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RESEARCH ARTICLE

Optimization and Application of APCI Hydrogen–Deuterium Exchange Mass Spectrometry (HDX MS) for the Speciation of Nitrogen Compounds

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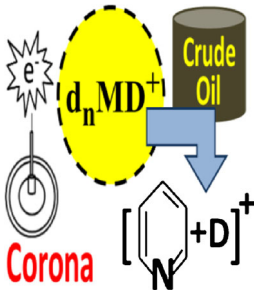

Abstract. A systematic study was performed to investigate the utility of atmospheric pressure chemical ionization hydrogen–deuterium exchange mass spectrometry (APCI HDX MS) to identify the structures of nitrogen-containing aromatic compounds. First, experiments were performed to determine the optimized experimental conditions, with dichloromethane and CH_3OD found to be good cosolvents for APCI HDX. In addition, a positive correlation between the heated capillary temperature and the observed HDX signal was observed, and it was suggested that the HDX reaction occurred when molecules were contained in the solvent cluster. Second, 20 standard nitrogen-containing compounds were analyzed to investigate whether speciation could be determined based on the different types of ions produced from nitrogen-containing compounds with various functional groups. The number of exchanges occurring within the compounds correlated well with the number of active hydrogen atoms attached to nitrogen, and it was confirmed that APCI HDX MS could be used to determine speciation. The results obtained by APCI HDX MS were combined with the subsequent investigation of the double bond equivalence distribution and indicated that resins of shale oil extract contained mostly pyridine type nitrogen compounds. This study confirmed that APCI HDX MS can be added to previously reported chemical ionization, electrospray ionization, and atmospheric pressure photo ionization-based HDX methods, which can be used for structural elucidation by mass spectrometry.

Keywords: Atmospheric pressure chemical ionization, Hydrogen/deuterium exchange, Mass spectrometry, High resolution mass spectrometry

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Introduction

Mass spectrometry has become one of the most important analytical tools in modern scientific studies and industrial applications [1–7]. The mass to charge ratios obtained from mass spectrometry data can be used to identify and detect modifications of compounds. By applying high-resolution mass spectrometry, elemental compositions can be calculated from accurate mass measurements, with some prior understanding of the elements to be included in the calculation [8–10]. There have been many studies in which high-resolution mass spectrometry has been used for the analysis of petrochemicals, biofuels, and biomolecules [11–14]. However, even with high-resolution mass spectrometry, there are limits to the structural information that can be obtained. The identification of chemical structures is important to understand the chemical properties of compounds.

Many techniques, including tandem MS, have been used to identify chemical structures by mass spectrometry (MS) [15–18]. The coupling of hydrogen–deuterium exchange (HDX) and MS is also a dynamic tool that has been used to identify the structures of compounds [19–22]. HDX MS has been coupled to various ionization methods, including chemical ionization (CI) and electrospray ionization (ESI). Hunt et al. reported use of HDX for determining the hydrogens bonded to heteroatoms in alcohols, phenols, carboxylic acids, amines, amides, and

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mercaptans by CI [23]. Later, the mass spectra of ESI HDX were used to study proteins, peptides, metabolites, and antibiotics [24–29]. Recently, atmospheric pressure photoionization (APPI) HDX coupled to MS has been reported [30], and its use for determining the structure of crude oil compounds has been explored [31].

Atmospheric pressure chemical ionization (APCI) is another important ionization source for modern MS techniques, and has been used to study a wide range of compounds [32–37]. As HDX MS with CI is effective, it is reasonable to expect that APCI would also be an effective ionization method for HDX. The use of HDX coupled with APCI MS has been reported for the analysis of monosaccharides [38]. APCI combined with ESI by utilizing a dual spray ionization source has been used to study some pharmaceuticals [39]. However, there have been limited reports regarding the use of APCI HDX MS compared with other ionization techniques.

The selected compounds offered a good platform for this study, since amine compounds and aromatic heterocycles containing nitrogens were the major prevailing structures among the nitrogeneous compounds in petroleomics [40]. In this study, APCI HDX MS analysis was performed systematically on various nitrogenous compounds with different structures using CH_3OD as the APCI reagent. The experimental results were used to perform speciation of nitrogen compounds in petroleum samples analyzed by ultrahigh resolution mass spectrometry.

