinger containing 20 mL MeOH that is immersed in a dry-ice/IPA bath. After smoking, the MS filter is weighed and transferred to the impinger. ISTD is added, the impinger is vortexed briefly and the extract subsequently analyzed by GC/MSD.

Individual volatile concentrations are determined by the internal standard method using gas-chromatography with mass-selective detection. The concentrations of the volatiles determined by the GC/MSD are reported in units of mass-to-volume (i.e., $\mu\text{g/mL}$). The measured concentration, the number of cigarettes smoked, and the sample solution volume(s) are used to calculate the total analyte mass on a per cigarette basis.

The analytical method used in this SOP has been fully validated. The validation included trapping efficiency, selectivity, method accuracy and precision, detection and quantitation limits, and sample stability. A flow diagram of the sample collection and workup procedure is given in Appendix A.

III. APPARATUS, CHEMICALS, AND LABORATORY SUPPLIES

A. Required Chemicals

See Appendix B:

B. Laboratory Apparatus and Supplies

- 70 mL glass impingers with coarse frits
- Dewar Flasks
- Thermometer (-100 to 40 °C)
- Volumetric flasks, calibrated "to contain" specified volumes, Class A
- Disposable Pasteur pipets
- Volumetric glass pipets, calibrated "to deliver" specified volumes, Class A
- Gas-tight syringe (500 or 1000 μL)
- 44-mm Cambridge filter
- Laboratory balance with 0.1mg accuracy
- 125mL PMP Erlenmeyer flasks
- Mechanical Dispenser (10 – 50 mL), Brand Tech model Dispensette III (Wertheim Germany)
- Automated liquid sampler (ALS) vials, with screw PTFE-lined lids.
- 5-mL Syringe, disposable
- Syringe Filter, 13 mm x 0.45 μm , PTFE

IV. Preparation of Solutions

A. Preparation of Dry-ice/IPA Slurry

Pour approximately 300 mL of IPA into a Dewar flask. Break off small chunks (1 to 3 cm in diameter) of dry ice and add to the IPA in each flask. Continue adding dry ice until IPA becomes very thick and bubbling slows. Monitor the temperature of the solution. Smoking can begin when the bath temperature is below $-70\text{ }^{\circ}\text{C}$.

B. Preparation of ISTD, Calibration and QC Solutions

See Appendix C.

V. Sample Collection

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.
- 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.
- Five cigarettes are smoked using ISO specifications² on a linear 20 channel smoking machine.

A flow diagram of the sample collection and workup procedure is given in Appendix A.

When collection of samples is planned, the lead chemist will specify the number of individual samples to be taken. For mainstream smoke collection, the appropriate sampling request form will be prepared and delivered to the laboratory supervisor. The chemistry technician will prepare impingers and deliver them to the smoking technician at the time of smoking.

For mainstream smoke collection, the sampling apparatus will consist of 1 impinger, containing 20 mL of MeOH, connected directly behind a 44-mm Cambridge filter pad. The MeOH is added to the impinger with a Mechanical Dispenser calibrated to deliver 20 mL. The mainstream sample train is depicted in Figure VII.1. Each mainstream sample will consist of the smoke collected from five cigarettes smoked under ISO conditions.

Immediately after the last clearing puff has been taken, the impingers should be disconnected, removed from the Dewar flasks and delivered to the chemistry technician.

NOTE: Coordination between the person who is smoking the cigarettes and the technician who is working up the samples must be made in advance to ensure that samples are prepared sequentially after collection. If samples cannot be processed

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

within 15 minutes after smoking is complete, the impingers may remain in the dry-ice/IPA slurry until processing can begin (but for no longer than one hour).

