

**Analysis of Mainstream Smoke for
Formaldehyde, Acetaldehyde, Acetone,
Acrolein, Propionaldehyde,
Crotonaldehyde, Methyl Ethyl Ketone &
Butyraldehyde**

Research, Development & Engineering
Philip Morris USA
Richmond, VA

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**Analysis of Mainstream Smoke for Formaldehyde,
Acetaldehyde, Acetone, Acrolein, Propionaldehyde, Crotonaldehyde,
Methyl Ethyl Ketone & Butyraldehyde**

I. PRINCIPLE OF MEASUREMENT

Mainstream smoke from the ISO-conditioned cigarettes, with 50% ventilation blocking, is generated under a puffing parameters of 45 mL puff volume with 2 second duration and once every 30 seconds as specified in the study protocol¹. The smoke is collected in an impinger assembly containing acidified 2,4-dinitrophenylhydrazine (DNPH) solution. After smoking, the combined solution from the impingers containing the derivatized carbonyls is stabilized by adding pyridine and subsequently analyzed by high performance liquid chromatography (HPLC). Individual aldehyde and ketone concentrations are determined by an external standard calibration using a diode array detector (DAD).

II. DEFINITIONS

1. HPLC: High Pressure Liquid Chromatograph
2. DNPH: 2,4- dinitrophenylhydrazine
3. MS: Mainstream smoke
4. TPM : Total Particulate Matter
5. Calibration Check Standard (CCS): Standard solution is prepared independent from the calibration standards. It is used to check the instrument stability after the calibration and during sample analysis. Minimum two concentrations, high and low, are prepared.
6. Percent relative concentration residue (%RCR) is calculated to show the degree of deviation of individual concentration points to the established calibration equation.
$$\%RCR = 100 * (RC - NC)/NC$$

RC = Back calculated concentration NC = Nominal concentration
7. RF = Response factor
8. CF = Cambridge Filter
9. RSD = Relative standard deviation
10. MW = Molecular weight
11. DAD = Diode Array Detector
13. THF = Tetrahydrofuran
14. IPA = Isopropanol

III. SAMPL REQUIREMENTS

Sample required per determination: 1 cigarette (TPM > 2 mg/cigt)
3 cigarettes (TPM < 2 mg/cigt)

IV. APPARATUS, CHEMICALS, REAGENTS AND INSTRUMENTATION

A. Apparatus and Instrument

1. Five-port smoking machine, KC Automation, Richmond, VA.
2. Hewlett-Packard 1090 HPLC equipped with autoinjector and a Diode Array Detector and a computerized data station (ChemStation A.05.01) for data acquisition and reduction, Hewlett-Packard Co., Wilmington, DE.
3. HPLC column: Columbus 5 μ C18, 250 x 4.6 mm, 00G-4108-E0, Phenomenex, Torrance, CA.
4. Smoke collection traps, part # 030496, glass impinger with 24/40 female inlet and standard insert with 12/5 ball and socket, 24/40 joint, and 1.6 mm delivery orifice, Research Glass, Richmond, VA.
5. Cambridge Filter disposable assembly, PN 6004300, Gelman Sciences, Ann Arbor, MI.
6. Eppendorf Pipetters: 10 - 100 μ l, 100 - 1,000 μ l and 500-2,500 μ l, Model # 4810, Fisher Scientific, Atlanta, GA
7. Amber autosampler vials with crimp tops, #5181-3376, Hewlett-Packard, Wilmington, DE
8. Plastic transfer pipets, FisherBrand, 13-711-5R, Fisher Scientific, Atlanta, GA
9. Brinkman Dispensette, 25mL, 13-688-84, Fisher Scientific, Atlanta, GA.
10. Amber glass vial with Teflon-lined screw cap, # 03-339-11B, Fisher Scientific, Atlanta, GA.

