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Ultra Performance Liquid Chromatography−Atmospheric Pressure Photoionization-Tandem Mass Spectrometry for High-Sensitivity and High-Throughput Analysis of U.S. Environmental Protection Agency 16 Priority Pollutants Polynuclear Aromatic Hydrocarbons

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In this work, we demonstrate the utility of ultra performance liquid chromatography−atmospheric pressure photoionization-tandem mass spectrometry (UPLC−APPI-MS/MS) for high-sensitivity and high-throughput analysis of United States Environmental Protection Agency (U.S. EPA) 16 priority pollutants polycyclic aromatic hydrocarbons (PAHs). Analyses were performed on a Waters Acquity-TQD equipped with Syagen's PhotoMate APPI source. All 16 PAHs were analyzed on column in approximately 3.5 min with excellent chromatographic separation for all PAH isomers and with low picogram detection limits on column for all analytes using chlorobenzene as a dopant. Dynamic linear ranges were evaluated and found to cover at least 3−4 orders of magnitude. In comparison with the existing U.S. EPA methods, this approach improves instrument sample throughput by at least 10-fold.

Polynuclear aromatic hydrocarbons (also termed as polycyclic aromatic hydrocarbons or PAHs) occur naturally in fossil fuels and are a byproduct of combustion processes involved in incineration and power generation. Many PAHs are carcinogens and are closely monitored in the environment. The United States Environmental Protection Agency (U.S. EPA) and other government agencies have formulated regulations for the monitoring and control of PAHs and have developed methods for their measurement in air, water, food, and other matrixes. The EPA has designated 16 PAHs as priority pollutants. EPA methods 550 and 8310 analyze these 16 PAHs in drinking water by high-performance liquid chromatography (HPLC) with UV and fluorescence detection (FLD).[1,2] Method 610 deals with the measurement of these PAHs in wastewater both by HPLC/UV and FLD and by gas chromatography with flame ionization detection (GC/FID).[3] Method 8100 analyzes many PAHs by GC/FID.[4] Methods 525, 625, and 8270 analyze several PAHs and many other environmental pollutants in drinking water, wastewater, and solid wastes, respectively, by gas chromatography/mass spectrometry (GC/MS).[5−7] Method 8275 is a thermal extraction capillary GC/MS procedure for the quantitative determination of targeted PCBs and the 16 EPA priority pollutants PAHs in soils, sludges, and solid wastes.[8]

Capillary GC offers high chromatographic resolving power, but GC analysis of PAHs has proven difficult for those analytes with molecular weight (MW) greater than 300 amu due to low volatilities of these compounds. GC analysis of high MW PAHs is subject to thermal decomposition and the adsorption to the GC inlet and column. GC/FID is not a very sensitive method and is subject to background interferences from other carbonaceous sources. In contrast, capillary GC/MS methods not only offer chromatographic separation for analyte identification but also give mass separation and chemical structure information, but this technique is limited to the analysis of those PAHs which are volatile and thermally stable. As a result, the most widely used

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technique for analysis of PAHs, especially those with high MW, is HPLC/UV and FLD.[1−3,9,10] HPLC/UV and FLD methods are more sensitive and selective than the GC/FID method. However, not all PAHs are highly fluorescent; for example, in the U.S. EPA methods 550, 610, and 8310, naphthalene, acenaphthylene, acenaphthene, and fluorene were determined using a UV detector and the rest of 16 PAHs were determined using a fluorescence detector.