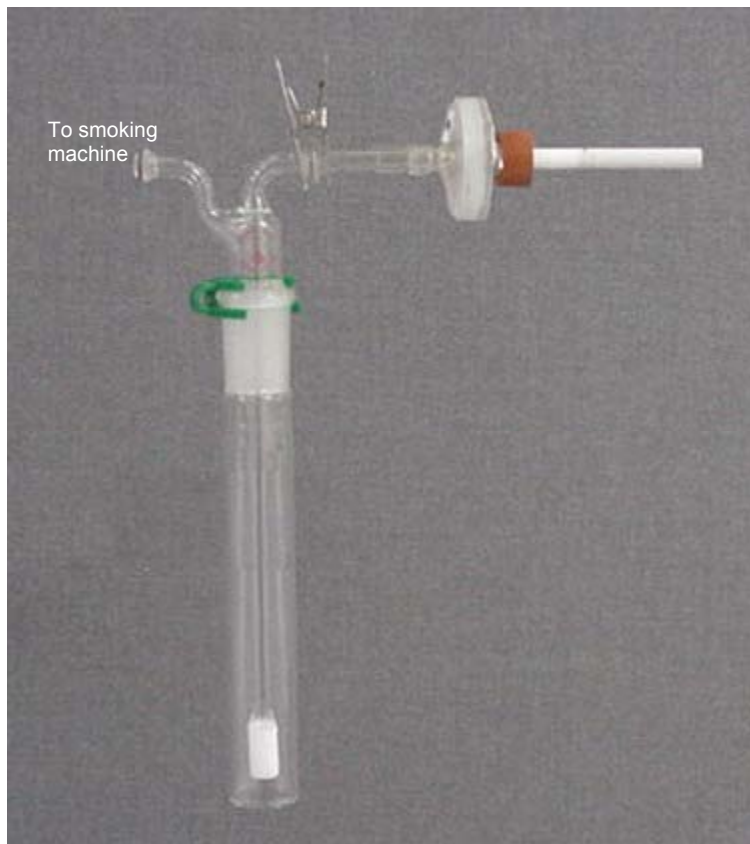


Figure VII.1 Mainstream Sample Train

VI. PROCEDURE

Mainstream Sample Workup

1. After smoking, weigh the MS pad and record the weight for MS TPM.
2. Add the pad to the impinger, pushing the pad down until it is immersed in the methanol. Rinse the inside of the impinger stem with two Pasteur pipettes full of clean methanol (approximately 2 x 1 mL) into the impinger. Blow any remaining MeOH out of the frits and into the impinger with a rubber bulb.
3. Using a Gas-tight syringe, add 200 μ L of the ISTD solution to the impinger.
4. Vortex the solution at high speed for 10 seconds. The pad and methanol in the impinger should be observed to mix freely. (If a vortexer is unavailable, a stopper may be placed on the impinger and it may be gently inverted 10 times to mix).
5. Attach a PTFE filter to a syringe and pour approximately 4 mL of the extract into the syringe.
6. Push the plunger into the barrel of the syringe and allow the first few drops to fall into waste. Filter approximately 1.5 mL of the extract into two ALS vials with Teflon-lined screw caps. Note: the solution in the vial should come up to the bottom of the vial neck.

7. Store the samples in the freezer (< - 5 °C) until analysis begins. Samples should be analyzed within 24 hours but are stable for up to 1 week, if kept in the freezer and septum is not pierced.

VII. INSTRUMENT ANALYSIS

A. Sample Run Order

Sampling analysis order is as follows:

- 1) Calibration standards = Calibration Curve Standards (S1 to S5)
- 2) Check Std = Quality Control check standard
- 3) Samples in sets of 10 to 15, check std between each set
- 4) Check Std at the end of the sequence

Analysis order will be designed so that an equal number of batched samples in groups of 15 or less be analyzed and bracketed by calibration check standards. For example, if there are 28 samples then 14 will be analyzed as the first batch of samples and 14 will be analyzed as the second batch.

The lead chemist will be contacted if there are any questions regarding the sample analysis order.

B. GC/MSD Apparatus and Operation Parameters

The conditions listed in Table IX.1 may be modified by the analyst, with the approval of the lead chemist in order to produce acceptable chromatography. While the polar pre-column listed in Table IX.1 is not essential, it does improve the chromatography and protects the column from build up from dirty samples.

This SOP assumes that the operator is familiar with the operation and maintenance of the GC/MSD.

Table IX.1 GC/MSD Parameters

Equipment / Parameter	Make	Model	
GC/MSD System Identification	Hewlett-Packard	HP 6890 Plus GC HP 5973 MSD HP 7683 Autosampler and Injector	
Data Acquisition Software	Hewlett-Packard	Chemstation	
Operating System Software	Windows NT		
Analytical Column:	J&W DB-5ms 60mx0.25mmx1.0µm	122-5563	
Pre-column (optional)	Supelco 1mx0.25mm Polar Deactivated Fused Silica PN 25712		
Injection type	Split (40:1)		
Injection Volume	1 µL		
Injection Liner	4 mm straight liner with glass-wool plug (deactivated)		
Injection port temperature	250 °C		
Column Flow	Constant Flow 1.0 mL/min (Helium)		
Column Temperature	35 °C for 10 minutes 20 °C per minute to 260 °C, hold 6.75 minutes		
Transfer Line temp.	250 °C		
Ionization Mode	Electron Impact		
Detection	Full-Scan (50 to 150 amu)		
MS Quad temperature	150 °C		
MS Source temperature	230 °C		
Tune parameters	Autotune		