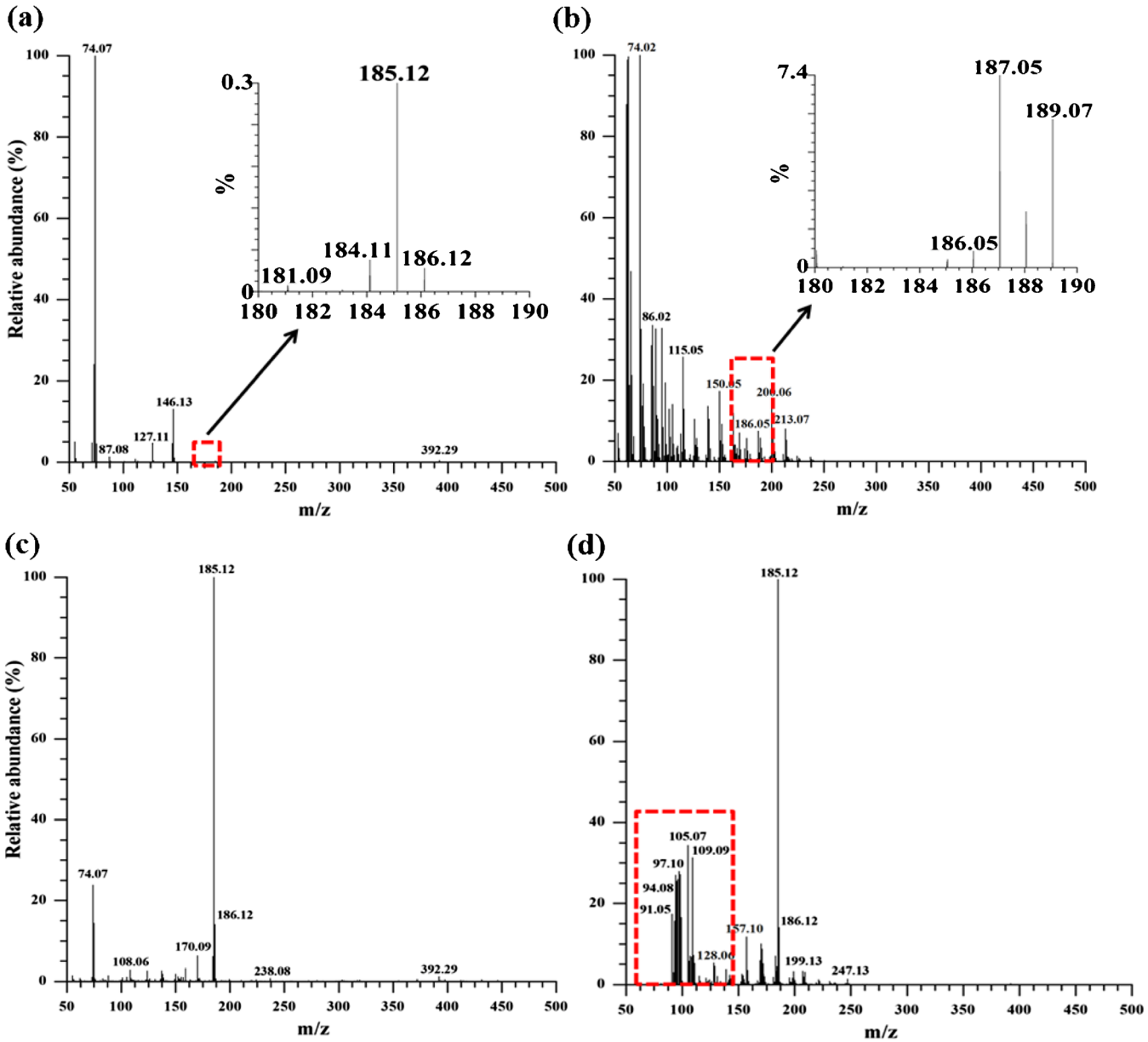

Figure 1. (+) APCI HDX mass spectra of 10 μM*N*-methyldiphenylamine, acquired with 30% CH_3OD mixed with (**a**) tetrahydrofuran, (**b**) benzene, (**c**) dichloromethane, and (**d**) toluene

Experimental

Preparation of Standards and Samples

HPLC grade solvents were purchased from J.T. Baker (Center Valley, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA). Twenty nitrogen standards were purchased from Sigma-Aldrich. A list of the standard compounds and their structures is provided in the Supplementary Figure 1S. Individual stock standard solutions were prepared in benzene, toluene, tetrahydrofuran (THF), or dichloromethane (DCM), to a final concentration of 10 µM. The HDX experiments