Liquid chromatography/mass spectrometry (LC−MS) is an analytical technique used for analysis of compounds that are thermally labile or nonvolatile and therefore not amenable to GC or GC/MS. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are two popular ionization sources used on LC−MS. However, ESI and APCI fail to ionize nonpolar compounds such as PAHs efficiently. APCI and ESI have been reported to analyze 10 of 16 EPA priority pollutants PAHs. This was accomplished by monitoring PAH−tropylium complexes[11] or PAH−silver complexes[12] by postcolumn addition of tropylium tetrafluoroborate or silver nitrate into ionization region. Both tropylium and silver cations are strong electron acceptors, which form the $[PAH + tropylium]^+$ and $[PAH + Ag]^+$ adducts. However, the sensitivity of this method heavily relies on the tropylium and silver affinities of the PAHs, and therefore this approach was found unsuitable for analysis of those PAHs with the number of aromatic rings less than four. These analytes, among the 16 EPA priority pollutants PAHs, include naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene, which have only two or three fused rings. This approach may also suffer potential source contamination after a prolonged period of introduction of nonvolatile metal salts into ionization region. APCI and ESI may also give limited dynamic linear ranges for quantification of these adducts ions such as $[PAH + tropylium]^+$ and $[PAH + Ag]^+$. APCI was also used for analysis of PAHs by monitoring protonated analyte molecules. Nevertheless, the applicability of this technique is limited only to those PAHs with high proton affinities. APCI was found to offer poor ionization efficiencies for analysis of PAHs with MW less than 300 amu. Theses PAHs usually have relatively lower proton affinity.[9] Unfortunately all the 16 EPA priority pollutants PAHs fall within this category, having MW less than 300 amu.

Atmospheric pressure photoionization (APPI) was initially developed to analyze those compounds not readily ionizable by ESI and APCI.[13,14] APPI extends the range of ionizable compounds, making it possible for analysis of many nonpolar molecules by LC−MS with high sensitivity. APPI has been used to analyze a broad spectrum of polar, medium polar, and nonpolar compounds such as carbohydrates,[15] surfactants,[16] sulfonamides,[17] mycotoxins,[18] drugs and drug candidates,[19] nucleic bases, ribonucleosides and ribonucleotides,[20] anthocyanins (wine fingerprints),[21] pesticides,[22] antibiotics,[23] quinones,[24] neurotransmitters,[25] steroids,[26] lipids,[27] PAHs,[28] petroleum mixtures,[29] peptides,[30] polymers,[31] oligodeoxyribonucleotides,[32] and halogenated compounds.[33] Additional references to APPI applications are available in a recently published review article.[34] APPI and APCI offer comparable dynamic linear ranges, but wider linear ranges, than ESI.[35,36] APPI also offers less ion suppression than APCI[18,37] and much less ion suppression than ESI.[34,35,38,39] APPI also gives no concern of explosion hazard when flammable solvents (e.g., hexane, heptane, and alcohol) are used as mobile phases for chromatographic separations such as for normal phase chiral analysis.[37,40,41]

Although small PAHs (MW < 300 amu) can be analyzed by GC/MS, LC−APPI-MS offers an alternative method for analysis of these compounds. In many applications, there are situations where polar and nonpolar compounds are required to be analyzed in the same samples. Polar compounds are usually much less volatile and thermally unstable, being not amenable to GC or GC/MS analysis without derivatization. LC−APPI-MS offers the advantage of simultaneously analyzing both polar and nonpolar analytes with a simplified sample cleanup procedure and the potential of lowering method detection limits.

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Table 1. PAH MRM Data Acquisition Method

analyte	parent ion type	parent ion (m/z)	daughter ion (m/z)	dwell time (s)	cone voltage (V)	collision energy (eV)	retention window (min)	function no.
naphthalene	M^+	128.20	102.1	0.06	54	24	0–1.34	1
acenaphthylene	M^+	152.21	126.1	0.06	90	32	0–1.34	1
acenaphthene	M^+	154.21	127.2	0.06	46	36	0–1.34	1
fluorene	M^+	166.22	115.1	0.06	42	38	0–1.34	1
anthracene	M^+	178.22	152.1	0.06	60	30	0–1.34	1
phenanthrene	M^+	178.22	152.1	0.06	60	30	0–1.34	1
fluoranthene	M^+	202.23	152.1	0.06	72	40	0–1.34	1
pyrene	M^+	202.23	152.1	0.06	72	40	0–1.34	1
benzo[*a*]anthracene	M^+	228.24	202.2	0.1	76	38	1.34–3.5	2
chrysene	M^+	228.24	202.2	0.1	76	38	1.34–3.5	2
benzo[*a*]pyrene	M^+	252.25	226.1	0.1	80	42	1.34–3.5	2
benzo[*b*]fluoranthene	M^+	252.25	226.1	0.1	80	42	1.34–3.5	2
benzo[*k*]fluoranthene	M^+	252.25	226.1	0.1	80	42	1.34–3.5	2
benzo[*ghi*]perylene	M^+	276.26	248.2	0.1	90	68	1.34–3.5	2
indeno[1,2,3-*cd*]pyrene	M^+	276.26	248.2	0.1	90	68	1.34–3.5	2
dibenzo[*a*,*h*]anthracene	M^+	278.33	250.2	0.1	82	58	1.34–3.5	2