Table IX.2 Quantitation and Confirmation Ions

Analyte	Retention Time	Quantitation Ion	Confirmation Ion
1,3-butadiene	5.40	54	53
Isoprene	8.15	67	68
Acrylonitrile	9.14	52	53
Benzene-d6	14.57	84	56
Benzene	14.64	78	77
Toluene	17.13	91	92
Styrene	19.07	104	78

Consult the lead chemist if instrument performance problems are encountered. Whenever routine or non-routine maintenance to this analytical system is performed, it must be logged into the GC/MSD Logbook. Documentation of any changes in system configuration must be recorded.

Example chromatograms are depicted in Figures IX.1 to IX.6.

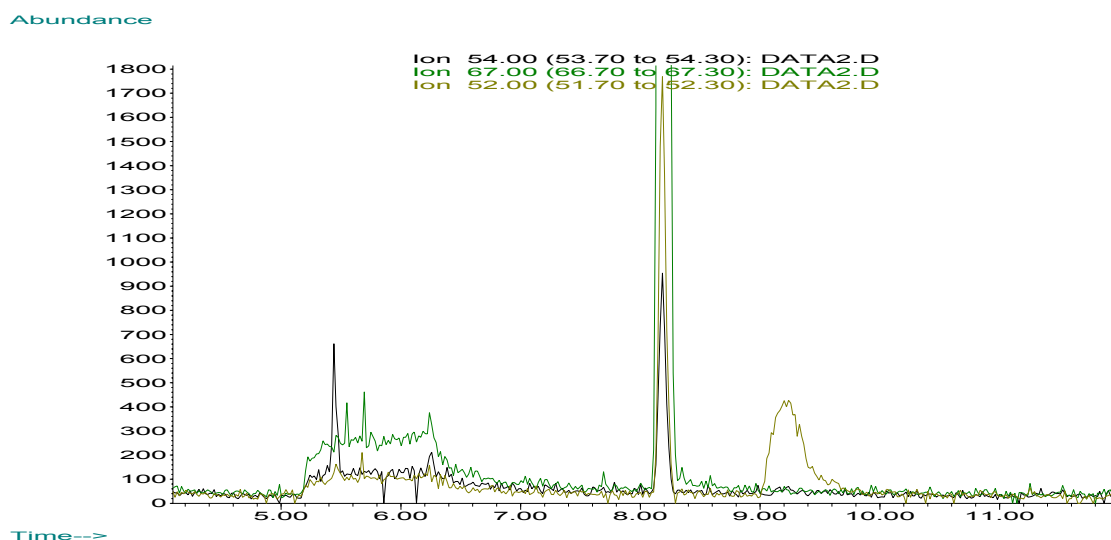


FIGURE IX.1 Example Ion Chromatogram: Low Standard. 1,3-butadiene (5.44 minutes), Isoprene (8.25 minutes) and Acrylonitrile (9.3 minutes)