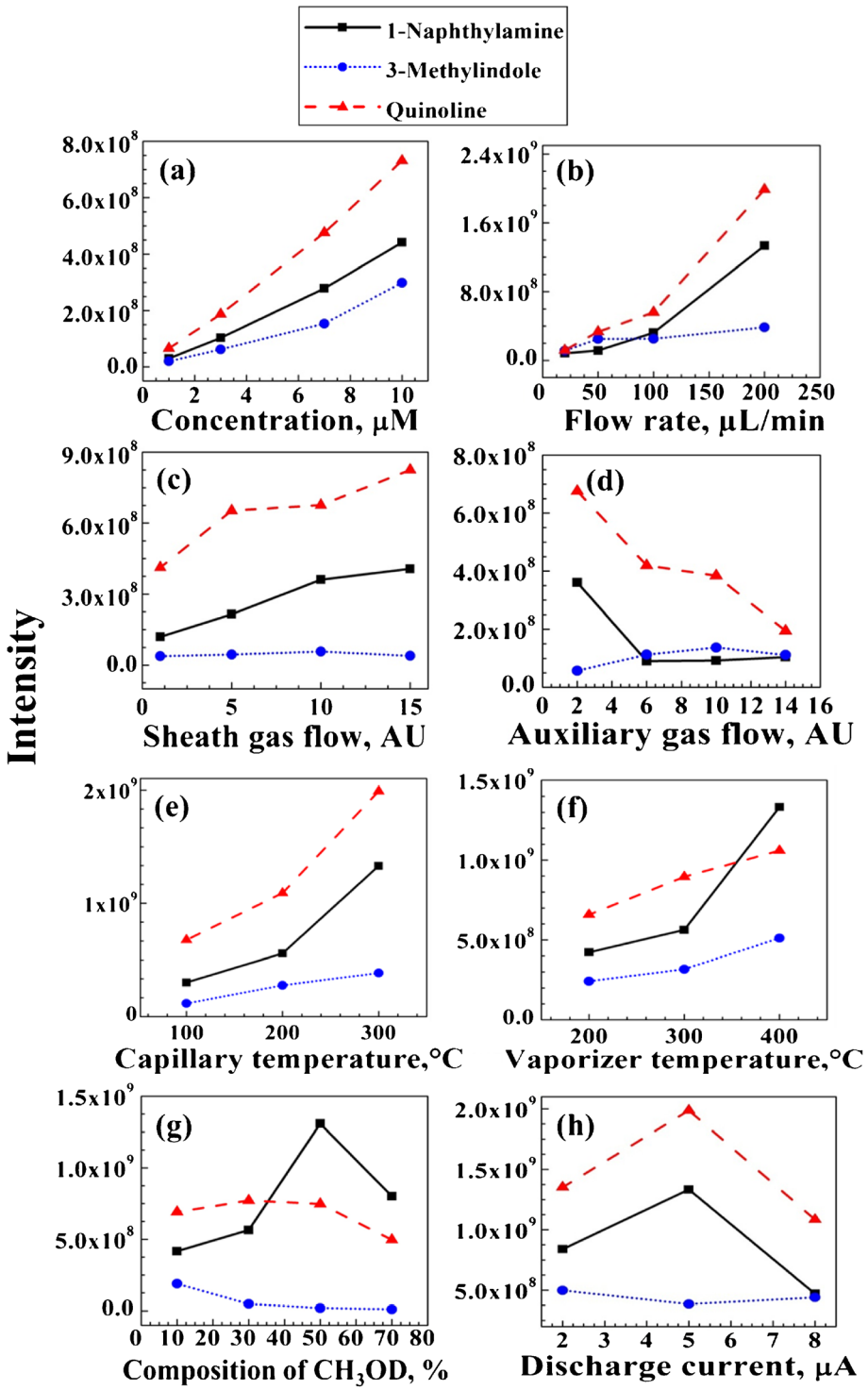

Figure 2. Plots showing the influence of (**a**) analyte concentration, (**b**) sample flow rate, (**c**) sheath gas flow rate, (**d**) auxiliary gas flow rate, (**e**) capillary temperature, (**f**) vaporizer temperature, (**g**) percentage concentration of CH_3OD, and (**h**) corona discharge current on the (+) APCI HDX MS signal abundance of 1-naphthylamine (black), 3-methylindole (blue), and quinolone (red)

were carried out by adding 30% deuterated methanol (CH_3OD) to each of analyte solution prior to injection in the ionization source. All the standards were directly infused into the APCI interface using a Harvard syringe pump model 11 (Harvard, Holliston, MA, USA) under atmospheric pressure at 200 μL/min. Shale oil samples from the Anvil Points Mine (APM) were used in this study. Detailed information regarding these samples was reported previously [31]. The shale oil was fractionated into saturated, aromatic, resin, and asphaltene (SARA) fractions [41]. Owing to the abundance of nitrogen-containing compounds in the resin fraction, it was used for the analysis [41, 42].