B. Chemicals and Reagents

1. Formaldehyde DNPH derivative, F2347, 99% purity, Chem Service, West Chester, PA
2. Acetaldehyde DNPH derivative, F2338, 99% purity, Chem Service, West Chester, PA
3. Acrolein DNPH derivative, F2340, 98% purity, Chem Service, West Chester, PA
4. Acetone DNPH derivative, F2339, 99% purity, Chem Service, West Chester, PA

5. Propionaldehyde DNPH derivative, F2345, 98% purity, Chem Service, West Chester, PA
6. Crotonaldehyde DNPH derivative, F2343, 99% purity, Chem Service, West Chester, PA
7. Butyraldehyde DNPH derivative, F2342, 99% purity, Chem Service, West Chester, PA
8. Methyl Ethyl Ketone DNPH derivative, prepared in-house using recrystallized DNPH with >98% purity.
9. Perchloric acid (60-62%, sp.gr ~1.54, ~9.1N), Reagent A.C.S., A228-6, Fisher Scientific, Atlanta, GA.
10. Methanol, HPLC grade, A452-4, Fisher Scientific, Atlanta, GA.
11. Water, HPLC grade, Fisher Scientific, Atlanta, GA
12. Acetonitrile, HPLC grade, A998-4, Fisher Scientific, Atlanta, GA
13. DNPH, #D19930-3, Aldrich Chemical, Milwaukee, Wisconsin.

The DNPH is purified to reduce the contaminants by an in-house procedure (See Appendix).

14. Perchloric Acid Solution (1.82 M): Add 40 mL of perchloric acid to a 200 mL volumetric flask (Class A) containing approximately 100 mL of water. Fill to volume with water and mix well. Store at room temperature in an amber bottle. This solution is expired after four weeks.
15. DNPH trapping and derivatization solution (25 mmole/L): Weigh approximately 5 ± 0.1 g, known exactly, of the purified DNPH and add to a 1,000 mL volumetric flask (Class A) containing approximately 700 mL acetonitrile. Add 15 mL of 1.82 M perchloric acid solution to the flask and swirl till the DNPH crystals dissolved. Fill to mark with acetonitrile and mix well. Transfer this solution to a 1 gallon amber bottle, fitted with a Brinkman Dispensette, pre-set at 30-mL dispensing volume. This solution, stored under room temperature, is expired after 5 weeks.
16. Pyridine, Certified ACS grade, P368-500, Class 1B, Fisher Scientific, Atlanta, GA.

C. Preparation of Standards

NOTE: All of the primary standards and solutions, stored at approximately -20 °C, must be allowed to warm up to ambient temperature prior to use.

1. Preparation of a stock solution by weighing the specified DNPH derivatives in Table 1 to ± 5 mg, known exactly, into a 10 mL volumetric flask (Class A)

with approximately 5 mL acetonitrile. Swirl the flask gently till all the crystals dissolve. Fill to the mark with acetonitrile and mix well. The stock solution, store in an amber bottle in the freezer at -20° C, expires after 6 months.

Table 1: Carbonyl Stock Solution

Compounds	Weight (mg)	Purity (%)	Volume (mL)	Stock Solution (µg/mL)
Formaldehyd-DNPH	37.6	99	10.0	528.86
Acetaldehyd-DNPH	31.1	99	10.0	601.63
Acetone-DNPH	21.0	98	10.0	498.57
Acrolein-DNPH	11.4	99	10.0	265.85
Pronionaldehyd-DNPH	23.2	99	10.0	558.48
Crotonaldehyd-DNPH	12.0	99	10.0	332.06
Methyl Ethyl Ketone-DNPH	15.1	98	10.0	426.02
Butyraldehyd-DNPH	14.1	99	10.0	398.11

Standard concentrations are calculated based on the molar percentage of the aldehyde in the DNPH derivative:

$$\text{Analyte } (\mu\text{g/mL}) = (\text{mg weight of DNPH derivative}) \times (\text{MW of the aldehyde} / \text{MW of aldehyde-DNPH}) \times \text{Purity}\% \times 1/10 \text{ mL} \times 1000 (\text{ug/mg})$$

2. HP7686 PrepStation is used to prepare the calibration standards by performing the functions of dispensing, dilution and mixing. All the calibration standard solutions are stored in amber autosampler vials with crimp tops and teflon liners at -20 °C in a freezer. They can be used daily and stored when not in use. Septum caps need to be changed regularly after use. They are expired 2 weeks from the date of preparation. The calibration standards can also be prepared by equivalent manual dilution procedures using volumetric flasks.
 - a. Allow the stock solution to warm up to room temperature before dispensing. Using Eppendorf pipetter, pipet 1.50 mL stock solution into an amber autosampler vial sealed with a crimp top. Prepare 9 empty amber vials fixed with crimp tops and sample labels.
 - b. Run the program, MACARBONYLS.TSP (See Appendix). Load the vials onto the tray according to the program instruction. For details, see HP7686 Operating Manual².
 - c. The concentrations of the resulting six calibration standards are listed in Table 2.

