UHPLC Separation with MS Analysis for Eight Carbonyl Compounds in Mainstream Tobacco Smoke

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Abstract

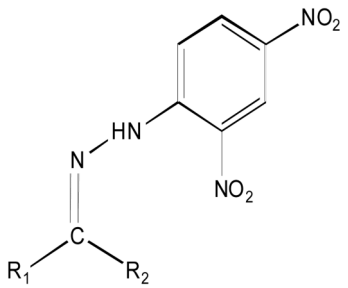
A method to quantify eight carbonyl compounds in mainstream cigarette smoke is presented using ultra-high pressure liquid chromatography (UHPLC). The combination of UHPLC and mass spectrometry (UHPLC–MS) dramatically reduces analysis times as compared to the current in-house high-performance liquid chromatography (HPLC)–UV method. In addition, improved detector selectivity and peak resolution are observed. Sample analysis times are reduced from 47 min with HPLC–UV to less than 5 min using this improved method. Atmospheric pressure chemical ionization, atmospheric pressure photo ionization, and electrospray ionization are directly compared to evaluate ionization potential and linear response range for the carbonyl 2,4-dinitrophenylhydrazine derivatives. Smoke extracts from three standard smoking protocols are analyzed by both UHPLC–MS and HPLC–UV for method comparison purposes.

Introduction

Quantitative analysis of trace level carbonyl compounds from mainstream smoke samples is especially complex and time-consuming. Complexities associated with the analysis of these compounds include volatility and instability at high temperatures or in acidic environments. Consequently, carbonyl compounds are typically derivatized with 2,4-dinitrophenylhydrazine (DNPH) to improve stability (Figure 1). These DNPH derivatives are more thermally stable, allowing for analysis by gas chromatography (GC). Quantitative analysis by GC coupled with mass spectrometry (MS) for as many as eleven carbonyl-DNPH derivatives in mainstream cigarette smoke was previously reported (1). In addition to increased stability, DNPH derivatization improves chromatographic properties and increases UV absorptivity for analysis by high-performance liquid chromatography (HPLC)–UV. However, most methods for the quantitation of multiple carbonyls-DNPH derivatives require long analysis times and have limited selectivity, especially in complex matrices. For example, lengthy chromatographic separations using HPLC–UV, which can be as long as 60 min, are described for the separation of DNPH derivatives of commonly occurring carbonyls in air and water (2–6). HPLC coupled with mass spectrometry (LC–MS) has better selectivity and sensitivity than UV absorbance, allowing for faster analysis times. Several methods have been published for the analysis of carbonyls in environmental samples using LC–MS with various modes of ionization (7–11).

Among the methods previously reported for the analysis of carbonyls in smoke, many issues remain unresolved (1). Smoke is an extremely complex matrix, reported to contain over 4000 compounds (1,12). The complexity of the sample matrix may result in possible interferences that are unresolved by HPLC–UV. In addition, the DNPH derivatization of carbonyls results in the formation of E and Z stereoisomers, which can be chromatographically separated by HPLC when the extracts are acidified (4,10). Studies indicate that these isomers are not formed in racemic amounts and have different UV absorbance maxima (4,10). If an HPLC–UV method only quantitates for a single isomer or if the detection wavelength is not optimized for each isomer, under-reporting may occur.

Jorgenson and colleagues reported high-resolution separations with a system capable of operating at ultra-high pressures and with sub-2 µm particle bonded-phase columns (13). UHPLC



	$-R_1$	$-R_2$
Formaldehyde	-H	-H
Acetaldehyde	-H	$-CH_3$
Acetone	$-CH_3$	$-CH_3$
Acrolein	-H	-CH=CH2
Propionaldehyde	-H	$-CH_2-CH_3$
Crotonaldehyde	-H	$-CH=CH-CH_3$
Methyl Ethyl Ketone	-CH3	$-CH_2-CH_3$
Butylaldehyde	-H	$-CH_2-CH_2-CH_3$

Figure 1. Structure of carbonyl compounds after derivatization with DNPH.

