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RESEARCH ARTICLE

Design and Validation of In-Source Atmospheric Pressure Photoionization Hydrogen/Deuterium Exchange Mass Spectrometry with Continuous Feeding of D_2O

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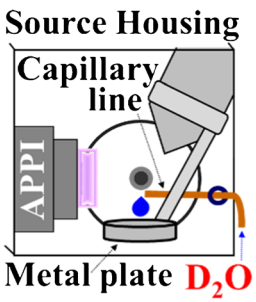

Abstract. In this study, continuous in-source hydrogen/deuterium exchange (HDX) atmospheric pressure photoionization (APPI) mass spectrometry (MS) with continuous feeding of D_2O was developed and validated. D_2O was continuously fed using a capillary line placed on the center of a metal plate positioned between the UV lamp and nebulizer. The proposed system overcomes the limitations of previously reported APPI HDX-MS approaches where deuterated solvents were premixed with sample solutions before ionization. This is particularly important for APPI because solvent composition can greatly influence ionization efficiency as well as the solubility of analytes. The experimental parameters for APPI HDX-MS with continuous feeding of D_2O were optimized, and the optimized conditions were applied for the analysis of nitrogen-, oxygen-, and sulfur-containing compounds. The developed method was also applied for the analysis of the polar fraction of a petroleum sample. Thus, the data presented in this study clearly show that the proposed HDX approach can serve as an effective analytical tool for the structural analysis of complex mixtures.

Keywords: Hydrogen/deuterium exchange, Mass spectrometry, Atmospheric-pressure photoionization

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Introduction

Hydrogen–deuterium exchange mass spectrometry (HDX-MS) is mainly used to count labile hydrogen atoms in molecules. Combined with other information such as elemental formulae provided by high resolution mass spectrometry and tandem mass spectrometry, the obtained information can be used to elucidate molecular structures [1–7]. Three types of HDX-MS experiments have been carried out. In the first, HDX is performed in solution for finding solvent exposed sites and thus gaining insights into the 3D structure of proteins and peptides [8].

The second type of HDX-MS is carried in the gas phase on mass selected ions via the use of gas-phase ion–molecule reactions [9–12]. The third type is carried out in the ionization source in gas phase either under reduced or atmospheric pressure. Conventionally, a deuterated gas such as ND_3 is introduced for chemical ionization (CI) in-source HDX MS [5–7, 13, 14]. With the recent development of atmospheric pressure ionization (API) sources, in-source API HDX-MS has been touted as a useful technique [1, 15, 16]. For example, HDX-MS coupled with electrospray ionization (ESI) have been developed and applied [16–21]. Deuterated solvents such as deuterium oxide (D_2O) were provided by a sheath or curtain gas flow for ESI HDX [3, 22]. In order to minimize the consumption of deuterated solvents, Hemling et al. proposed switching air and deuterated gas in the source interface [1], and Wolff et al. introduced deuterated solvents through a dual sprayer [17].

Atmospheric pressure photo ionization (APPI) is another important API technique that has been used to study the structures of nonpolar molecules [23–26]. APPI has been used for HDX-MS.[15, 27] In such applications, deuterated solvents were mixed with the sample solutions and the mixed solutions were subsequently injected into the ion source. This approach was successfully used to study the chemical structures of aromatic compounds in crude oils [27–30]. However, mixing sample solutions and deuterated solvents has two potential limitations. First, protic solvents cannot be used in the sample solutions because they can cause back exchange, thus limiting the choice of solvent; Supercritical fluid chromatography was combined with APPI HDX-MS to overcome this limitation

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[31]. The second potential limitation is that mixing deuterated solvents can limit analyte solubility and cause precipitation. For example, mixing heavy crude oil solutions with deuterated methanol can result in precipitation.

Recently, Kostyukevich et al. demonstrated a simple way to achieve in-source ESI HDX-MS [16, 32]. In this design, a drop of D_2O was added to a plate located in the ESI source. Exchanged compounds were observed from humic substances, proteins, peptides, and carbohydrates [33–39]. The major advantage of this method is that deuterated solvents can be added regardless of the composition of the sample solution. Therefore, it is reasonable to expect that this method could be successfully combined with APPI-MS to overcome the potential limitations of previous approaches [27, 30]. However, direct application of the method also suffers from some limitations, such as the time requirements of APPI HDX. Moreover, only fixed amounts of D_2O can be mounted on the plate, and D_2O evaporates faster in APPI than ESI because of the temperature and gas flow in the source.

In this study, an experimental setup for H/D-exchange with a continuous supply of D_2O was developed using a capillary line on a metal plate installed between the APPI lamp and nebulizer. The proposed method was optimized and validated using standard compounds containing heteroatoms and a petroleum sample.