Table 2: MS Aldehyde Calibration standards

Compounds	Std1 (ug/mL)	Std2 (ug/mL)	Std3 (ug/mL)	Std4 (ug/mL)	Std5 (ug/mL)	Std6 (ug/mL)
Formaldehyde	0.18	1.76	3.53	7.05	10.58	21.15
Acetaldehyde	0.20	2.01	4.01	8.02	12.03	24.07
Acetone	0.17	1.66	3.32	6.65	9.97	19.94
Acrolein	0.09	0.89	1.77	3.54	5.32	10.63
Propionaldehyde	0.19	1.86	3.72	7.45	11.17	22.34
Crotonaldehyde	0.11	1.11	2.21	4.43	6.64	13.28
Methyl Ethyl Ketone	0.14	1.42	2.84	5.68	8.52	17.04
Butyraldehyde	0.13	1.33	2.65	5.31	7.96	15.92

3. Preparation of Calibration Check Standards (CCS): Same procedure is used as in C.2.a – C.2.c to prepare the high and low check standards. The check standards are separately prepared from the stock solution.

Table 3: Concentrations of the CCS

Compounds	CCSL (ug/mL)	CCSH (ug/mL)
Formaldehyde	1.76	10.58
Acetaldehyde	2.01	12.03
Acetone	1.66	9.97
Acrolein	0.89	5.32
Propionaldehyde	1.86	11.17
Crotonaldehyde	1.11	6.64
Methyl Ethyl Ketone	1.42	8.52
Butyraldehyde	1.33	7.96

4. Calculate the concentration in $\mu\text{g/mL}$ for each calibration standard as follows:

$$\frac{(\text{concentration of the stock solution}) \times (\text{Volume of the stock solution used by HP7686})}{\text{Final volume used by HP7686}} = \mu\text{g/mL standard}$$

D. Instrument Conditions

1. HPLC Column: Columbus C18, 250 mm x 4.6mm
2. Flow rate: 1.2 mL / min.
3. Column temp: Ambient

4. Mobile Phase A: Acetonitrile/Water/THF/IPA³ (30/59/10/1%)
Mobile Phase B: Acetonitrile/Water (65/35%)
Mobile Phase C: Acetonitrile (100%)

5. HPLC mobile phase gradient:

Time (min)	A (%)	B (%)	C (%)
0	100	0	0
20	60	40	0
25	60	40	0
35	0	100	0
37	0	0	100
42	0	0	100

6. Stop and Post Time: 42 min / 5 min
7. Injection volume: 20 μ L
8. DAD setting: Sample 355 nm with bandwidth 4 nm / Reference 550 nm with bandwidth 80 nm
9. Retention time & integration:

Typical retention time of the analytes are shown in the chromatograms from the standard 2 (Figure 1) and a MS sample extract from 1R4F reference cigarettes (Figure 2). Typical integration parameters used to generate peak height data for quantitation are tabulated in Table 3

Table 3: Typical Integration Parameters

Events	Value	Time (min)
Peak width	0.18	initial
Slope sensitivity	0.45	initial
Height reject	0	initial
Integrator OFF		0.00
Integrator ON		10.0
Fixed peak width	0.5	29
Integration OFF		37

Under the HPLC conditions, the syn and anti forms of acetaldehyde are separated (See Figures 1 & 2), the sum of their response is used in the quantitation.