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has been employed to achieve higher-resolution separations in shorter analysis times when compared to conventional HPLC (13–16). Analysis of carbonyls in tobacco smoke could be achieved with greater efficiency, selectivity, and higher sensitivity through the use of UHPLC coupled with MS.

This is the first reported demonstration of UHPLC–MS applied to the analysis of carbonyl compounds in mainstream cigarette smoke. This method includes the analysis of eight carbonyl compounds: formaldehyde, acetaldehyde, acetone, acrolein, crotonaldehyde, butylaldehyde, and methyl ethyl ketone (MEK). Data is presented for a direct comparisons of quantification, precision, and reproducibility between HPLC–UV and UHPLC–MS. Although this paper is focused on the analysis of mainstream tobacco smoke, the chromatographic separation and detection techniques could be applied to the analysis of carbonyls in environmental samples and consumer related products.

Experimental

Reagents

A combined stock standard solution containing DNPH derivatives of formaldehyde, acetaldehyde, acetone, acrolein, propionaldehyde, crotonaldehyde, methyl ethyl ketone, and butylaldehyde was purchased from ChemService (West Chester, PA). DNPH derivatives of isotopically labeled internal standards acetone-D6 and methyl ethyl ketone-D3 were also obtained from ChemService. Isobutylaldehyde and methacrolein standards from ChemService were derivatized with DNPH and used as chromatographic interference checks. DNPH (97% purity) was purchased from Aldrich Chemical Company (Milwaukee, WI). All other chemicals were purchased from Fisher Scientific (Atlanta, GA), which includes anhydrous ammonium acetate, HPLC-grade tetrahydrofuran (THF), Optima-grade acetonitrile (ACN), HPLC-grade methanol, HPLC-grade isopropanol (IPA), ACS reagent-grade perchloric acid (60–62%, ~ 9.1 N), and certified ACS-grade pyridine.

Table I. Gradient Conditions for UHPLC Separation

Time (min)	10 mM Ammonium Acetate(%)	ACN (%)
0.0	65	35
3.2	40	60
3.7	40	60
4.0	65	35

Table II. Selective Ions Monitored for DNPH Derivatives

Analytes	m/z	Analytes	*m/z*
Formaldehyde-DNPH	209.1	Propionaldehyde-DNPH	237.1
Acetaldehyde-DNPH	223.1	Crotonaldehyde-DNPH	249.2
Acrolein-DNPH	235.1	Methyl ethyl ketone-DNPH	251.1
Acetone-DNPH	237.1	Butylaldehyde-DNPH	251.1
Internal standards			
Acetone-d_6-DNPH	243.1		
Methyl ethyl ketone-d_3-DNPH	254.1		

Table III. Gradient Conditions for HPLC Separation

Time (min)	ACN–Water–THF–IPA (30:59:10:1, %)	ACN–Water (65:35, %)	ACN (%)
0.0	100	0	0
20	60	40	0
25	60	40	0
35	0	100	0
37	0	0	100
42	0	0	100

Instrumentation

For UHPLC–MS analysis, a Waters Acquity Ultra-Performance LC (Milford, MA) with a Sample Manager and Binary Solvent Manager interfaced to a Waters Micromass Quattro Premier triple quadrupole mass spectrometer was used. Ionization sources included electrospray (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo ionization sources (APPI). UHPLC separations were conducted using a Waters Acquity UPLC BEH C_{18} column (2.1 × 50 mm, 1.7 µm) under the mobile phase and gradient conditions shown in Table I. LC method parameters include a flow rate of 0.5 mL/min and a 5 µL injection volume.

Negative electrospray ionization was used under single MS mode with selective ion recording (SIR) at the appropriate mass-to-charge ratio (*m/z*) (Table II). Mass spectrometer settings were optimized for each compound to ensure optimal sensitivity: capillary voltage, cone voltage, corona voltage, desolvation temperature.