Materials and Methods

Chemicals

HPLC-grade solvents, D_2O (99.9 atom %D), and nitrogen-, oxygen-, and sulfur-containing compounds were purchased from J. T. Baker (Center Valley, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA). The list of standard compounds used in this study is provided in the Supporting Information (Supplementary Table S1). All standards were dissolved in toluene to make 1 mM stock solutions, and were diluted to a final concentration of 10 μM. D_2O was previously used to create an atmosphere of D_2O vapor in the ESI region [16]. MeOD was used for in-source HDX [15, 27, 30], but MeOD clusters significantly reduced the APPI signal and D_2O clusters had less of a negative effect on the APPI signal [40]. In addition, D_2O evaporates slowly because of its high boiling point (101.4 °C) and hence remains in the hot ionization source for a longer period. Therefore, D_2O was used as a deuterating agent in this study.

Preparation of Oil Sample for HDX-MS Study

A crude oil sample from Kuwait was used in this study. Detailed sample information is provided in Supplementary Table S2. First, 10 g of the oil was dissolved in 1 mL of hexane. Next, the sample solution was sonicated for 5 min and filtered with a syringe filter (diameter 25 mm, pore size 0.2 μm). The oil was fractionated using a previously reported HPLC method [41, 42]. The fractionation system consisted of two automated six-port valves (Rheodyne, Rohnert Park CA, USA), a propylaminocyano column (250 × 4.6 mm, Phenomenex, CA, USA), and a dinitroanilinopropyl column (250 × 4.6 mm, ES Industries, Berlin, NJ). Five different fractions were obtained and the collected polar fraction was dried and redissolved in toluene for analysis.

HDX Design for APPI Source

The design used for continuous H/D exchange MS experiments with the APPI ionization source is presented in Figure 1. The detailed specifications of the metal plate and capillary line are provided in Supplementary Table S3. First, the circular metal plate was installed just beneath the nebulizer using a holder attached to the probe. The purpose of the metal plate was to hold the drop of deuterating-solvent (D-solvent). The plate was fixed in such a way that the drop of the D-solvent could flow on it and would remain on the plate during the experiment. The end of capillary tubing (inner diameter, 250 μm) was inserted into the ion-source housing through a hole on the front glass window and was placed approximately in the center of the plate. This capillary line was used to deliver the D-solvent continuously onto the plate. Thus, the D-solvent was added to the plate by connecting the other end of the capillary line with a syringe mounted on a syringe pump. As there is limited space in the source housing, the size of the plate was kept at 15 mm^2. The plate size should also be such that it can hold a sufficient volume of the D-solvent for HDX during ionization. As shown in Figure 1, the distance of the plate from the nebulizer, APPI lamp, and capillary entrance should be small enough to ensure sufficient contact between D_2O and analyte molecules during the experiment.

Mass Spectrometry

A Q Exactive quadrupole Orbitrap mass spectrometer (Thermo Fisher Scientific Inc., Rockford, IL, USA) and an APPI source (Krypton vacuum UV lamp) were utilized for the in-source APPI HDX MS. A Harvard stainless steel syringe (5 mL) and syringe pump (model 11; Harvard, Holliston, MA, USA) were utilized to deliver the standard and

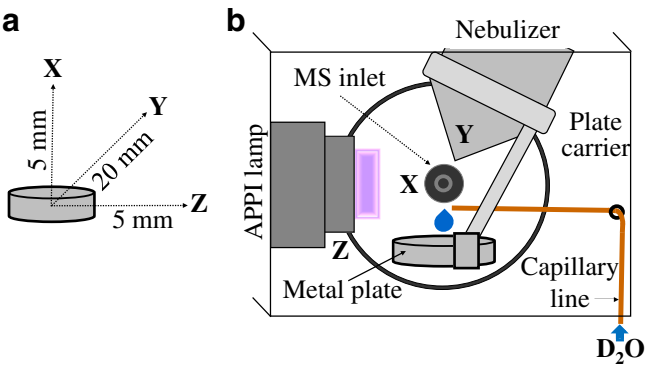


Figure 1. (**a**) Two-dimensional layout showing the distance of the metal plate from the heated capillary or MS inlet (X), nebulizer sprayer (Y), and the APPI UV lamp (Z); (**b**) combined design modified for continuous in-source APPI HDX-MS set up including the insertion of a capillary line inside the ion-source house (by piercing a hole on the front window glass of the source house) and the placement of a metal plate at the middle position among APPI lamp, MS capillary and nebulizer

sample solutions. A Harvard stainless steel syringe (1 mL) and KD Scientific syringe pump (model 100, Holliston, MA, USA) were used to deliver the D-solvent. Typical APPI (+) conditions were as follows: tube lens radio frequency (rf) level, 50 Hz; tube lens voltage, 25 V; skimmer voltage, 15 V; C-Trap rf, 550 V; sweep gas flow, 0 (arbitrary units); sheath gas flow, 10 (arbitrary units), and auxiliary gas flow, 5 (arbitrary units). High-purity (99%) nitrogen was obtained upon evaporation of liquid nitrogen and was used as the source gas. External positive calibration was carried out using (+) Pierce Velos solution (Thermo Fisher Scientific) in the ESI source. The data acquisition parameters for the standard compounds were as follows: *m/z* range, 50–500; maximum injection time, 500 ms; 1 micro scan; automatic gain control (AGC) setting 5×10^5; resolution, 140,000. The data acquisition parameters for the oil fraction were as follows: *m/z* range, 450–660; maximum injection time, 500 ms; 1 micro scan; AGC ON; resolution, 140,000.