With the introduction of LC column with sub-2 μm particle size, the column separation efficiencies, sensitivity, sample throughput, and peak capacity have been dramatically improved. Ultra performance liquid chromatography (UPLC) combined with tandem mass spectrometry (MS/MS) offers unprecedented on-column resolving power, sensitivity, speed of analysis, and mass selectivity. In this work, we demonstrate the utility of UPLC−APPI-MS/MS for high-sensitivity and high-throughput analysis of the 16 EPA priority pollutants PAHs. With conventional HPLC column, the injection to injection cycle times usually exceed 45 min for the analysis of 16 EPA priority pollutants PAHs.[1−3] In contrast, this method allows analysis of the 16 PAHs with run to run cycle times of approximately 3.5 min, improving instrumental sample analysis throughput by at least 10-fold with limits of detection (LODs) at low picogram level on column.

EXPERIMENTAL SECTION

Chemicals and Reagents. All PAH individual component stock solutions were purchased from AccuStandard (New Haven, CT). These individual component stock solutions were predissolved in either acetonitrile or methanol when purchased, and have a concentration of 500, 1000, 2000, or 5000 μg/mL. HPLC water and acetonitrile (Optima grade, 0.2 μm prefiltered) were both purchased from Thermo Fisher Scientific (Fair Lawn, NJ). Chlorobenzene (99.5%, ACROS Organics), used as an APPI dopant, was also purchased from Thermo Fisher Scientific (Pittsburgh, PA).

Instruments. The liquid chromatograph−mass spectrometer (LC−MS) used was Waters Acquity UPLC−TQD equipped with ESI, APCI, and Syagen's PhotoMate APPI source, Acquity UPLC column manager, sample manager, binary solvent manager, and photodiode array (PDA) UV detector. The LC column used was an Agilent Zorbox Eclipse PAH, 2.1 mm × 50 mm, 1.8 μm particle size, UPLC column.

Preparation of Standards. A mixed PAH stock solution at a concentration of 50 ng/μL (ppm) each of target analyte was prepared in acetonitrile using the above individual component stock solutions. Working standard solutions were prepared from stock solutions by series dilution method in acetonitrile.

Optimization of Tune Page Parameters. Many tune page parameters were automatically optimized using the "IntelliStart" program of Acquity UPLC console. The tuning solution used was Waters API Calibration (NaCsI) Solution (Rev. B, cat. no. 700001593), placed in reservoir A of the Acquity-TQD syringe pump system. The MS/MS target resolution was set at 1.00 Da on the Instrument Setup tab of IntelliStart for tune page parameter optimization. Several other parameters, not tuned by the IntelliStart, were manually optimized on the Masslynx tune page using target analyte standards. Below are the final parameters used on Masslynx tune page for MS/MS data acquisition.

The parameters for APPI Source tab are the following: repeller, 1.00 kV; extractor, 3 V; rf lens, 0.1 V; source temp, 120 °C; APCI probe temp, 550 °C; desolvation gas flow, 900 L/h; cone gas flow, 0 L/h. The MS/MS parameters for Analyzer tab are the following: LM resolution 1, 2.8; HM resolution 1, 15.7; ion energy 1, −0.1; entrance, 1; collision, 20; exit, 0.5; LM resolution 2, 2.7; HM resolution 2, 15.2; ion energy 2, 0.5; gain, 1.00; collision gas Ar flow, 0.17 mL/min (equivalent to collision cell MKS reading of about 3.2×10^{-3} Torr).

The acquisition interscan delay settings (under interscan setup menu) were MS interscan, 0.01 s; polarity/mode switch interscan, 0.02 s; interchannel delay, 0.005 s.