The comparative HPLC method employed an Agilent Technologies HP1100 LC consisting of a low-pressure quaternary pump, autosampler, and diode array detector. HPLC separations were conducted using a Columbus C_{18} column (4.6 × 250 mm, 5 µm, Phenomenex, Torrence, CA) under mobile phase and gradient conditions shown in Table III. UV data was collected for formaldehyde at 355 nm with a 4 nm bandwidth and for the other carbonyls at 370 nm with a 4 nm band width. A 5 min post-run column re-equilibration time with Mobile Phase A was used. A 20 µL injection volume was analyzed for each sample.

Smoke Collection

A linear 5-port smoking machine was used for smoke collection (K.C. Automation; Richmond, VA). For each sample, the smoke from a single cigarette is drawn through two glass impingers (Part number 030496, Research Glass; Richmond, VA). Each impinger contained 30 mL of DNPH derivatization solution, which consists of 0.025 M DNPH and 0.027 M perchloric acid in acetonitrile. Before smoking, all cigarettes were conditioned at 75 ± 2°F with 60 %RH ± 2 %RH for 24 h prior to smoking (17). The laboratory conditions during smoking were 75 ± 2°F with 60% RH ± 5%RH (17). The following standardized smoking parameters were used for testing. Federal Trade Commission (FTC): Puff volume 35 mL ± 0.3; puff duration 2.0 ± 0.1 s with a 60.0 ± 1 s puff interval. No cigarette ventilation holes were blocked. A Sine wave puff profile was used (17).

Massachusetts Department of Public Health (MDPH): Puff volume 45 mL ± 0.5, puff duration 2.0 ± 0.1 s with a 30.0 ± 1 s

puff interval. There was 50% blocking of cigarette ventilation holes. A Sine wave puff profile was used (18).

Health Canada Intense (HC): Puff volume 55 mL ± 0.5, puff duration 2.0 ± 0.1 s with a 30.0 ± 1 s puff interval. There was 100% blocking of ventilation holes. A Sine wave puff profile was used (19).

Immediately after smoking each sample, the two impingers were combined and mixed thoroughly. For the HPLC analysis, a 1000 µL aliquot of the sample is transferred into an autosampler vial and stabilized with the addition of 50 µL of pyridine. For analysis by UHPLC, a 100 µL aliquot of the sample is transferred into a separate autosampler vial and diluted with 900 µL of internal standard working solution (1 µg/mL) in acetonitrile containing 0.6% pyridine.

A 2R4F Kentucky reference cigarette obtained from the University of Kentucky, Kentucky Tobacco Research and Development Center was used for smoke collection using both methods. This reference cigarette was designed to replace the 1R4F Kentucky reference cigarette used by the tobacco industry for the analysis of compounds found in tobacco and mainstream smoke (20). There are several references that report data for specific constituents found in mainstream cigarette smoke using these reference cigarettes (1,20).

Results and Discussion

Chromatography

Due to limited detector selectivity between the carbonyl DNPH derivatives, previous HPLC–UV methods required lengthy run times to separate all analytes and structural isomers (1,7). In the analysis of mainstream smoke samples, there is the need for additional separation from matrix-related components and other peaks, some of which have been identified as stereoisomers. It has been previously reported that stereoisomers of the carbonyl DNPH derivatives are formed during derivatization and are chromatographically separated when acidified (4). Analysis of mainstream smoke samples for carbonyl-DNPH derivatives by the in-house HPLC–UV method (Figure 2) has a 47 min total analysis time. In this chromatogram of a mainstream smoke sample, the peaks of the individual constituents and their stereoisomers are identified, and unidentified peaks are related to the smoke matrix. Previous HPLC–UV methods did not account for the presence of these stereoisomers except for acetaldehyde, whose isomer is not fully baseline resolved and is quantified based on the sum of the two peak areas. The high degree of complexity associated with the analysis of smoke matrix and the need for adequate resolution between analyte peaks and interferences is apparent from this chromatogram. Long HPLC analysis times and the poor selectivity of the UV detection made it desirable to develop a more selective, accurate, and faster method for the quantitation of carbonyls in mainstream smoke.