Data Analysis

The mass spectra of the standard compounds obtained from HDX-MS experiments were processed using Xcalibur 2.2 SP1.48 software (Thermo Fisher Scientific). The HDX-MS mass spectra of the polar oil fractions were processed using Xcalibur software and an in-house-developed software from Statistical Tool for Organic Mixtures' Spectra for Hydrogen/Deuterium eXchange (STORMS-HDX) [43, 44]. The molecular formulae of the HDX peaks were assigned within a 1 ppm mass error range.

Notation for Exchanged Ions

The following notations were used to designate exchanged ions, where (1) the molecular ion, (2) the protonated ion, (3) the radical ion with n number of H atoms exchanged with n deuterium atoms (n = 0, 1, 2, 3…), and (4) the exchanged ion with an additional deuterium ion (D^+).

$$M^{+\cdot} \ldots\ldots\ldots\ldots\ldots \quad (1)$$

$$MH^+ \ldots\ldots\ldots\ldots\ldots \quad (2)$$

$$d_nM^{+\cdot} \ldots\ldots \quad (3)$$

$$d_nMD^+ \ldots\ldots \quad (4)$$

Results and Discussion

Validation and Optimization of APPI HDX-MS with Continuous D_2O Feeding

The proposed experimental setup was initially tested on three nitrogen-containing standard compounds (p-toluidine, aniline,

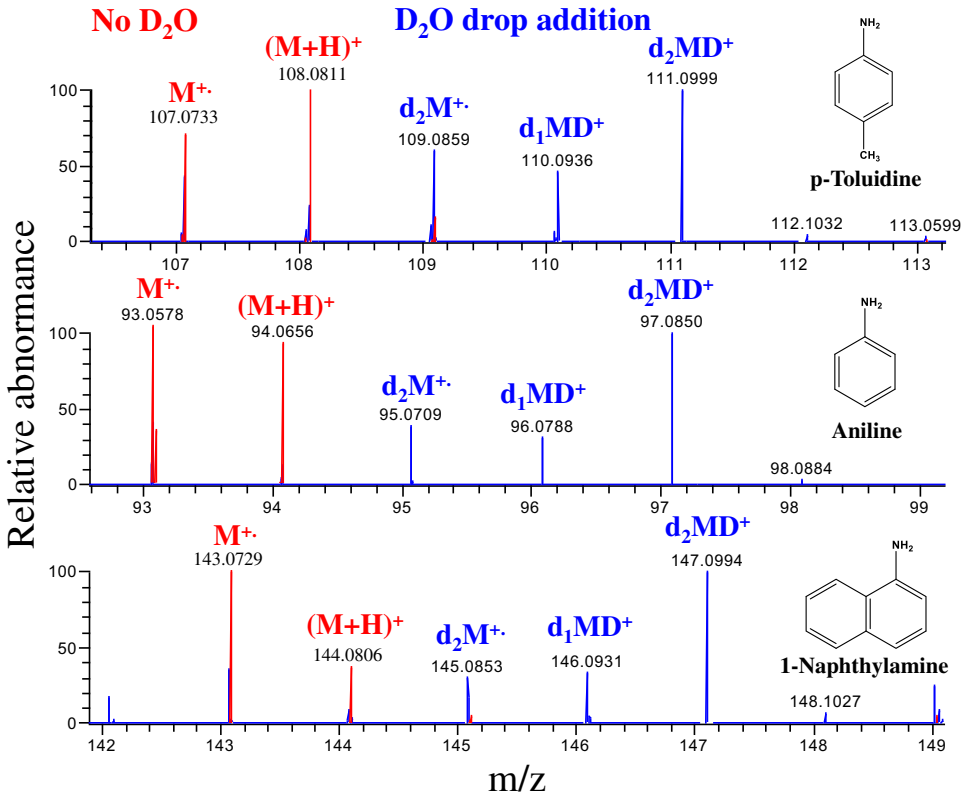


Figure 2. (+) APPI MS spectra from 10 μM solutions with p-toluidine, aniline, and 1-naphthylamine obtained without (red spectra) and with (blue spectra) D_2O. The flow rate of the analyte solution and D_2O drop addition was maintained at 50 μL/min and 20 μL/min, respectively. The source and capillary temperature was set at 300 °C

and 1-naphthylamine) with two exchangeable hydrogen atoms. The mass spectra of the tested compounds before and after the addition of D_2O (red and blue, respectively) are shown in Figure 2. Prior to the addition of a drop of D_2O, the tested compounds produced abundant molecular and protonated ions. Upon continuous addition of D_2O onto the plate by the capillary line, abundant d_2MD^+ and d_2M^+ ions were observed (Figure 2). Upon comparison of the mass spectra shown in Figure 2, it was confirmed that the design for APPI H/D-exchange was applicable for full HDX.