Optimization of Multireaction Monitoring Data Acquisition Parameters. The parameters such as multireaction monitoring (MRM) transition (major parent ion > daughter ion), cone voltage, and collision energy were optimized by a flow injection using the IntelliStart program of Acquity UPLC console. Several PAH tuning standard mix solutions were prepared in pure chlorobenzene with approximately 10−20 ng/μL of each analyte. Each tuning mix contained PAH analytes with distinctive MWs. The PAH isomers sharing the same MW were not grouped together in the same mix. The tuning standard mix, placed in reservoir A of the Acquity-TQD syringe pump system, was infused at a default flow rate 10 μL/min. The water/acetonitrile (1:9, v/v) mobile phase flow rate was set at 200 μL/min. Table 1 lists MRM data acquisition parameters based on the UPLC column separation gradient elution program presented in Table 3.

Dopant Selection and Introduction Method. APPI is able to ionize nonpolar analytes through charge exchange with dopant radical cations. Attempts have been made to optimize the dopants

Table 2. Method Events for Dopant Delivery

initial settings		time (min)	event	action
flow state:	combined	0.00	reservoir	B
flow rate μL/min:	65	0.00	refill flow	refill
reservoir:	B	0.00	state flow	combined
refill:	refill	0.00	rate	65
		0.01	infusion	start

Table 3. UPLC Gradient Elution Program

time (min)	flow (mL/min)	% A1	% B1	curve
initial	0.65	30.0	70.0	initial
0.30	0.65	30.0	70.0	6
1.50	0.65	0.0	100.0	6
3.50	0.65	0.0	100.0	6
3.51	0.65	30.0	70.0	11

for PAH analysis by LC−APPI-MS.[28,42] It was believed that an effective dopant for charge exchange to occur must meet two criteria: (1) the ionization energy (IE) of the dopant must be greater than that of the analyte, and (2) for ionization to be efficient, dopant radical cations must not react with the LC solvents, dopant neutrals, or solvent impurities. The commonly used dopants for charge exchange are toluene and anisole,[43] but none of them is ideal for this role: toluene has a high IE (8.83 eV), but its photoions react with mobile phase solvents such as methanol or acetonitrile, making it poorly suited for reversed-phase (RP) applications; anisole's photoions are stable in the RP solvents, but it has a low IE (8.20 eV), limiting the analytes it can be used for.[34] Anisole/toluene mixture (0.5:99.5, v/v) was demonstrated to give enhanced overall performance compared to either pure anisole or toluene in the analysis of the 16 U.S. EPA priority pollutants PAHs.[28] In addition to anisole and toluene, several other solvents or their mixtures have been identified as effective dopants for analysis of nonpolar compounds such as PAHs under reversed-phase LC conditions because their photoions are stable in RP solvents and they have relatively high IEs.[44] These compounds include chlorobenzene (IE = 9.07 eV), bromobenzene (IE = 9.00 eV), 2,4-difluoroanisole (IE > 8.2 eV), and 3-(trifluoromethyl)anisole (IE > 8.2 eV) diluted in either chlorobenzene or bromobenzene (0.5:99.5, v/v).

Chlorobenzene was used as a dopant in this work. The optimized dopant flow rate was found to be approximately 10% of mobile phase flow rate. The dopant flow was combined with mobile phase effluent flow by post column addition using Masslynx integrated fluidics. The introduction of chlorobenzene was automatically controlled by Masslynx software based on the method event parameters listed in the Table 2. The method event allows the dopant to be delivered and combined with mobile phase effluents at preset "start" and "stop" times and to be refilled in the syringe pump at a particular preset moment during UPLC column separation. The chlorobenzene dopant was contained in reservoir B of the integrated fluidics. For continuous dopant supply or overnight sample analysis, the default reservoir B was replaced with a 100 mL bottle with an extended length of peek tubing for dopant delivery.