Mass Spectrometry

Analysis of all the standards was performed on a Q Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Rockford, IL, USA) equipped with a commercially available APCI source. Samples were directly infused into the APCI interface at varying flow rates from 20 to 200 μL/min. The regular operating parameters were as follows: tube lens rf level, 50 Hz; tube lens voltage, 25 V; skimmer voltage, 15 V; C-Trap RF, 550 V; and sweep gas flow, 0 (arbitrary units). The following source conditions were varied for optimization: discharge current 2–8 μA; heated capillary and vaporizer temperatures, 100°C–300°C and 200°C–400°C, respectively; sheath and auxiliary gas, 1–15 and 2–14 (arbitrary units), respectively. High-purity (99%) nitrogen was used as the source gas (sheath/auxiliary/sweep gas) obtained from the evaporation of liquid nitrogen stored in a pressurized, stainless steel Dewar. MS external calibration was conducted in positive ion mode with infusion of Pierce Velos solution (Thermo Fisher Scientific) into the ESI source. All mass spectra were collected in positive ion mode. For data acquisition in full scan mode, the mass spectral range from *m*/*z* 50 to 500 was scanned with a maximum injection time of 500 ms, 1 microscan, automatic gain control (AGC) ON, and FT resolution of 140,000.

A 15-T Fourier transform ion cyclotron resonance mass spectrometer (FTICR MS; Bruker Daltonics, Billerica, MA, USA) was used for crude oil analysis. A nebulizing gas temperature of 450°C, flow rate of 2.0 L/min, drying gas temperature of 210°C at a flow rate of 2.3 L/min, and capillary voltage of 3600 V were used. The collision cell radio frequency (rf) voltage and energy were 1500 V and –3.0 eV, respectively. Spectra were acquired with a 4-MW transient size and summed over 150 time-domain transients to improve the signal-to-noise ratio. Resolution of ~500,000 at 400 *m*/*z* was routinely obtained. Internal calibration was performed using the radical cations of the N_1 and N_1D_1 series in (+) mode.

Data Analysis

Spectra obtained from the quadrupole mass spectrometer were processed using Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific). Spectral interpretation of FT-ICR MS data was performed using software developed in-house (Statistical Tool for Organic Mixtures' Spectra for Hydrogen/Deuterium eXchange: STORMS-HDX) with an automated peak-picking algorithm for more reliable and faster results [43, 44]. Elemental formulas were calculated from the calibrated peak list and the list of mass and relative abundance obtained from the software were exported into Microsoft Excel and OriginLab for further processing.

Nomenclature

The following notation is used to indicate the deuterium exchanged ions:

$$d_nMD^+ \quad (1)$$

d_n, number of hydrogen–deuterium exchanges (n=0, 1, 2, 3…)
D^+, deuterium ion
M, analyte

Results and Discussion

Optimization of APCI HDX Conditions and Implications for the Exchange Mechanism

Deuterated methanol (CH_3OD) was chosen as the deuterium reagent for HDX in this study because it is an effective isotopic exchange agent with CI, ESI, and APPI [45, 46]. To increase the solubility of aromatic compounds, four common solvents (THF, benzene, DCM, and toluene) were tested as cosolvents for HDX. *N*-methyldiphenylamine was dissolved in each mixture of CH_3OD and the four solvents, and then analyzed by (+) APCI. The spectra obtained are shown in Figure 1. THF and benzene were excluded as cosolvents because very little HDX product (*m*/*z*=185.12) was observed and/or significant extra peaks were observed (refer to Figure 1a and b). Exchanged ion was observed with DCM and toluene, and they were therefore determined to be good cosolvents for (+) APCI HDX (Figure 1c and d). However, the mass spectra obtained with

Table 1. Optimized Operational Conditions for (+) APCI HDX MS

Parameters	Values considered for optimization	Optimized value
Concentration, μM	1,3,7,10	10
Flow rate, μL/min	20, 50,100, 200	200
Sheath gas[a]	1,5,10,15	10
Auxiliary gas[a]	2,6,10,14	2
Capillary temperature, °C	100,200,300	300
Vaporizer temperature, °C	200,300,400	400
Composition of CH_3OD in DCM,%	10,30,50,70	30
Discharge current, μA	2,5,8	5

[a]Nitrogen was used for nebulizer gas and auxiliary gas. All gas flow settings represent arbitrary units

toluene contained many peaks at *m/z* lower than 200 originating from toluene (refer to the red box in Figure 1d). Therefore, it was determined that THF and benzene were poor cosolvents for (+) APCI HDX, whereas toluene and DCM were good cosolvents for (+) APCI HDX, although toluene may not be a good cosolvent for compounds with a molecular weight less than 200 Da.

Takáts et al. used DCM as a solvent in ESI and APCI and obtained high sensitivity for both polar and apolar aromatic compounds [47]. In addition, DCM is suitable for HDX because it does not have exchangeable H. Therefore, DCM was used as the cosolvent for analysis of standard compounds.