After establishing the design for H/D-exchange, the method was further optimized to improve the HDX efficiency. Optimization of the HDX-MS system was also necessary to allow for the accurate determination of exchangeable hydrogen atoms in analytes of interest. Key parameters that have a major impact on the current HDX approach, such as the flow rate of analyte solution, D_2O addition flow rate, and change in capillary and vaporizer temperatures were examined.

First, the effect of flow rate of the analyte solution on HDX efficiency was examined. For these experiments, the flow rate of D_2O was maintained at 20 μL/min and the vaporizer and capillary temperatures were set at 200 °C and 300 °C, respectively. The flow rate of the analyte solutions was varied from low to high values (1, 5, 10, 25, 50, 75, and 100 μL/min). Figure 3a demonstrates the HDX behavior of the exchanged ($d_2M^{+\cdot}$ + d_2MD^+) ions of the tested compounds at different analyte solution flow rates. The accurate number of labile hydrogen atoms was easily identified at low and high analyte solution flow rate and the degree of exchange was same at different flow rate of analyte solution. The total abundance of molecular and protonated ions ($M^{+\cdot}$ + MH^+) is shown in Figure 3a. The total abundance of the exchanged ions ($d_2M^{+\cdot}$ + d_2MD^+) at an analyte solution flow rate of 5 μL/min was higher than that at 1 μL/min. As shown in Figure 3a, the total abundance of the exchanged ions was highest at an analyte solution flow rate of 10 μL/min. It is reasonable to expect that the increase in signal is per increase of mass transfer rate of analyte solutions. However, the HDX efficiency decreased at 25 μL/min and was the lowest at an analyte solution flow rate of 100 μL/min. This may be attributed to the fact that overall ionization efficiency decreases at very high flow rates. It has been reported that solvent clusters can reduce APPI signals [40]. Therefore, a sample flow rate of about 10 μL/min was determined to be optimal when 20 μL/min of D_2O was used. This suggested that the best results could be obtained with a 2:1 ratio between the D_2O and sample flow rates.

Next, the optimum flow rate of D_2O was investigated with a fixed sample flow rate. Since the plate used in this experiment has a capacity of about 500 μL, an excessive flow rate of D_2O can result in overflow from the plate. The