A UHPLC–MS method for the analysis of mainstream smoke samples was developed using a sub-2 µm particle column and a triple quadrupole mass spectrometer. Narrow analyte elution bands resulting from small particle size and

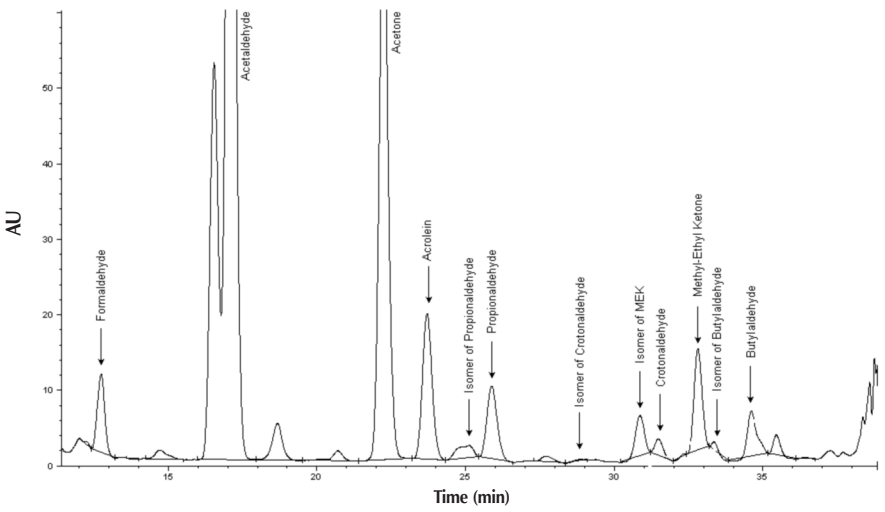

Figure 2. A HPLC–UV chromatogram DNPH derivatized sample extract of a mainstream smoke sample collected by FTC parameters for a reference 2R4F cigarette at 370 nm.

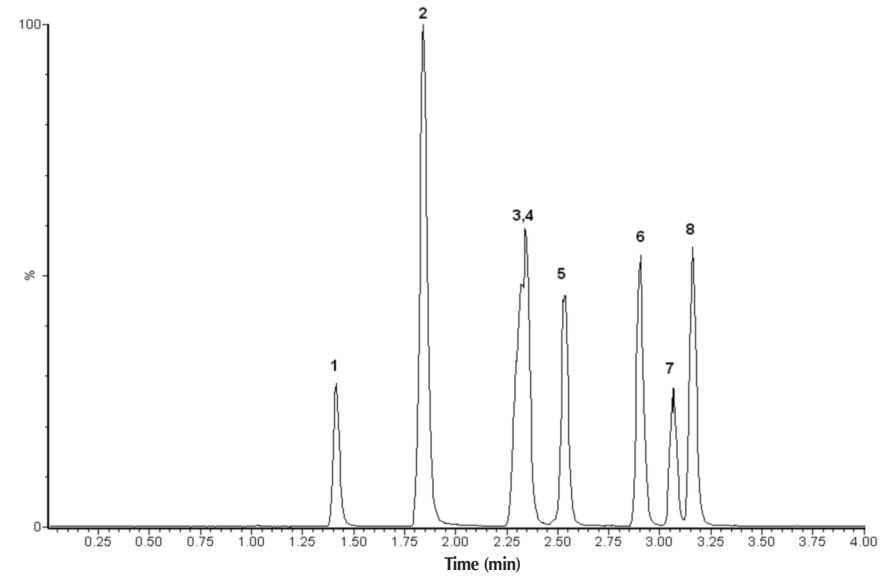

Figure 3. A SIM chromatogram of a DNPH derivatized sample analyzed by UHPLC with negative electrospray ionization: 1, formaldehyde; 2, acetaldehyde; 3, acetone; 4, acrolein; 5, propionaldehyde; 6, crotonaldehyde; 7, methyl ethyl ketone; and 8, butylaldehyde.