Table 4. PAH Dynamic Linear Ranges

analyte	linear range (pg)	equation	R^2
naphthalene	24.4−100 000	$y = 5.8517x$	0.9979
acenaphthylene	97.7−100 000	$y = 1.8015x$	0.9963
acenaphthene	97.7−100 000	$y = 1.4906x$	0.9969
fluorene	6.10−50 000	$y = 1.9887x$	0.9968
phenanthrene	6.10−100 000	$y = 5.6371x$	0.9975
anthracene	6.10−100 000	$y = 7.9816x$	0.9961
fluoranthene	6.10−100 000	$y = 2.4828x$	0.9969
pyrene	12.2−100 000	$y = 0.7456x$	0.9972
benzo[*a*]anthracene	6.10−100 000	$y = 2.3576x$	0.9983
chrysene	6.10−100 000	$y = 1.0066x$	0.9991
benzo[*b*]fluoranthene	6.10−100 000	$y = 1.757x$	0.9980
benzo[*k*]fluoranthene	6.10−100 000	$y = 1.7928x$	0.9984
benzo[*a*]pyrene	6.10−100 000	$y = 1.1809x$	0.9995
benzo[*ghi*]perylene	6.10−100 000	$y = 0.6587x$	0.9994
indeno[1,2,3-*cd*]pyrene	6.10−100 000	$y = 0.6384x$	0.9992
dibenzo[*a*,*h*]anthracene	6.10−100 000	$y = 0.965x$	0.9994

Table 5. On-Column Detection Limits

analyte	injection amount (pg)	detection limits (pg)
naphthalene	48.8	34.9
acenaphthylene	195.4	158.4
acenaphthylene	97.7	91.6
fluorene	6.1	6.2
phenanthrene	12.2	4.3
anthracene	6.1	3.1
fluoranthene	6.1	3.4
pyrene	24.4	10.3
benzo[*a*]anthracene	6.1	5.4
chrysene	24.4	11.4
benzo[*b*]fluoranthene	6.1	2.5
benzo[*k*]fluoranthene	6.1	2.5
benzo[*a*]pyrene	6.1	3.3
benzo[*ghi*]perylene	6.1	2.8
indeno[1,2,3-*cd*]pyrene	6.1	4.4
dibenzo[*a*,*h*]anthracene	6.1	1.7

High-Throughput Column Separations and Analysis. Column separation was performed using an Agilent Zorbox Eclipse PAH 600Bar, 2.1 mm × 50 mm, 1.8 μm, LC column (Agilent P/N, 959741-918). An Acquity VanGuard precolumn, BEH C18, 2.1 mm × 5 mm, 1.7 μm (Waters P/N, 186003975) and a guard filter (0.2 μm frit, Waters P/N, 289002378) were both used. They were assembled in the sequence of the guard filter followed by the guard column and then the analytical column. Column separation was achieved using water/acetonitrile as a binary mobile phase system. The mobile phase solvents were 90:10 water/acetonitrile (A1) and 100% acetonitrile (B1). The mobile phase flow rate was 650 μL/min. The dopant chlorobenzene flow rate was 65 μL/min. The gradient elution program is described in detail in Table 3. The column temperature was set at 15 °C. A column temperature at 15 °C provides slightly better baseline separations for PAH isomers than a column temperature at 25 °C. An amount of 2 μL of standard solutions was injected on column using a 10 μL loop with "partial loop with needle overfill" method. With such a high mobile phase flow rate, column re-equilibrium time is not required