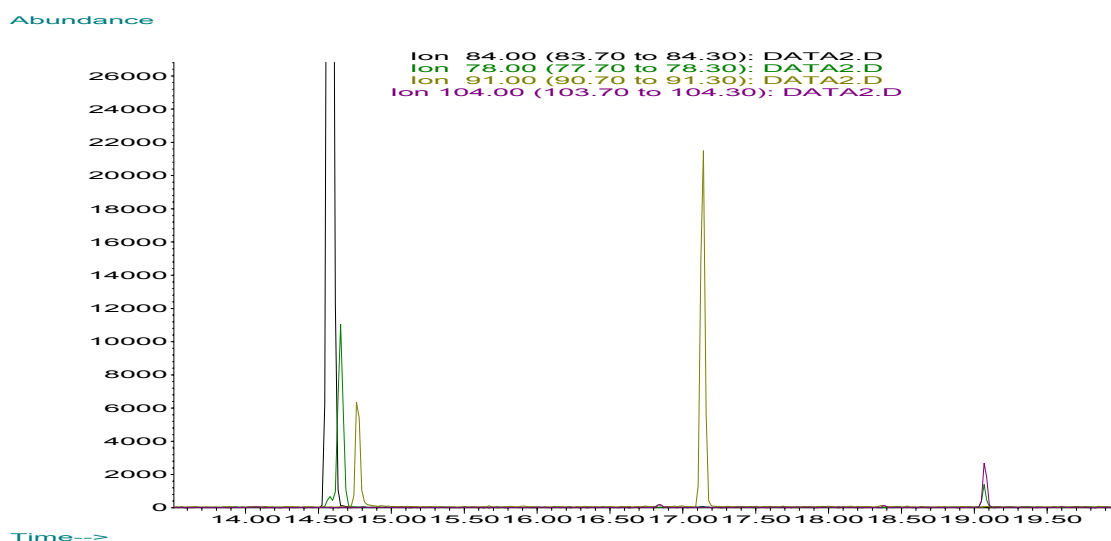


FIGURE IX.2 Example Ion Chromatogram: Low Standard. d6-benzene (14.6 minutes), Benzene (14.7 minutes), Toluene (17.2 minutes) and Styrene (19.1 minutes)

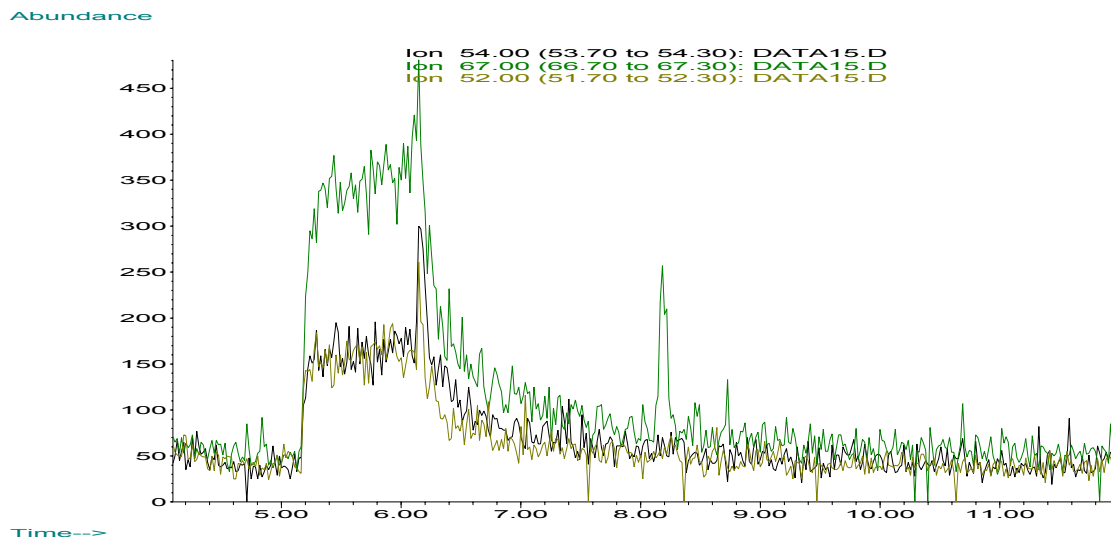


FIGURE IX.3 Example Ion Chromatogram: Mainstream Method Blank.

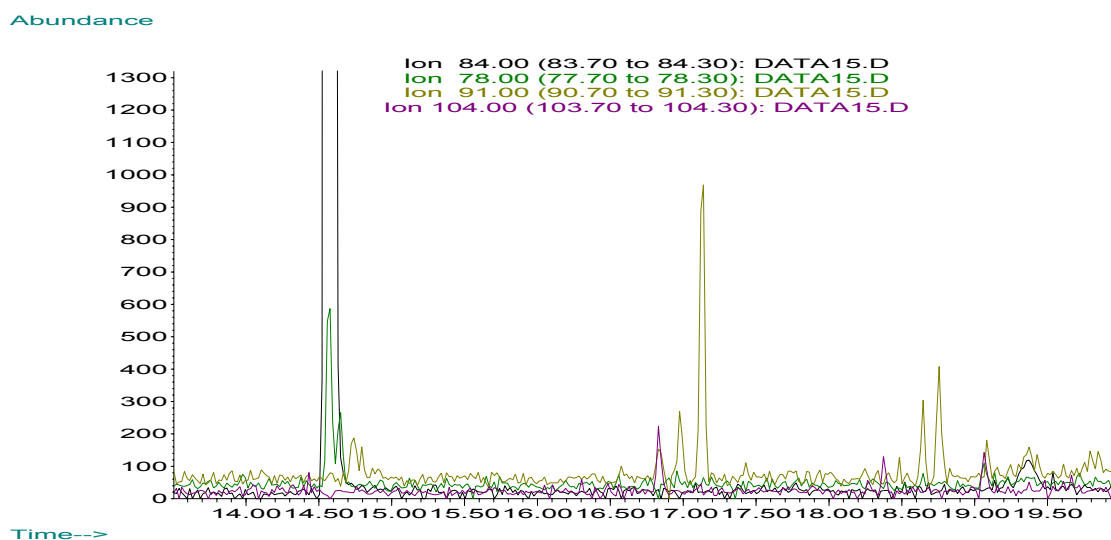


FIGURE IX.4 Example Ion Chromatogram: Mainstream Method Blank.