To investigate the influence of the operating parameters on (+) APCI HDX, data were obtained with varying parameters, such as sample concentration, flow rate, concentration of methanol-d in solvent, nebulizer gas flow, discharge current, and APCI desolvation temperature. 1-Naphthylamine, 3-methylindole, and quinolone were used as standard compounds. The data obtained are summarized and presented in Figure 2. The raw data used to generate Figure 2 are shown in Supplementary Figure 2S.

The abundance of the HDX peak improved with increasing analyte concentration (Figure 2a), flow rate of sample (Figure 2b), sheath gas flow rate (Figure 2c), and capillary

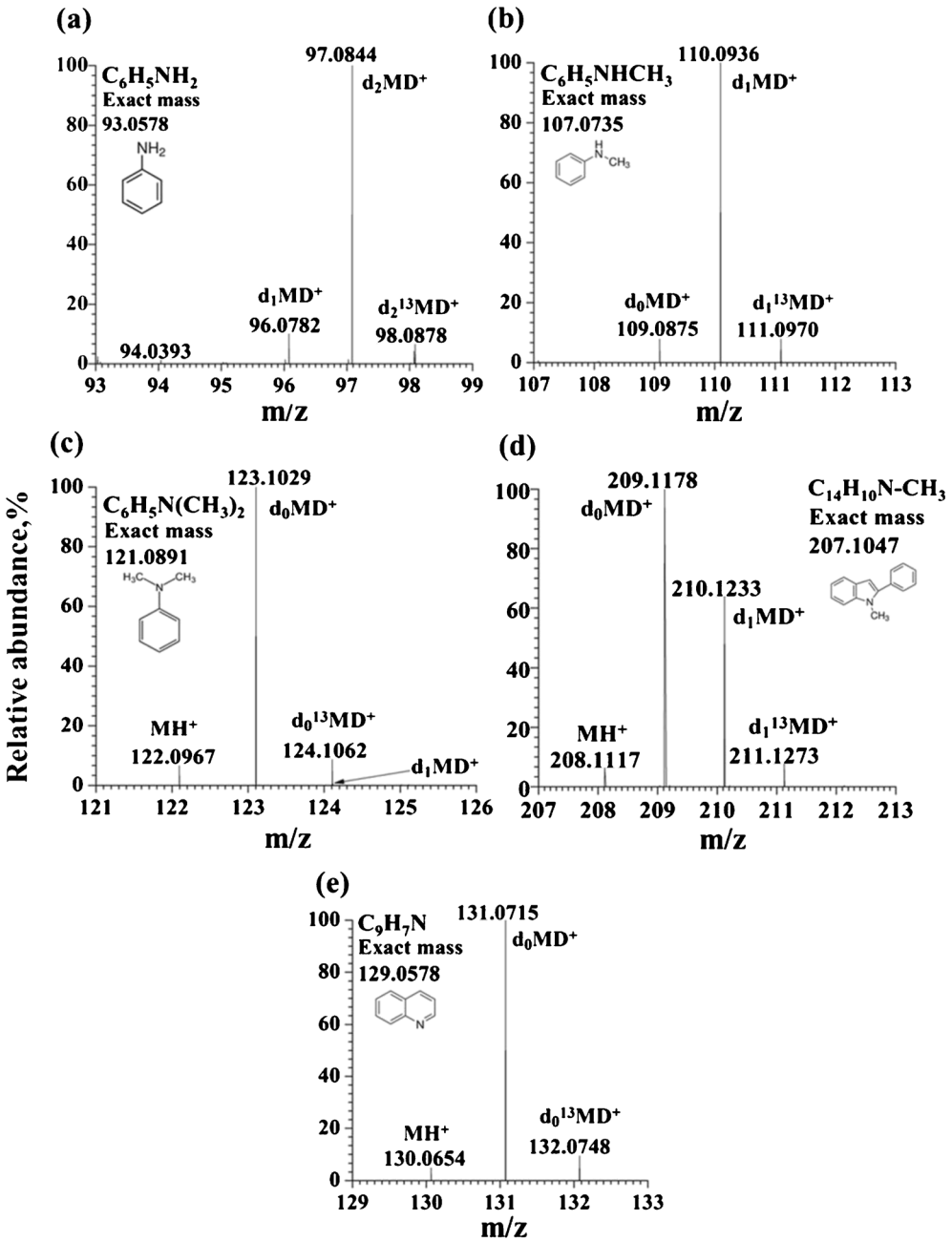

Figure 3. (+) APCI HDX mass spectra of (**a**) aniline, (**b**) *N*-methylaniline, (**c**) *N*,*N*-dimethylaniline, (**d**) 1-methyl-2-phenylindole, and (**e**) quinoline showing a different number of exchanged peaks based on their chemical structures

and vaporizer temperatures (Figure 2e and f). The signal was decreased with higher auxiliary gas flow (Figure 2d). It is reasonable to suggest that the increase in signal per increase of mass transfer rate resulted from the increases of analyte concentration and flow rate. The sheath gas was used to pneumatically assist the formation of the sprayed droplets [48], and auxiliary gas was introduced to achieve very efficient desolvation [49]. The positive correlation between the sheath gas flow rate and peak abundance indicates that an increase in droplet formation helps the HDX reaction. A low flow rate of auxiliary gas was sufficient to assist the nebulizer gas.