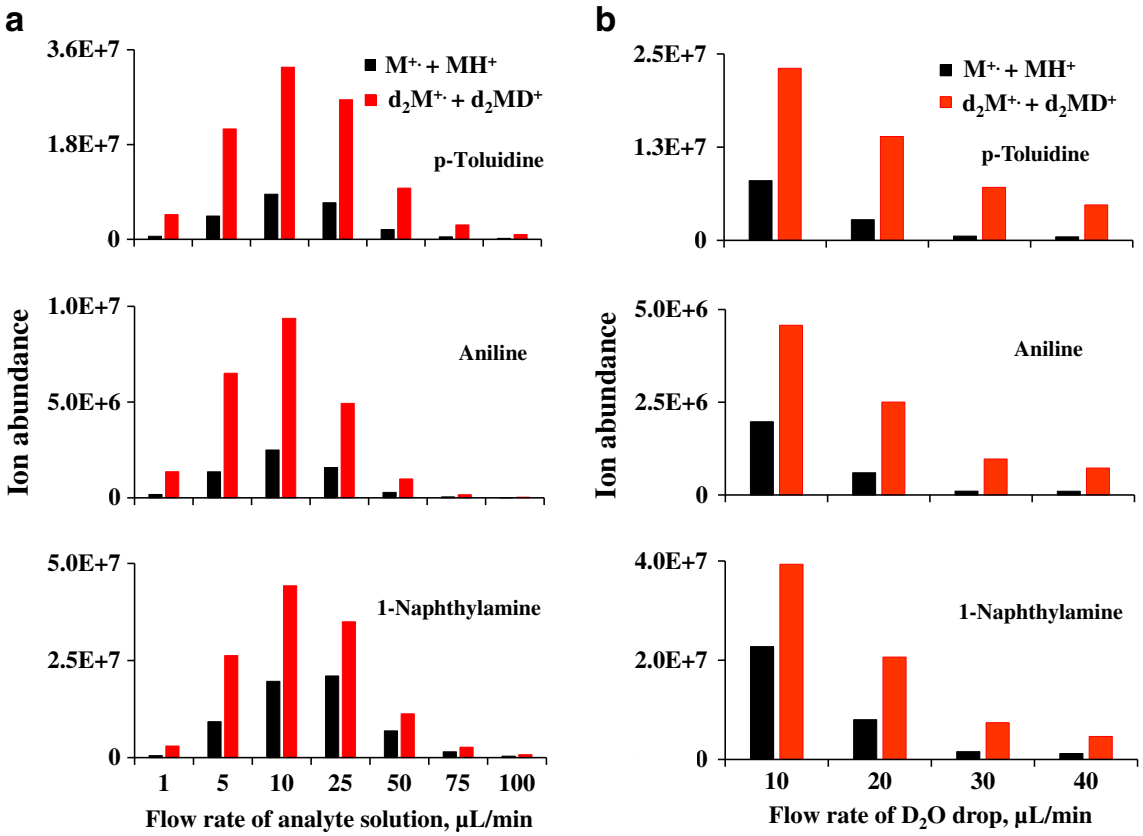


Figure 3. Plots showing summed abundances of exchanged and unexchanged ions observed by (+) APPI HDX-MS as a function of (**a**) analyte solution flow rate with D_2O flow rate of 20 μL/min; (**b**) D_2O flow rate with analyte solution flow rate of 5 μL/min. For all the data presented in Figure 3, the vaporizer and capillary temperature was 200 °C and 300 °C, respectively

highest flow rate of D_2O without concomitant overflow was determined to be about 40 µL/min. Therefore, the range of D_2O flow rates examined in this study was 10 to 40 µL/min. Figure 3b illustrates the dependence of HDX on D_2O flow rate at low sample flow rate. The accurate number of labile hydrogen atoms was easily identified even at low D_2O drop addition flow rate such as 10 µL/min. The analyte solution flow rate was kept at 5 µL/min and abundant exchanged ions were observed with a D_2O flow rate of 10 µL/min. The abundance of the exchanged peaks did not improve with increased D_2O flow rates. Supplementary Figure S1 presents the dependence of HDX on D_2O flow rate at high sample flow rate. The major HDX ions at low D_2O flow rate like 10 µL/min were $M^{+\cdot}$ and $d_2M^{+\cdot}$. Changing the flow rate of D_2O addition from 10 to 40 µL/min, d_2MD^+ was the most abundant ion (refer to Supplementary Figure S1). However, the sensitivity of d_2MD^+ ion was also low at higher D_2O addition flow rate. In summary, the D_2O flow rate should be larger than the sample flow rate for efficient HDX MS experiments (Figure 3a and b), and a 2:1 ratio between the D_2O and sample flow rates was optimal in the current design.

Previous studies of atmospheric pressure in-source HDX methods [27, 45] revealed that capillary and vaporizer ionization temperatures affected HDX efficiency. HDX MS spectra were obtained at various vaporizer temperatures; the spectra are presented in Supplementary Figure S2. The number of labile hydrogen atoms could not be identified at low vaporizer temperature like 50 °C because the major HDX ions were $M^{+\cdot}$, $d_2M^{+\cdot}$, and d_2MD^+. The summed abundance of exchanged ions ($d_2M^{+\cdot} + d_2MD^+$) observed at different vaporizer temperatures is presented in Figure 4a. The abundances of exchanged ions at a vaporizer temperature of 50 °C and 400 °C were the low. Dominant exchanged ions were obtained at 100 °C (Figure 4a). The abundance of exchanged ions decreased from 200 °C. Therefore, the vaporizer temperature was set at 100 °C in subsequent experiments.

The major role of capillary temperature in the current HDX method was to improve the sensitivity of HDX ions. The number of labile hydrogen atoms was found to be accurate at any temperature of the desolvating capillary as HDX in this method mainly occurs before the final desolvation stage. Figure 4b shows the summed abundance of ($d_2M^{+\cdot} + d_2MD^+$) ions at various capillary temperatures. Overall, the most abundant HDX signal was observed at a capillary temperature of 250 °C. The observed abundance decreased at capillary temperatures above 250 °C. However, when the same HDX approach was used previously with an ESI source, samples showed increased incorporated deuterium atoms with increasing capillary temperatures [33]. Notably, H/D-exchange reactions occurring at atmospheric pressure can also exchange non-labile H-atoms at high capillary temperatures such as 400 °C during ionization [22, 46]. Hence, a capillary temperature of 250 °C was determined to be optimal in the current H/D exchange experiments, in order to avoid the exchange of non-labile H-atoms in the target molecules.