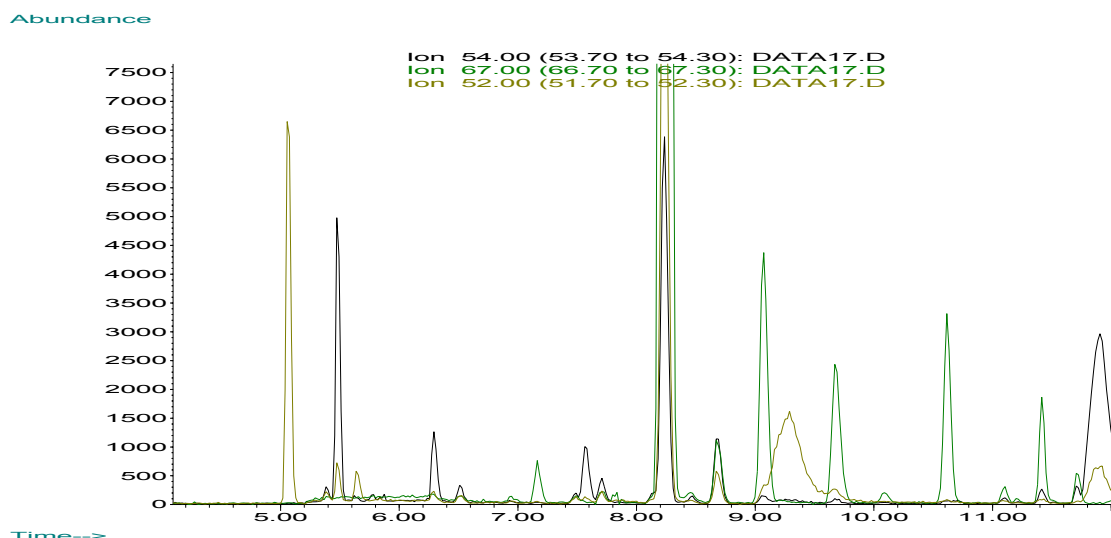


FIGURE IX.5 Ion Chromatogram of MS Sample Extract for 1,3-butadiene (5.44 minutes), Isoprene (8.25 minutes) and Acrylonitrile (9.3 minutes)

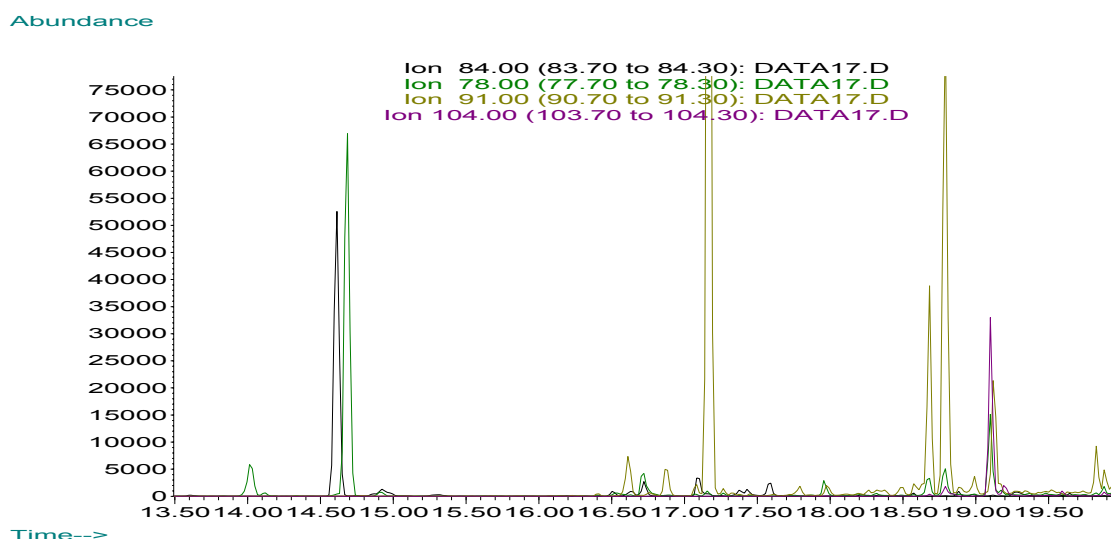


FIGURE IX.6 Ion Chromatogram of MS Sample Extract for d6-benzene (14.6 minutes), Benzene (14.7 minutes), Toluene (17.2 minutes) and Styrene (19.1 minutes)