V. PROCEDURE

A. Preparation of Calibration Curve

1. Transfer the six calibration standards, prepared by the HP7686 PrepStation, to the sample tray of the HP1090 HPLC. For details, consult the HP1090 user manual⁴.
2. Using Eppendorf Pipetter, transfer 1.0 mL of DNPH reagent to an amber autosampler vial and add 50.0 uL of pyridine. Cap the vial. This is the DNPH reagent blank.
3. At the ChemStation, construct a sequence beginning with an injection of the DNPH reagent blank and follow by the calibration standards arranged from low to high concentrations. Sample names and the HPLC method are specified. "Replace" is selected for retention time and RF update for each level of calibration. Linear regression ($y = ax + b$) with equal weighting and inclusion of origin (intercept) are specified in the instrument method. Y is the peak height and X is the concentration (ug/mL) of the standards
4. Load the HPLC method from ChemStation and turn on the pump and DAD detector. Wait till a stable baseline is observed. This will take approximately 15 minutes. Start the sequence for calibration.
5. The acceptance or rejection of the calibration run is determined by calculating the %RCR, examining the correlation coefficient and visual inspection of the calibration curves. %RCR shall be within $\pm 10\%$ and the correlation coefficient no less than 0.999. Calibration that do not meet the requirements shall be brought to the attention of the senior laboratory personnel for proper corrective action.
6. The instrument is calibrated at least every 2 to 3 days. Calibration check standards are always analyzed after every 6 smoke samples to ensure the validity of the calibration. Smoke samples are bracketed by the low and high CCS. %RCR for the check standards shall be within $\pm 10\%$ or the problem shall be brought to the attention of the senior laboratory personnel for proper corrective action.

B. Sample Analysis

1. Follow the random run order of the samples as specified in the study protocol¹.
2. Prior to smoking, cigarettes must be conditioned as specified in ISO 3402. Fifty percent of the cigarette ventilation shall be blocked by taping.

3. Set up the 5-port smoking machine with the puffing parameters¹ of 45 ± 0.5 mL puff volume, 2 ± 0.1 seconds puff duration and 30 ± 1 seconds puff interval.. For operational details, see 5-port operation manual⁵.
5. Dispense 30.0 mL of DNPH solution to each trap and fasten the two impingers with a clamp. Connect the impinger assembly to the smoking machine. Prior to each smoking, a leak and puff volume check must be performed and recorded on the smoking request record sheet.
6. Start smoking machine and light the cigarette with matches. The smoking is terminated when tipping + 3 mm line is reached. Record the puff count.
7. After removal of the cigarette, take 6-8 clearing puffs. Disconnect the impingers from the machine and shake gently to rinse the inside wall
8. Pipet 1.0 mL the smoke extract into an amber vial with teflon-lined screw cap and add 50 μ L pyridine using the appropriate Eppendorf Pipetters. Cap the vial and mix well.
9. Inject the sample by initiating 'RUN SEQUENCE' command at the ChemStation after loading the sample vial to the autosampler tray.
10. Blank samples shall be analyzed regularly (2-3 times per week). It is generated by puffing unlit 1R4F cigarette under identical conditions. A typical chromatogram of a blank sample is shown in Figure 3.
11. Acceptance of the results from test cigarettes relies on the internal quality control of the calibration standards and CCS. See VI.A.5 and 6 for acceptance criteria.

C. Calculations

The analyte concentration is determined by an external standard method using the regression equation derived from the calibration curve. Calculation of each specific analyte is obtained using the following equation:

Analyte (μ g/cigt) =

$$\frac{\mu\text{g/mL (from instrument report)} \times 1.05 \text{ (dilution factor)} \times 60\text{mL (volume from traps)}}{(\# \text{ of cigarette})}$$

VI. REFERENCES

1. Massachusetts Constituents Testing Study Protocol, April 1999.
2. HP7686 PrepStation Operating Manual, Hewlett-Packard, March 1994.
3. Determination of Selected Carbonyls in Mainstream Smoke, No. MS-14, Proposed Analytical Methods, Health Canada.
4. HP1090L Operating Manual, Hewlett-Packard, April 1990.
4. Five-port Smoking Machine Manual, KC Automation, July 1997.

VI. REFERENCES

Figure 1: A typical chromatogram from the calibration standard.

Figure 2: A typical mainstream chromatogram for 1R4F

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Figure 3: A Typical system blank by puffing the unlit 1R4F

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