reduced dead volume pre- and post column improve the response of analytes independent of detection technique. The combination of UHPLC–MS dramatically improves sensitivity and selectivity over the previously used UV detection method. For example, in the improved method the samples and standards required a 10-fold dilution to remain within the linear dynamic range of the LC–MS detector. Figure 3 is a typical chromatogram for a smoke extract injected under UHPLC conditions with negative electrospray ionization and using a mass selective detector in SIM mode. A major difference seen between this chromatographic separation and the HPLC–UV separation shown in Figure 2 is the stereoisomers formed during the derivatization co-elute, and there are no visible matrix related peaks. Although some analytes and matrix components co-elute under these conditions they can be separated by their different *m/z*. Figure 4 shows a chromatogram after extraction of the individual mass ions for each analyte. The analytes that co-eluted are separated by *m/z*, acetone-DNPH (237.1 *m/z*) and acrolein-DNPH (235.1 *m/z*). Constituents with the same *m/z* ratio (MEK and butylaldehyde, 251.1 *m/z*) are sufficiently separated chromatographically. The total analysis time is only 4.5 min compared to 47 min with the HPLC–UV method. The analysis time is also four times faster than a previously reported LC–MS method with a similar number of analytes (8). Faster chromatography is a direct result of higher efficiencies obtained at faster flow rates using a 1.7-µm particle column. The flow rate used with a sub-2 µm particle column is optimal at higher linear velocities than observed with larger particle columns (13). The optimal mobile phase flow rates for a typical UHPLC separation are between 0.5–1.2 mL/min, which results in back pressures dramatically higher than for columns packed with larger particles. For this analysis, a flow rate of 0.5 mL/min was used, and back pressures as high as 10,000 psi were observed.

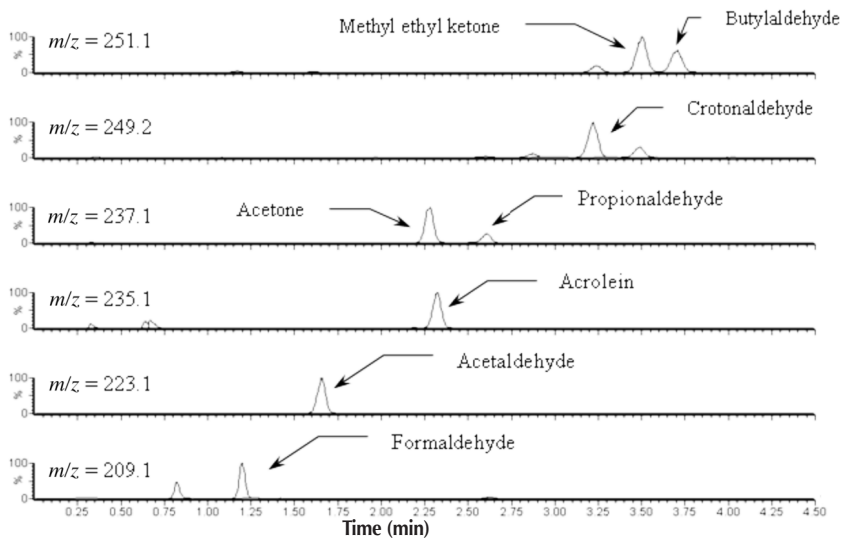

Figure 4. Extracted ion chromatograms of a DNPH derivatized mainstream smoke sample collected by FTC parameters for a reference 2R4F cigarette. Separation between all analytes and structural isomers has been achieved.