The abundance of the HDX peak was improved with increasing capillary temperature (refer to Figure 2e), which provided important insight regarding the HDX mechanism. If the exchange reaction occurs exclusively at the source region in the gas phase, the capillary temperature would not have an impact on the exchanged ion signal. Therefore, the large increase in the exchanged signal with increasing capillary temperature suggested that the HDX reaction occurs not only at the ionization source but also in the capillary region. The influence of capillary temperature on ESI HDX in case of proteins and peptides was investigated, and the increase of HDX signal by the increase of capillary temperature was also observed [50, 51]. The main role of capillary temperature is to evaporate solvents from clusters. Therefore, the effect of capillary temperature on the HDX signal indicates that HDX products are abundant in the solvent cluster when it enters the capillary. This strongly suggests that the HDX reaction occurs when molecules are contained in the solvent cluster. Therefore, the mechanism of APCI HDX may be different from that of HDX under CI conditions, which occurs in the gas phase [52, 53].

Figure 2f shows the marked effect of APCI vaporizer temperature on HDX signal intensity. The signal was increased by about 2-fold as the vaporizer temperature was increased from 200°C to 400°C. An inverse correlation was observed for two of the three compounds between the percentage concentration of CH_3OD and the HDX signal abundance (Figure 2g). It is well known that the addition of methanol can decrease the (+) APCI signal [54]. In addition, the deuterated solvent is more expensive than regular solvent. Therefore, it is advantageous to minimize the CH_3OD concentration. However, it is also advantageous to have enough CH_3OD in the solution to facilitate its use as an exchange reagent. Therefore, a concentration of 30% was used in the experiments described here.

A corona discharge current above or below 5 μA led to severe fluctuations at the highest HDX peak intensity (Figure 2h). At the chosen discharge current of 5 μA, sheath and auxiliary gas flows of 10 and 2 units, respectively, were found to deliver the highest intensity of HDX peak. The optimized experimental conditions are summarized in Table 1.

Analysis of Standard Compounds Containing Nitrogen

Twenty standard nitrogen-containing compounds were analyzed under the optimized (+) APCI HDX conditions described in the previous section. The spectra obtained from all 20 compounds are listed in the Supplementary Figure 3S. Some of the representative spectra are presented in Figure 3. The overall results obtained from the 20 compounds are summarized in Table 2, including the relative abundance of the peaks originating from the different types of ions. The nomenclature used for the different ions was presented in the previous section.

Compounds in group I in Table 2 have two exchangeable hydrogen atoms attached to a nitrogen atom and also have aromatic amines containing the R-NH_2 functional group. These

Table 2. Observed HDX Peaks and Their Relative Abundance Observed from (+) APCI HDX MS

Group	Category	No.	Compound	DBE value	Relative abundance (%)[a]				
					d_0MD^+	d_1MD^+	$d_2M^{+\cdot}$	d_2MD^+	d_3MD^+
I	Primary aromatic amine	1	Aniline	4		10.11	0.13	100	0.43
		2	p-Toluidine	4		11.15	0.24	100	0.28
		3	1-Naphthylamine	7		10.86	0.94	100	01
		4	2-Aminoanthracene	10		10.31	0.69	100	56.85
		5	1-Aminopyrene	12		10.81	3.01	100	
II	Secondary aromatic amine	6	N-Methylaniline	4	7.86	100		1.14	
		7	Diphenylamine	8	17.7	100			
	Pyrrolic/indolic heteroarene	8	3-Methylindole	6	18.33	100		6.41	
		9	2-Phenylindole	10	39.24	100		16.02	
III	Tertiary aromatic amine	10	*N*,*N*-Dimethylaniline	4	100				
		11	Triphenylamine	12	100				
		12	*N*-Methyl diphenylamine	8	100				
	Pyrrolic/indolic heteroarene	13	*N*-Methylpyrrole	3	100	28.28		3.21	0.76
		14	9-Phenylcarbazole	13	100	92.29		1.68	2.03
		15	1-Methyl-2-phenylindole	10	100	75.51			
	Pyridinic heteroarene	16	Quinoline	7	100				
		17	Acridine	10	100				
		18	2-Ethylpyridine	4	100				
		19	7-Methylquinoline	7	100				
		20	2-phenylpyridine	8	100				

[a]Calculated from mass spectral data

compounds showed the exchange of two hydrogen atoms with deuterium atoms (denoted as d_2) as well as the adduction of one deuterium ion (denoted as D^+). For example, d_2MD^+ (m/z= 97.084) was the most abundant ion for aniline (m/z=93.058) in Figure 3a. Other types of ions, such as d_1MD^+, was observed but it had a lower abundance than d_2MD^+.