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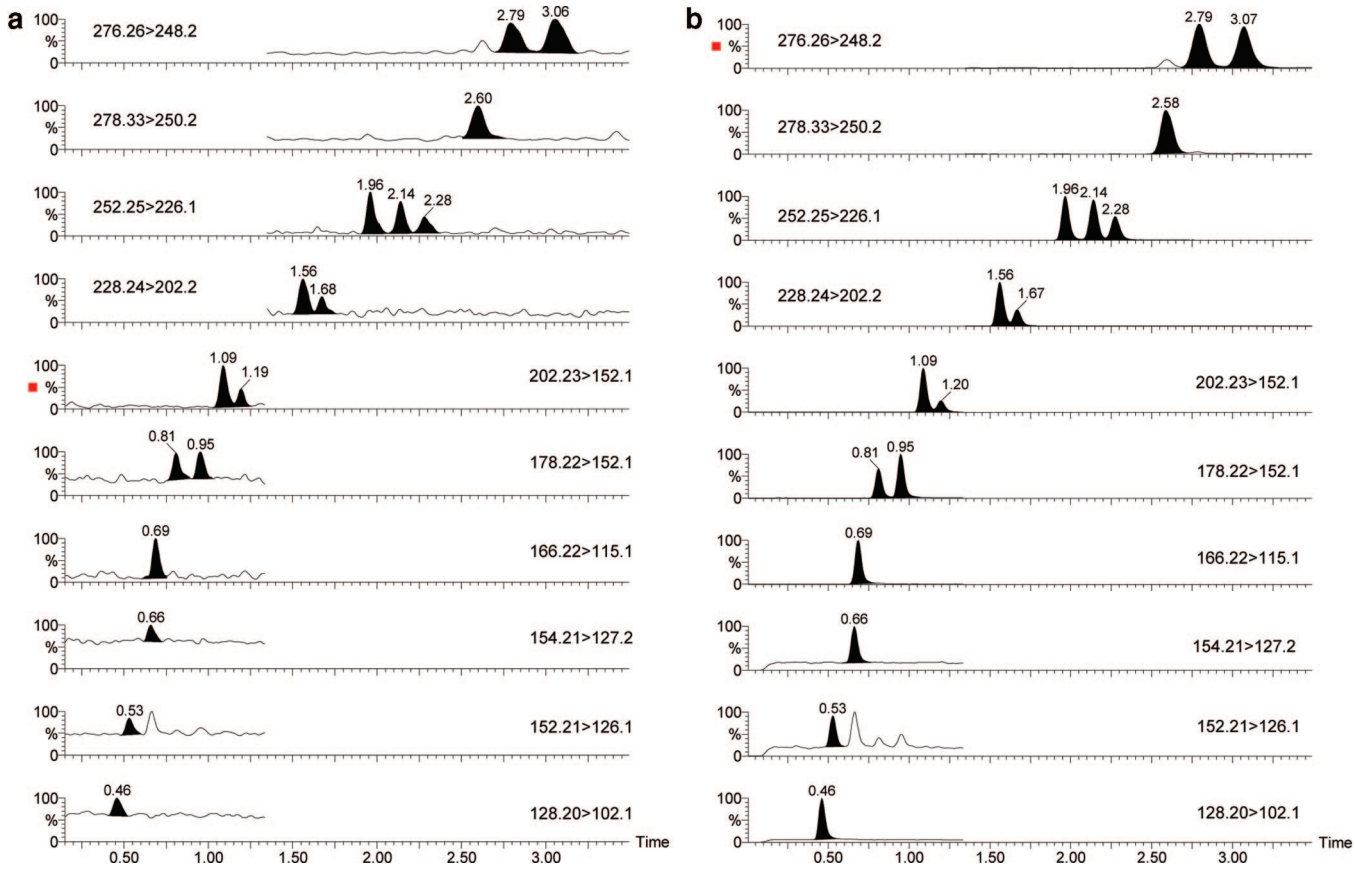

Figure 1. (a) PAH MRM chromatograms for RT 0.46, naphthalene (48.8 pg); 0.53, acenaphthylene (390 pg); 0.66, acenaphthene (195.4 pg); 0.69, fluorene (24.4 pg); 0.81, phenanthrene (12.2 pg); 0.95, anthracene (12.2 pg); 1.09, fluoranthene (24.4 pg); 1.19, pyrene (24.4 pg); 1.56, benzo[*a*]anthracene (12.2 pg); 1.68, chrysene (12.2 pg); 1.96, benzo[*b*]fluoranthene (12.2 pg); 2.14, benzo[*k*]fluoranthene (12.2 pg); 2.28, benzo[*a*]pyrene (12.2 pg); 2.60, dibenzo[*a,h*]anthracene (12.2 pg); 2.79, benzo[*ghi*]perylene (12.2 pg); 3.06, indeno[1,2,3-*cd*]pyrene (12.2 pg). Injection: 2 μL. Peak top labels denote retention time on column. (b) PAH MRM chromatograms. Injection amount: 1.56 ng for each analyte. Parameters and conditions same as panel a.