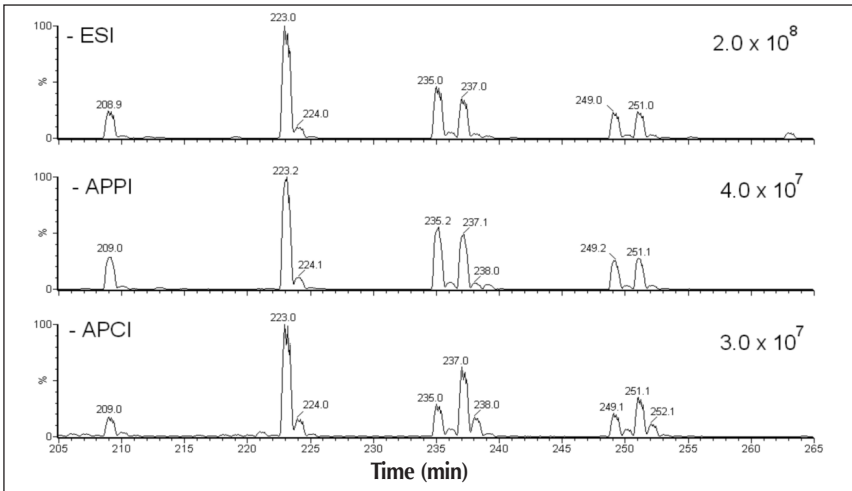

Figure 5. Comparison of negative ion mass spectra with different ionization techniques by direct infusion of carbonyl-DNPH tune solution at 10 µL/min with mobile phase. These mass spectra include the *m/z* range of the derivatized analytes and the absolute response in counts.

Ionization Comparisons

Several references have previously reported the use of APCI, APPI, and ESI as modes of ionization for DNPH derivatives of carbonyl compounds (7–11). None of the previous published work has compared all three ionization techniques to determine the differences in ionization potential. In this paper, we evaluated each ionization technique to determine which provided optimal sensitivity with the largest linear dynamic range. Levels of carbonyl constituents present in mainstream smoke vary dramatically, requiring the ability to quantify lower concentration constituents along with higher concentration constituents within the same analysis. Therefore, it was desirable to have a linear response across three orders of magnitude.

Figure 5 is a comparison of the analyte responses with different ionization modes by direct infusion of a tune solution into the LC–MS system. It is apparent that APPI without the use of a dopant is more sensitive than APCI under the same mobile phase conditions. Experiments conducted with negative electrospray ionization (ESI) and 10 mM ammonium acetate–acetonitrile as the mobile phase had the most intense response for all analytes and almost 10-fold higher intensities over APCI and APPI. Careful examination of Figure 5 shows differences in individual mass spectra for absolute peak intensities of individual ions as well. Although negative ESI is much more sensitive for ionization, there was lower background observed when using APPI ionization, which might be the result of a more selective mode of ionization. In addition, the APCI spectrum does not produce a very stable mass spectrum and shows an increase in M-ions.

The linearity of analyte response for dif-

ferent modes of ionization was evaluated to determine ionization efficiency. A linear response is desirable for accurate quantitation of analytes across a given range. In order to overcome non-linearity issues related to ionization inefficiency, samples could be diluted or the concentration range could be truncated. However, for this application, it was desirable to have a single preparation for all samples collected under both FTC and Health Canada smoking regimes. Further, the current range required for the analysis of carbonyls in whole smoke is different for each analyte due to higher levels of some constituents in cigarette smoke than others. The resulting dynamic range required for a single analysis from a smoke sample is actually large. The levels of acetaldehyde detected in mainstream smoke under HC conditions for 2R4F are approximately 1370 µg/cig, but the levels of formaldehyde under FTC conditions are 65 times lower at approximately 21 µg/cig. The desired calibration range for formaldehyde was 0.022–4.48 µg/mL and 0.134–26.7 µg/mL for acetaldehyde, taking into account the collection of one cigarette into 60 mL of DNPH solution. Therefore, the actual range of quantitation that is needed must encompass a limit of quantitation for formaldehyde that is 0.022 µg/mL while still being practical for the quantitation of acetaldehyde at 26.7 µg/mL for a total of three orders of magnitude.