Group II in Table 2 includes compounds with one exchangeable hydrogen atom attached to the nitrogen atom. They have secondary amine (R-NH) or *N*-heteroarene functional groups. This group of compounds showed the exchange of one hydrogen atom with a deuterium atom (denoted as d_1) as well as the adduction of one deuterium ion (denoted as D^+) in most cases. In the case of *N*-methylaniline, the d_1MD^+ ion, resulting from the exchange of one active hydrogen atom attached to an N atom with one deuterium added through ionization was most abundant (refer to Figure 3b).

Compounds with no exchangeable hydrogen atoms are included in group III in Table 2. They have tertiary aromatic amine, *N*-alkylated *N*-heteroarene, or pyridine functional groups. In most cases, deuterium adduct ions were observed for this group of compounds. For example, in the APCI spectrum of *N*,*N*-dimethylaniline, d_0MD^+ was most abundant (refer to Figure 3c). It should be noted that d_1MD^+ was also abundant for compounds with *N*-alkylated *N*-heteroarenes (refer to No. 13–15 in Table 2). It is well documented that exchange can occur on hydrogen atoms in aromatic rings [55]. Indole compounds may be more prone to exchange because of the high electron density of the aromatic ring attributable to electron donation from the non-basic indole nitrogen.

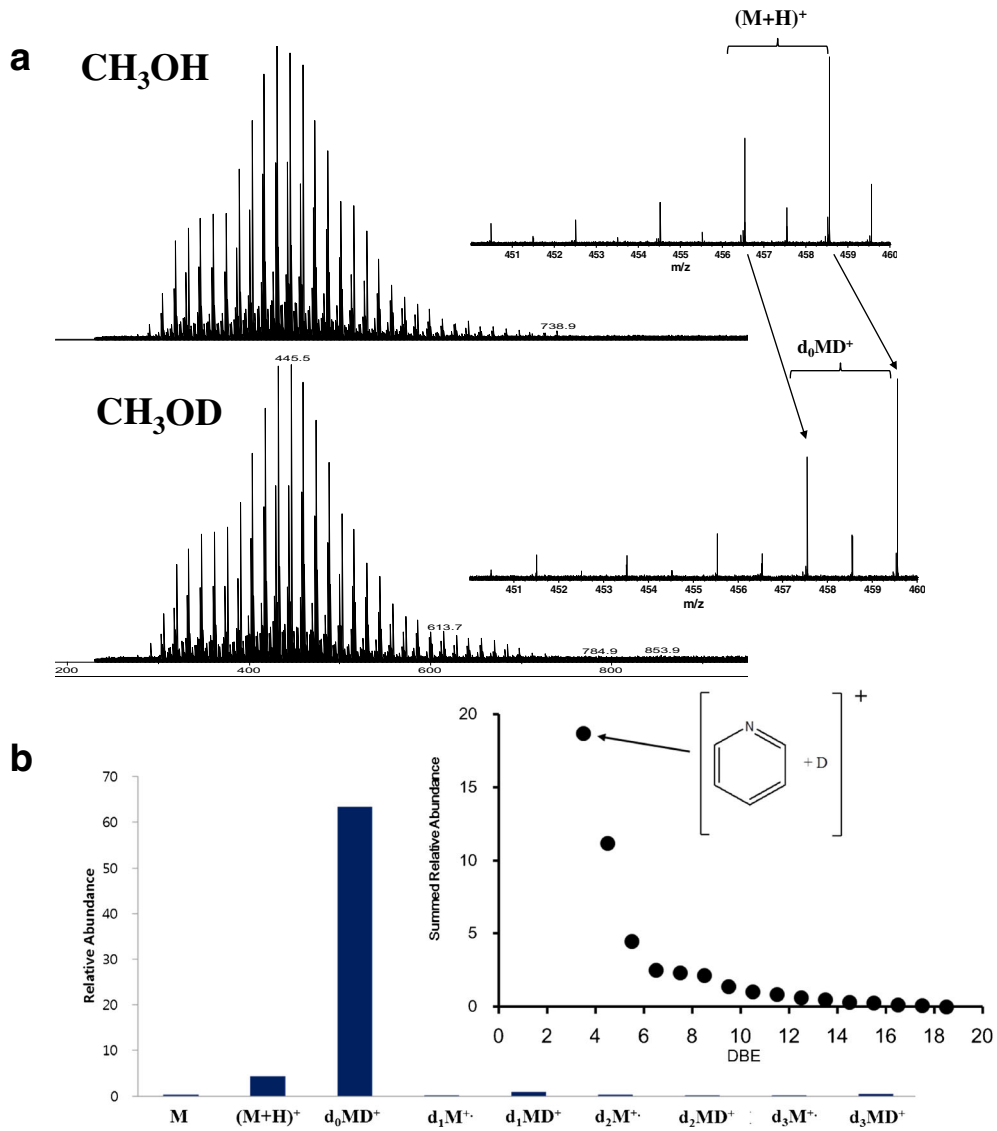

Figure 4. (**a**) Broad band and expanded (+) APCI high resolution mass spectra of APM resins using CH_3OH (top) and CH_3OD (bottom) as solvents. (**b**) The sum of the relative abundances of various types of N_1 peaks observed from the (+) APCI HDX spectrum shown in the bottom of (**a**). The double bond equivalent (DBE) distribution of the d_0MD^+ ions is presented as an inset

Comparing the results obtained from the 20 standard compounds, it was deduced that the number of HDXs observed correlated well with the number of hydrogen atoms attached to nitrogen. This result agreed well with those obtained using other ionization techniques, such as CI and (+) APPI HDX [30, 45, 56, 57]. It is important to note that the (+) APCI HDX has a limitation to distinguishing the compounds without exchangeable hydrogen. In case of (+) APPI HDX, compounds with pyridine functional groups could be distinguished from the ones with tertiary aromatic amine and *N*-alkylated *N*-heteroarenes.

Application of (+) HDX APCI MS

The resin fraction of shale oil, with a high nitrogen content, was dissolved in solutions of CH_3OH and CH_3OD using DCM as a cosolvent, as explained in the previous section. The solutions were analyzed by (+) HDX APCI MS. FT-ICR MS was used previously for this application because crude oil analysis typically requires high resolution. The resulting high-resolution mass spectra and subsequent processed data are shown in Figure 4. Figure 4a shows broad band and expanded spectra. After switching to CH_3OD, an increase of mass by 1 was observed in the overall spectra. The mass increase could be assigned to the difference between deuterium and hydrogen atoms (refer to the expanded spectra in Figure 4a).