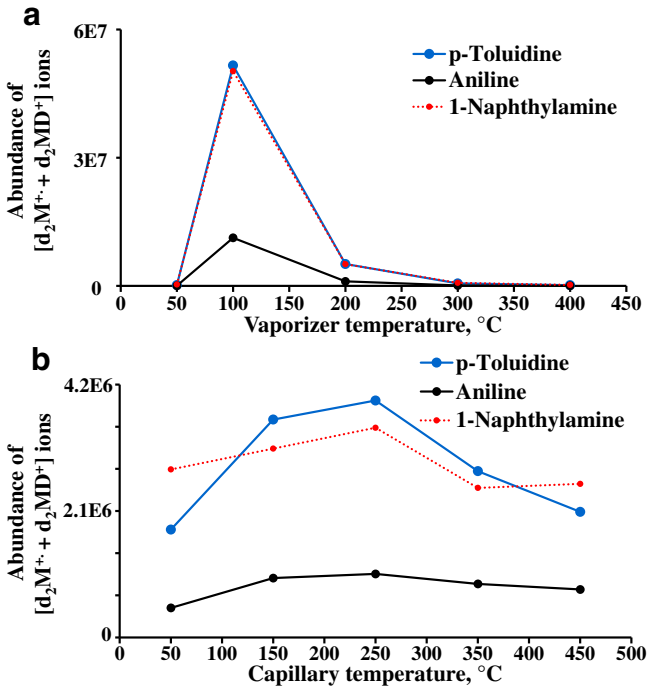


Figure 4. Plots showing summed abundance of exchanged ions observed by (+) APPI HDX-MS as a function of (**a**) vaporizer temperature with capillary temperature set at 300 °C; (**b**) capillary temperatures with vaporizer temperature set at 100 °C. The flow rates of the analyte solution and D_2O were maintained at 5 µL/min and 10 µL/min, respectively

Application of the Proposed Design to Standard Compounds

The optimized (+) APPI HDX MS procedure was applied to the analysis of standard compounds to further evaluate the effectiveness of the proposed method. Thirty-one nitrogen-, oxygen-, and sulfur-containing compounds with different numbers of exchangeable H-atoms in their structures were analyzed. Since APPI HDX-MS in positive mode was not sensitive enough for oxygen-containing compounds that also contained carboxylic acids [27], carboxylic acids were not analyzed.

The *m/z* values and assignments of most abundant ions observed before and after the addition of D_2O on the metal plate are listed in Table 1. Raw HDX-MS spectra of all compounds are provided in the Supporting Information (Supplementary Figure S3). For all compounds, the number of H/D-exchanges correlated well with the number of labile H-atoms present (Table 1 and Supplementary Figure S3). Moreover, the results summarized in Table 1 facilitated the unambiguous quantification of exchangeable hydrogens in each compound, which agreed with previous results obtained using in-source HDX MS methods [5, 15, 27, 30, 45]. Thus, the proposed HDX method can be used

Table 1. Summary of the Results for 31 Standard Compounds Analyzed by the Proposed Continuous In-Source (+) APPI HDX MS Method

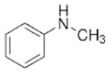
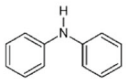
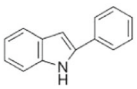
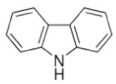
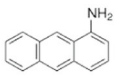
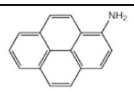
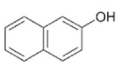
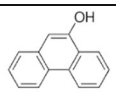
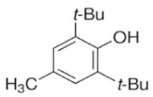
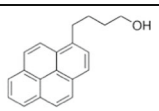
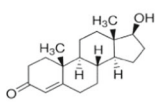
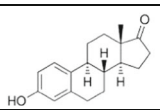
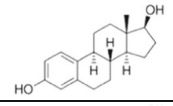
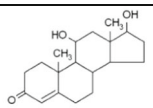
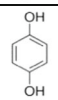
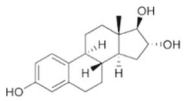
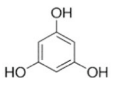
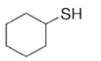
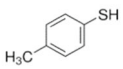
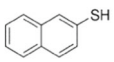
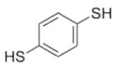
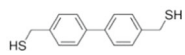
S. No	Name of compounds	Structure	No. of labile H	Relative abundances of most abundant peaks without D_2O addition		m/z and assignment of most abundant peak with D_2O addition	
				$(M^{+\cdot})$, %	(MH^+), %	Theoretical m/z	Actual m/z
1	N-Methylaniline		1	107.0734 100	108.0811 15.90	110.0933	110.0937 (d_1MD^+)
2	Diphenylamine		1	169.0885 100	170.0963 11.92	172.1089	172.1089 (d_1MD^+)
3	3-Methylindole		1	131.0730 100	132.0807 8.51	134.0933	134.0933 (d_1MD^+)
4	2-Phenylindole		1	193.0886 100	194.0963 16.99	196.1089	196.1089 (d_1MD^+)
5	Carbazole		1	167.0730 100	168.0807 11.25	168.0792	168.0791 $(d_1M^{+\cdot})$
6	2-Aminoanthracene		2	193.0885 100	194.0961 58.90	197.1152	197.1152 (d_2MD^+)
7	1-Aminopyrene		2	217.0885 100	n.o.	219.1011	219.1012 $(d_2M^{+\cdot})$
8	2-Aminopyridine		2	94.0534 0.26	95.0595 100	98.0792	98.0798 (d_2MD^+)
9	m-Phenylenediamine		4	108.0686 100	109.0766 23.55	114.1074	114.1077 (d_4MD^+)
10	2-Naphthol		1	144.0569 100	145.0649 1.22	145.0632	145.0632 $(d_1M^{+\cdot})$
11	9-Phenanthrol		1	194.0722 2.41	195.0804 100	195.0788	195.0798 $(d_1M^{+\cdot})$
12	2,6-Di-tert-butyl-4-methyl phenol		1	220.1829 100	n.o.	221.1884	221.1899 $(d_1M^{+\cdot})$
13	1-Pyrenebuthanol		1	274.137 100	275.1454 7.65	275.1415	275.1431 $(d_1M^{+\cdot})$
14	Testosterone		1	288.2084 1.22	289.2180 100	291.2287	291.2296 (d_1MD^+)
15	Estrone		1	270.1611 14.21	271.1689 100	271.1677	271.1674 $(d_1M^{+\cdot})$