VIII. Data Reduction and Example Calculations

This section describes the calculations for the calibration of the GC/MSD and the method used for determining the amount of each analyte per cigarette.

A. Instrument Calibration Calculations

The analysis is carried out using the calibration capabilities of the Hewlett-Packard Chemstation software and assumes the operator is already familiar with the procedure for setting up a calibration. The linear regression ($y = ax + b$) is used with equal weighting.

B. Data Reduction of Raw Data from GC/MSD

The analyte concentration is determined by the internal standard calibration method using the regression equation derived from the calibration curve. The concentration of each analyte is obtained by using the calculation capabilities of the HP Chemstation software and assumes the operator is already familiar with the software operation. The concentration of each analyte is manually entered into an Excel Spreadsheet where the number of cigarettes and the dilution volume are used to calculate the analyte delivery on a per cigarette basis.

Example Calculation:

$$\text{Analyte Delivery } (\mu\text{g/cig}) = \frac{\text{Analyte Conc. } (\mu\text{g/mL}) \times \text{Volume (mL)}}{\text{Number of Cigarettes}}$$

C. Data Acceptance

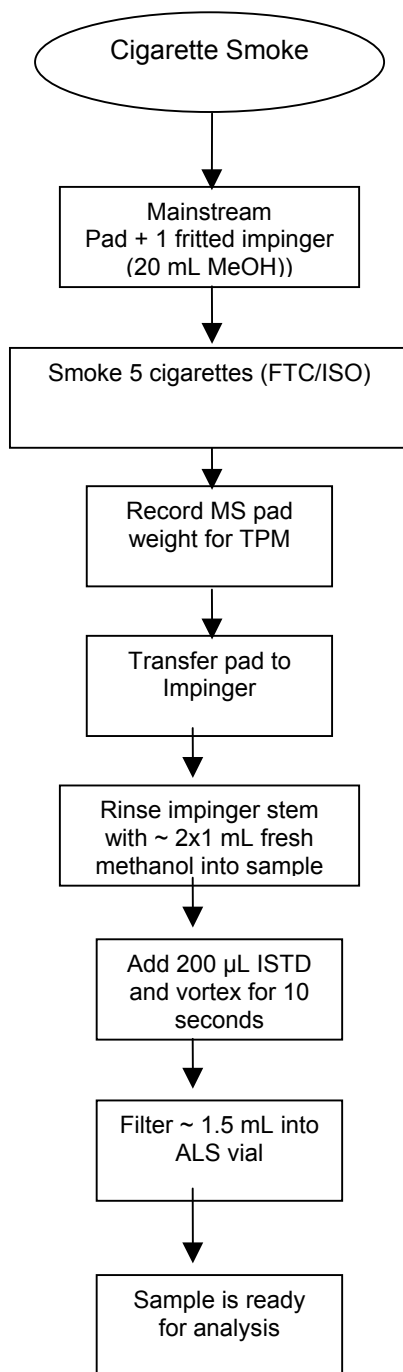
Each chromatogram is reviewed to confirm peak identification and integration using the Qedit software in the Chemstation package. If a peak must be manually integrated, the corrected value will be noted on the sample summary sheet.

D. Calibration and Quality Control Standard Acceptance Criteria

Each calibration curve must have a correlation coefficient (r^2) of 0.995 or better. Calibrations that do not meet these requirements should be brought to the attention of the lead chemist immediately. The analysis must stop, the problem must be corrected and the instrument re-calibrated.

All quality control standards must be within $\pm 10\%$ 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 $\pm 10\%$ 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 $\pm 10\%$ RE of its calculated value then sample analysis may continue. If the new quality control standard is not $\pm 10\%$ 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.

APPENDIX A SAMPLE COLLECTION AND WORKUP PROCEDURE



**APPENDIX B
REQUIRED CHEMICALS**

Use Table 1 for documentation of all required chemicals. Check labels carefully to ensure that all materials are current.