between injections. Figure 1a shows MRM ion chromatograms of 16 PAHs with on-column injection amounts ranging from 12.2 to 390 pg on column using the parameters and conditions described in Table 1. Figure 1b shows MRM ion chromatograms of the 16 PAHs with an identical injection amount of 1.56 ng for each analyte under the same parameters and conditions as those of Figure 1a. These results show that all the PAH isomers sharing the same mass transitions are well separated by retention times and are quantified in approximately 3.5 min. The peak next to acenaphthylene (152.21 > 126.1, RT = 0.53) is the mass interference from acenaphthene (154.21 > 127.2, RT = 0.66). This peak response (from precursor ion $[M - 2]^+$ of acenaphthene) is always approximately 1.5 times higher than the response from the quantification precursor ion $[M]^+$ of acenaphthene and therefore can be used as a confirmation ion for this analyte. The peak response proceeding benzo[*ghi*]perylene (276.26 > 248.2, RT = 2.79) is the mass interference generated from dibenzo[*a,h*]anthracene (278.33 > 250.2, RT = 2.60). These mass interference ion responses are well separated from their affected adjacent analytes. No other mass interferences were observed affecting the peak responses of analytes when high levels of individual analyte standards were analyzed.

RESULTS AND DISCUSSION

Evaluation of Linearity. An amount of 50 ng/μL of calibration standard mix was prepared from individual component stock solutions described in the above Chemicals and Reagents section. This standard mix was diluted further using acetonitrile by series dilution method, resulting in the following calibration standard solutions: 50 000, 25 000, 12 500, 6250, 3125, 1562.5, 781.3, 390.6, 195.3, 97.7, 48.8, 24.4, 12.2, 6.1, and 3.1 pg/μL. An amount of 2 μL of each standard solution was injected on column five times using a 10 μL loop with "partial loop with needle overfill" method. The calibration standards were analyzed using water/acetonitrile as a binary mobile phase in an injection sequence starting from the lowest (e.g., 3.1 pg/μL) to the highest (50 000 pg/μL) concentrations. Table 4 shows the linear ranges, linear regression equations, and correlation coefficients (R^2) in terms of analyte peak area as a function of absolute injection amount (pg) on column. These results show that all the R^2 are higher than 0.996 with calibration ranges from LODs up to 50 000 or 100 000 pg on column. Upper calibration ranges were not calibrated over 100 000 pg. These results show APPI offers at least 3−4 orders of dynamic linear ranges for PAH analysis under tested conditions.

On-Column Instrument Detection Limits. The instrument detection limits (IDL in pg, peak to peak signal-to-noise ratio (S/N) = 3) on column based on the gradient elution program (Table 3) were calculated from the lowest injection amounts (pg) being able to generate peak responses with a peak to peak S/N ratio of 3. The instrument detection limits (average of five replicate analyses) and near-to-IDL injection amounts used to generate the instrument detection limits are presented in Table 5. These results show that Acquity UPLC−APPI-TQD offers low picogram on-column detection limits for all 16 PAH analytes under the tested conditions.

Several earlier on-column eluters such as naphthalene, acenaphthylene, and acenaphthene give relatively higher peak responses, as indicated by the slopes of the calibration curves (Table 4), but lower sensitivity or higher instrument detection limits due to their elevated baseline noise. This is probably due to the fact that these earlier eluters have lower masses, appearing in a mass region which generally has a higher chemical background in LC−MS.

CONCLUSION

In this work, we demonstrate the utility of UPLC−APPI-MS/MS for high-sensitivity and high-throughput analysis of U.S. EPA 16 priority pollutants PAHs. The ACQUITY TQD system offers high throughput in both method development and sample analysis. The cycle time for this analysis is approximately 3.5 min using an Agilent Zorbax Eclipse PAH, 2.1 mm × 50 mm, 1.8 μm, UPLC column and a water/acetonitrile binary mobile phase with no column re-equilibration time required between sample injections. The on-column instrument detection limits averages 21.6 pg for 16 PAHs using chlorobenzene as a dopant. Removing naphthalene, acenaphthylene, and acenaphthene yields an average of 4.7 pg on-column limits of detection. Dynamic linear ranges cover at least 3−4 orders of magnitude. In comparison with the existing U.S. EPA methods, this method improves instrument sample throughput by at least 10-fold. ACQUITY TQD also offers a unique, intelligent, and flexible way of dopant introduction for APPI PAH analysis via its integrated fluidics system.

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