To further analyze the molecular composition, elemental compositions of the peaks were calculated. The Kendrick mass plot was included to provide overall distribution of peaks (refer to Supplementary Figure 4S). The N_1 class was most abundant and the sum of the relative abundances of various types of N_1 peaks observed from the (+) APCI HDX spectrum (bottom of Figure 4a) were obtained and plotted in Figure 4b. In the figure, it is clear that d_0MD^+ was the most abundant ion. From the data presented in Table 2, it can be concluded that the crude oil analyzed here contains N_1 compounds and does not have exchangeable hydrogen atoms.

To further identify the structures of the observed ions, the double bond equivalent (DBE) values were calculated from the assigned formulas using the following equation:

$$\mathrm{DBE} = \mathrm{c} - (\mathrm{h} + \mathrm{d})/2 + \mathrm{n}/2 + 1 \quad (2)$$

(for $C_cH_hD_dN_n$ elemental formulas)

The DBE distribution of the d_0MD^+ ions is presented as an inset in Figure 4b. The DBE distribution started from 3.5. As the attached deuterium ion contributes –0.5 to the DBE value, the DBE value of neutral molecules starts from 4. Pyridine (C_5H_5N) has a DBE value of 4 and does not have an exchangeable hydrogen atom attached to the nitrogen atom. Therefore, the DBE distribution strongly suggests that the N_1 compounds observed in the oil sample have pyridine type structures. This agrees well with the observations reported previously for (+) APPI MS analysis of the sample [31].

Conclusions

This study confirmed that (+) APCI HDX can be used to identify the number of active hydrogen atoms in nitrogen-containing compounds. The concept was confined using 20 standard nitrogen-containing compounds. (+) APCI HDX was applied to determine the chemical structures of nitrogen-containing compounds in the shale oil sample. The results obtained with (+) APCI HDX agreed well with those obtained with (+) APPI HDX, and clearly showed that (+) APCI HDX is a powerful method for studying nitrogen-containing compounds. It has been well documented that CI and APPI HDX can be used to identify the structures of nitrogen-containing compounds [30, 45, 57].

As all of the currently available ionization techniques are selective, it is advantageous to have an additional ionization technique for structural analysis.

Acknowledgments

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