Table 1 – Chemicals

Chemical	Supplier	Grade or Purity
Methanol	EM Science or Equivalent	Distilled-in-Glass
Benzene-d6	CDN Isotopes Catalog # D-6	≥ 99.5 %
1,3-butadiene	Sigma-Aldrich Catalog # 29,503-5	≥ 99.0 %
Isoprene	Acros Catalog # 12267-0050	≥ 99.0 %
Acrylonitrile	Acros Catalog # 14963-0050	≥ 99.5 %
Benzene	Acros Catalog # 16766-0250	≥ 99.5 %
Toluene	Acros Catalog # 42455-0250	≥ 99.5 %
Styrene	Acros Catalog # 22053-0050	≥ 99.5 %

- IPA (Isopropanol)
- Dry-Ice
- Ice cubes
- Water

APPENDIX C:
Part 1: PREPARATION OF INTERNAL STANDARD SOLUTION

1. Weigh approximately 1.0 gram of d6-benzene into a 10 mL volumetric flask. Record the actual amount to 0.0001 g. Dilute to volume with methanol and shake well. Store in freezer. Expires in 6 months.

Weight of d6-benzene: _____ Concentration: _____

2. ISTD Spiking Solution:

Allow the stock to warm to room temperature. Transfer 1.0 mL to a 100 mL volumetric flask and dilute to volume with methanol. Mix well. Transfer to four 25 mL amber vials with open caps and Teflon-lined septa. Store in freezer when not in use. Expires in 2 months.

Concentration of ISTD Solution: _____

3. Concentration of d6-benzene in samples:

Using a gas-tight syringe, or volumetric pipette, add 100 μ L of the ISTD spiking solution to every 10 mL of solution. For example, add 100 μ L to each calibration standard, 200 μ L to each MS sample and 1.0 mL to each SS sample. The concentration of ISTD in all samples and standards will be approximately 10.00 μ g/mL.

Concentration of ISTD in samples and standards: _____

An Excel spread sheet data file is used to calculate standard concentrations. Tables 2 through 4 are examples of spreadsheets.

Part 2: PREPARATION OF CALIBRATION STANDARDS

A. Preparation of Volatile Stock Standard Solutions (except 1,3-butadiene)

The storage conditions for the standard solutions are in amber vials with Teflon-lined caps at $\leq -5^{\circ}\text{C}$. The stocks expire 2 months after preparation.

Weigh the approximate amounts listed below to the nearest 0.0001 g into separate 10-mL volumetric flasks (Class A). Fill each to the mark with methanol and mix well. Note: Isoprene, especially, should be weighed while still COLD. Perform dilutions according to the table below. Note: Volumes may be adjusted so that the calibration ranges remain consistent from batch to batch.

Table 2 – Excel Spreadsheet for recording Volatiles Standards Solutions

Volatiles Standards Solutions							
1° Stock Solutions							
	<u>Target Wt.</u>	<u>Wt (mg)</u>	<u>Purity</u>	<u>Volume ml</u>	<u>Conc ug/ml</u>		
Isoprene	100.0	153.8	0.99	10	15226.2		
Acrylonitrile	100.0	156.1	0.995	10	15532.0		
Benzene	100.0	142.7	0.999	10	14255.7		
Toluene	100.0	175.3	0.999	10	17512.5		
Styrene	100.0	158.1	0.999	10	15794.2		
2° Stock Solution							
	<u>Vol 1° Stock (mL)</u>			<u>2° Stock Volume ml</u>	<u>2° Stock Conc ug/ml</u>		
Isoprene	7.0			100	1065.8		
Acrylonitrile	0.5			100	77.7		
Benzene	1.0			100	142.6		
Toluene	2.0			100	350.2		
Styrene	0.5			100	79.0		
Working Standards (ug/mL)				Add 100 uL ISTD to each			
<u>Label</u>	<u>Volume 2° Stock(mL)</u>	<u>Volume ml</u>	<u>Isoprene ug/ml</u>	<u>Acrylonitrile ug/ml</u>	<u>Benzene ug/ml</u>	<u>Toluene ug/ml</u>	<u>Styrene ug/ml</u>
S-1	0.05	10	5.329	0.388	0.713	1.751	0.395
S-2	0.1	10	10.66	0.777	1.426	3.502	0.790
S-3	0.5	10	53.29	3.883	7.128	17.51	3.949
S-4	1	10	106.6	7.766	14.26	35.02	7.897
S-5	2	10	213.2	15.53	28.51	70.05	15.79

Part 3: PREPARATION OF 1,3-BUTADIENE STANDARDS

A. Preparation of 1,3-butadiene Stock Solution

Make fresh stock solution weekly and any time fresh calibration standards are required.