Table 1. (continued)

16	3-Estradiol		2	272.1767 100	273.1845 1.60	274.1896	274.1894 ($d_2M^{+\cdot}$)
17	4-androsten-11β,17β-Diol-3-one		2	304.2033 1.32	305.2120 100	308.2299	308.2309 (d_2MD^{+})
18	Hydroquinone		2	110.0367 100	111.0445 27.72	112.0487	112.0492 ($d_2M^{+\cdot}$)
19	1,5-Dihydroxynaphthalene		2	160.0529 100	161.0607 6.64	162.0644	162.0654 ($d_2M^{+\cdot}$)
20	Estriol		3	288.1716 100	289.1794 3.48	291.1908	291.1908 ($d_3M^{+\cdot}$)
21	Phloroglucinol		3	126.0326 17.25	127.0401 100	131.0641	131.0652 (d_3MD^{+})
22	1-Decanethiol	$CH_3(CH_2)_8CH_2SH$	1	174.1435 100	n.o.	175.1499	175.1494 (d_1M^{+})
23	1-Dodecanethiol	$CH_3(CH_2)_{10}CH_2SH$	1	202.1752 100	n.o.	203.1812	203.1809 ($d_1M^{+\cdot}$)
24	1,8-octanedithiol	$HSCH_2(CH_2)_6CH_2SH$	2	178.0841 100	179.0927 33.57	182.1111	182.1104 (d_2MD^{+})
25	Cyclopentanethiol		1	102.0491 0.19	103.0554 100	105.0701	105.0711 (d_1MD^{+})
26	Cyclohexanethiol		1	116.0649 100	n.o.	119.0858	119.0867 (d_1MD^{+})
27	4-Methylbenzenethiol		1	124.0352 100	125.0434 0.10	125.0404	125.0415 ($d_1M^{+\cdot}$)
28	p-Xylene-alpha-thiol		1	138.0493 100	n.o.	141.0701	141.0709 (d_1MD^{+})
29	2-Naphthalenethiol		1	160.0353 100	161.0423 1.33	161.0404	161.0416 ($d_1M^{+\cdot}$)
30	Benzene-1,4-dithiol		2	141.9916 100	n.o.	144.0031	144.0042 ($d_2M^{+\cdot}$)
31	4,4'-bis(mercaptomethyl) biphenyl		2	246.0534 100	247.0595 3.6	248.0657	248.0675 ($d_2M^{+\cdot}$)

for the identification of nitrogen-, oxygen-, and sulfur-containing compounds with diverse structures.

Application of the Proposed Design to Oil Sample

The polar fraction of a crude oil sample was analyzed using the proposed HDX method in order to illustrate the viability of the method in determining the number of exchangeable H-atoms in analytes. Instead of carrying out a thorough investigation of the structures of compounds in the oil sample, only the exchanged peaks were interpreted. Figure 5 shows the APPI HDX MS broad band and expanded spectra with and without addition of D_2O obtained during APPI HDX MS analysis of the oil sample. As