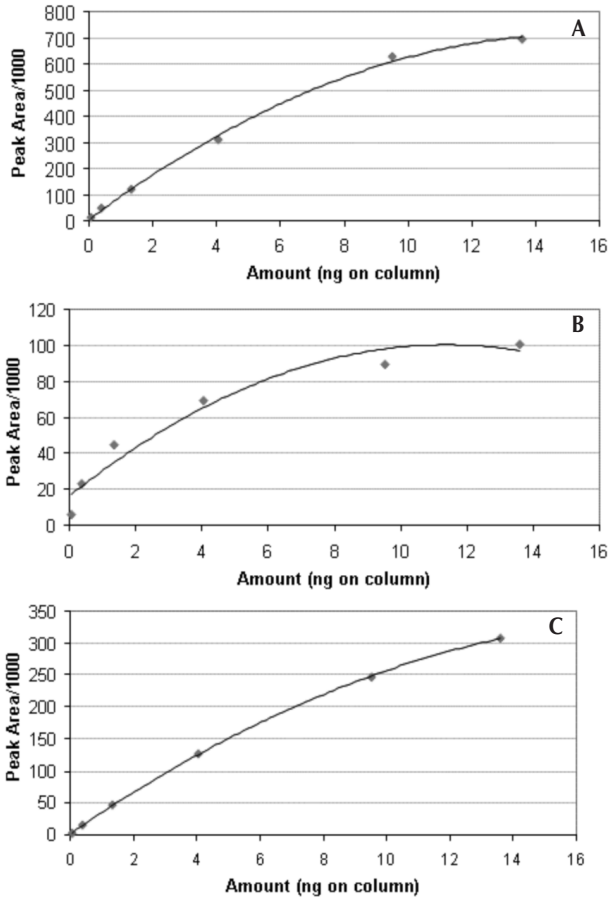

Figure 6. Plots of external calibrations of acetaldehyde using different ionization modes for sample introduction to the mass spectrometer: (A) APCI, (B) APPI, and (C) ESI.

Prior to the investigation of the linear response, the lowest calibration standard was injected to ensure adequate sensitivity was achieved. The linear response for acetaldehyde was evaluated because this analyte is at the highest concentration and is most affected by ionization saturation. The other analytes are less affected by ionization saturation due to their lower concentrations levels. APPI appears to have the shortest dynamic linear range regardless of concentration of the standards. Negative APCI has a wider dynamic range; however, the signal-to-noise (s/n) ratio at the limit of quantitation (LOQ) for formaldehyde is less than 10:1, which is undesirable. The best results were obtained under negative ESI and the dynamic range is almost linear for acetaldehyde with acceptable s/n for all analytes at the LOQ level. Figure 6 shows a comparison of standards injected under the three different modes of ionization. A quadratic fit with $1/x$ concentration weighting was used for each calibration model. This provided correlation coefficients greater than 0.9940 and had average percent difference from theoretical values less than 15% for all analytes.

Method Comparison

The HPLC–UV method for the analysis of carbonyls in mainstream smoke was previously validated with respect to precision, recovery, selectivity, and stability of extracts. To ensure that the constituents could be quantified with different smoking regimes, the method was validated using 2R4F reference cigarettes smoked according to three different smoking regimes; FTC, Massachusetts Department of Public Health, and Health Canada Intense. In order to compare the new method to the previously validated method, six replicates of smoke samples were collected and analyzed over three days under all three smoking regimes. Samples were analyzed using both the HPLC–UV and UHPLC–MS methods. The calculated concentrations for each

Table IV. Method Comparison Between UHPLC–MS and HPLC–UV*

Smoking Regime	Analysis	Formaldehyde (µg/cig)	Acetaldehyde (µg/cig)	Acetone (µg/cig)	Acrolein (µg/cig)	Propionaldehyde (µg/cig)	Crotonaldehyde (µg/cig)	MEK (µg/cig)	Butylaldehyde (µg/cig)
FTC	UHPLC–MS	23.9 (± 3.17)	601 (± 41.6)	356 (± 22.1)	65.0 (± 6.39)	47.9 (± 3.52)	17.6 (± 2.34)	83.3 (± 6.67)	31.7 (± 2.47)
	HPLC–UV	21.3 (± 2.75)	610 (± 47.5)	330 (± 23.8)	57.7 (± 5.54)	45.5 (± 3.20)	14.5 (± 1.85)	80.9 (± 8.20)	32.8 (± 5.78)
MDPH	UHPLC–MS	46.9 (± 6.56)	1059 (± 62.2)	635 (±39.2)	131 (± 13.2)	95.4 (± 6.30)	51.9 (± 4.58)	166 (± 10.4)	62.5 (± 3.38)
	HPLC–UV	42.1 (± 4.49)	1182 (± 65.6)	603 (± 30.9)	119 (± 7.19)	88.7 (± 4.58)	40.3 (± 3.05)	181 (± 15.4)	66.4 (± 11.7)
Health Canada	UHPLC–MS	74.3 (± 6.50)	1369 (± 59.1)	773 (± 34.9)	168 (± 17.4)	120 (± 11.2)	73.0 (± 6.30)	206 (± 9.12)	77.9 (± 6.73)
	HPLC–UV	68.8 (± 5.38)	1530 (± 56.5)	742 (± 26.1)	156 (± 5.71)	112 (± 4.12)	56.3 (± 3.10)	228 (± 18.5)	91.3 (± 19.7)