1. Add 20 mL of methanol to a 50-mL volumetric flask. Stopper the flask and place on a tared balance. Wait approximately 10 minutes to ensure the weight is stable. Record the initial weight.
2. Attach a piece of tygon tubing to the 1,3-butadiene cylinder. Attach a fresh Pasteur pipette to the end. Turn on the valve very slightly and slowly lower the pipette tip into the methanol in the flask.
3. Allow the 1,3-butadiene to bubble gently for 1 minute. NOTE: If the bubbling is too vigorous, methanol may be lost.
4. Remove the tip from the solution, gently touching the sides of the flask to allow any methanol to drain back into the solution.
5. Turn off the valve and stopper the flask.
6. Swirl gently to mix the contents and place on the balance.

7. Watch until the weight is stable for 10 to 15 second.
8. Record the final weight and make to volume with methanol. Mix well.

B. Preparation of 1,3-butadiene Secondary Stock Solution

Calculate the concentration of the stock solution prepared in A. Dilute approximately 1.0 mL of the stock to 10 mL with methanol and record the volume of stock used. The exact volumes will depend on the concentration of the stock. Mix well. The calibration standards may be made separately from the other volatiles, or may be combined. Use the Excel Spreadsheet in Table 5 to calculate the concentrations. Volumes should be adjusted to maintain calibration range from approximately 1 to 30 µg/mL.

Table 3 – Excel Spreadsheet for recording 1,3-butadiene Standard Solutions

1,3-butadiene Standard Solutions				
1° Stock Solution				
	<u>Wt (g)</u>	<u>Purity</u>	<u>Volume mL</u>	<u>Conc ug/mL</u>
Flask + MeOH	56.5620			
+ 1,3-butadiene	56.8633			
1,3-butadiene(mg)	301.3	0.99	50	5965.7
2° Stock Solution				
	<u>Vol 1° stock(mL)</u>		<u>2° Stock Volume mL</u>	<u>2° Stock Conc ug/mL</u>
1,3-butadiene	0.5		10	298.3
Working Standards (ug/mL)				
	<u>Volume 2° Stock (mL)</u>		<u>Volume mL</u>	<u>1,3-but ug/mL</u>
<u>Label</u>				
S-1	0.05		10	1.491
S-2	0.1		10	2.98
S-3	0.25		10	7.46
S-4	0.5		10	14.91
S-5	1		10	29.8

Part 4: PREPARATION OF QC STANDARDS

Volatiles QC Stock Solution expires when there is no longer agreement or there is a discernible change in the agreement between the calibration standards and the QC standards. (Maximum 2 months) QC Check standards are made weekly using the current QC Stock Solution and a fresh stock 1,3-butadiene solution.

Use Class A pipettes or Gas-tight syringe to prepare the QC standards in 50-mL volumetric flasks. Make to mark with methanol and mix well. All standards are stored in amber vials with Teflon-lined caps at $\leq -5^{\circ}\text{C}$.

Table 4 – Excel Spreadsheets for recording QC Stock and Check Standard Solutions

4 QC Stock Solution					QC stock	Aliquot	Date		
	<u>Target</u>	<u>Wt (mg)</u>	<u>Volume</u>	<u>Purity</u>	Conc	of Stock	QC check	vol of QC soln	50
					<u>ug/ml</u>		Conc	500 uL ISTD	
							<u>ug/ml</u>		
Isoprene	500.0	369.0	100	0.99	3653.1	1.0	73.1		
Acrylonitrile	50.0	55.5	100	0.995	552.2	1.0	11.0		
Benzene	100.0	100.0	100	0.999	999.0	1.0	20.0		
Toluene	200.0	219.6	100	0.999	2193.8	1.0	43.9		
Styrene	50.0	49.3	100	0.999	492.5	1.0	9.85		

4QC Stock Solution					QC stock	Aliquot	QC check		
	<u>Target Wt</u>	<u>Wt (mg)</u>	<u>Volume</u>	<u>Purity</u>	Conc	of Stock	Conc	vol of QC soln	50
					<u>ug/ml</u>	<u>mL</u>	<u>ug/ml</u>	500 uL ISTD	
1,3-butadiene	100	239.2	50	0.99	4736.2	0.1	9.47		
Flask + MeOH		56.7820							
+ 1,3-butadiene		57.0212							
1,3-butadiene(mg)		239.2							