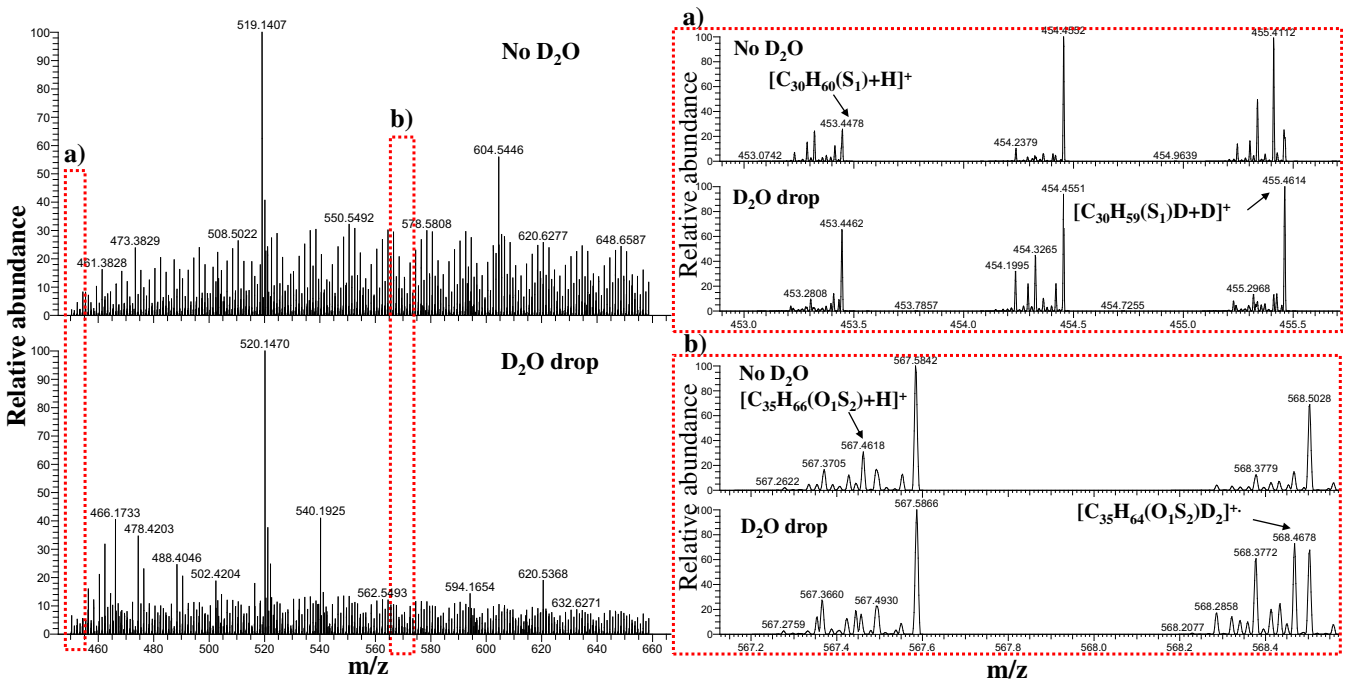


Figure 5. (+) APPI MS spectra obtained from polar fraction of oil sample without and with addition of D_2O in *m/z* window between 450 and 660 (left column). Expanded spectra of narrow *m/z* windows marked as (**a**) and (**b**) are presented in the right column

shown in Figure 5 (left side-top and bottom), there was a significant difference in the *m/z* values in the overall spectra with and without D_2O, indicating the presence of HDX product ions. S_1 and O_1S_2 were the major chemical classes observed during (+) APPI HDX MS analysis. As a result, the HDX examples of S_1 and O_1S_2 species are shown in expanded spectra in Figure 5 (red box-a, b).

The red box-a in Figure 5 shows the presence of $[C_{30}H_{59}(S_1)D_2]^+$ upon continuous addition of D_2O, which was denoted by the d_1MD^+ ion. Further, H/D-exchange was also confirmed by calculating the non-exchanged mass of the $[C_{30}H_{60}(S_1)+H]^+$ ion (MH^+ ion) in the absence of D_2O. Thus, the d_1MD^+ ion represents the presence of one exchangeable H atom attached to the sulfur atom. The red box-b in Figure 5 shows the presence of the $[C_{35}H_{64}(O_1S_2)D_2]^{+\cdot}$ ion upon continuous addition of D_2O, which was denoted by the $d_2M^{+\cdot}$ ion. The $d_2M^{+\cdot}$ ion indicates the exchange of two H-atoms with two deuterium atoms, which was confirmed by the presence of the non-exchanged $[C_{35}H_{66}(O_1S_2)+H]^+$ ion (MH^+ ion) in the absence of D_2O. Thus the data presented in Figure 5 strongly suggested that two exchangeable H atoms were present in $[C_{35}H_{66}(O_1S_2)+H]^+$ ion. Therefore, the proposed HDX-MS method can be viable for the structural elucidation of various compounds in complex mixtures. It should be noted that the proposed design is the inauguration of continuous HDX-MS system. Applicability of the continuous HDX-MS system needs to be further tested and confirmed by using ultrahigh resolution mass spectrometry. In that sense, it would be more informative to adopt this method to Fourier transform ion cyclotron resonance-mass spectrometer (FTICR-MS) for HDX analysis of complex mixture such as crude oil.

Conclusions

In this study, a continuous in-source APPI H/D exchange MS technique was developed. Considering the capabilities of the current HDX method and major parameters influencing HDX-behavior, some experimental conditions were optimized for accurate HDX data interpretation and to improve HDX efficiency. The method was used to distinguish the number of labile hydrogens in diverse nitrogen-, oxygen-, and sulphur containing compounds, in addition to a crude oil sample. The primary advantage of this method is that H/D-exchange can take place regardless of the composition of the sample solution. Owing to the HDX efficacy at low vaporizer temperatures, it is expected that this technique will be suitable for the analysis of thermally sensitive compounds, as well as those with high volatility. The current HDX-MS method is expected to be used in combination with liquid chromatography systems.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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