* Reporting average inter-assay means and standard deviations for a 2R4F cigarette smoked under specified conditions (n = 6): † Mean (± 1 SD).

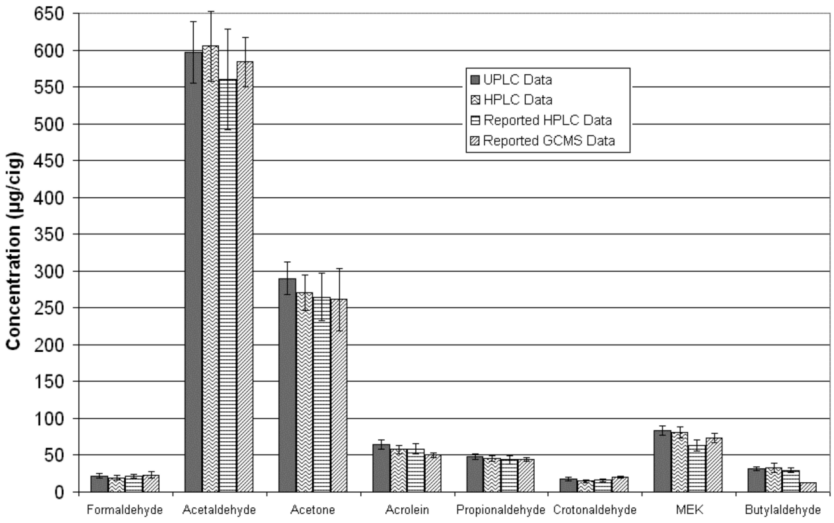

Figure 7. FTC, 2R4F data from precision experiments on UHPLC and HPLC systems. Comparison data shown is from previously reported results for HPLC and GC–MS studies under FTC conditions. Error bars represent one standard deviation.

constituent in mainstream smoke are presented in Table IV for all three smoking regimes. The data show good agreement between the two techniques. Further the results have good agreement and consistency for all constituents with previously reported results (Figure 7). The only difference is apparent in the GC–MS data for butylaldehyde, which is lower than for the other techniques and has been linked to the separation between isobutylaldehyde and butylaldehyde structural isomers (1). These isomers are not separated by HPLC or UHPLC. Additional work was conducted to try to resolve these isomers chromatographically but was unsuccessful. Because they have the same molecular ions and the same product fragments, they cannot be resolved by tandem MS. While it is not shown, there was acceptable separation between two other structural isomers methacrolein and crotonaldehyde.

Conclusions

A UHPLC–MS method for the analysis of selected carbonyl compounds from mainstream cigarette smoke was developed. This method provides several advantages over the previous HPLC–UV method including shorter analysis times, the use of an internal standard for quantitation and improvements in chromatographic conditions. The analysis time was significantly reduced from 47 min to 4.5 min with a 10-fold increase in sensitivity. There were no apparent interferences, and stereoisomers previously separated now co-elute resulting in reduced chromatographic complexity. Further chromatographic improvements achieved with the UHPLC method include the use of a simple binary mobile phase gradient instead of the complex tertiary mobile mixtures used for the HPLC analysis. In our experiments, electrospray ionization provided an increased dynamic range over APPI and APCI ionization techniques. The increased dynamic range is necessary with constituent and sample concentrations that may vary by three orders of magnitude. Results from the analysis of 2R4F reference cigarette extracts demonstrate that the new UHPLC–MS method performs similarly to previously reported HPLC–UV and GC–MS methods with a significant decrease in sample analysis times.

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