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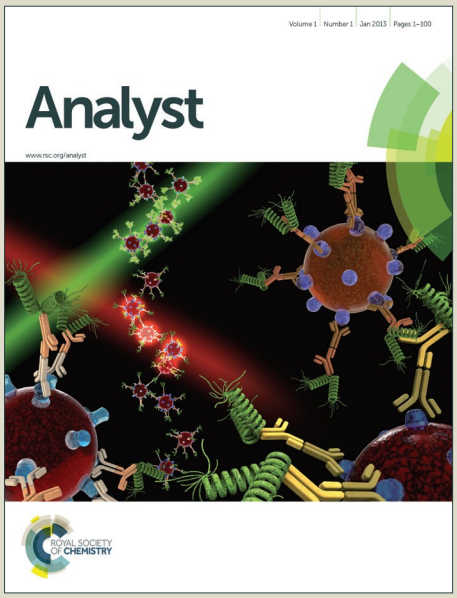

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High desolvation temperature facilitates in ESI-source H/D exchange at non-labile sites of hydroxybenzoic acids and aromatic amino acids

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KEYWORDS

H/D exchange, non-labile protons, desolvation temperature, mass spectrometry, ESI, natural organic matter

ABSTRACT

Hydrogen/Deuterium exchange coupled to high-resolution mass spectrometry has become a powerful analytical approach for structural investigations of complex organic matrices. Here we report feasibility of the site-specific H/D exchange of non-labile hydrogens directly in the electrospray ionization (ESI) source, which was facilitated by an increase in the desolvation temperature from 200 ^{o}C up to 400 ^{o}C. We have found that the exchanges at non-labile sites were observed only for the model compounds capable of keto-enol tautomeric transformations (e.g., 2,3-, 2,4-dihydroxybenzoic acids, gallic acid, DOPA), and only when water was used as a solvent. We hypothesized that the detected additional exchanges were induced by the presence of hydroxyls in the sprayed water droplets generated in the negative ESI mode. It was indicative of the exchange reactions rather taking place in the sprayed droplets than in the gas phase. To support this hypothesis, the H/D exchange experiments were run in deuterated water under base-catalyzed conditions for three model compounds, which showed the most intensive exchanges in the MS experiments: DOPA, 2,4-DHB, and 5-acetylsalycilic acid. 2H NMR spectroscopy has confirmed keto-enolic transformations of the model compounds leading to the specific labeling of the corresponding non-labile sites. We believe that the proposed technique will be useful for structural investigations of natural complex mixtures (e.g. proteins, humic substances) using site-specific H/D exchange.

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Introduction

Hydrogen/deuterium exchange (HDX) is an analytical approach, which is widely used for structural studies of proteins.[1,2] The labile hydrogens attached to heteroatoms such as oxygen, nitrogen, sulfur, can be easily replaced on deuterium by incubation with deuterated solvents such as D_2O, MeOD, and others.[3] Mass measurement of the target molecule is a method of choice to monitor HDX reactions.[4–7] A use of soft ionization techniques, such as electrospray ionization (ESI), coupled to HDX allows for identification of labile hydrogens in proteins[2,8,9] and other polymers[10–12]. That is why numerous studies were performed on H/D exchange in ESI[13–17], APPI[18,19], APCI[20], and others[21]. Specific option of atmospheric pressure HDX mass-spectrometry is a facile conversion of folded to unfolded proteins during ionization, which can be achieved by heating desolvation capillary.[22] This conformational change gives rise to a number of exchanged labile hydrogens which are easily registered by mass-spectrometer and used for data interpretation. However, some studies on gas phase H/D exchange at the atmospheric pressure reported deuterium enrichment exceeding the maximum amount of labile hydrogens present in the system.[4,23,24] The authors related these extra exchanges to contribution from C-H acidity, which can become profound under conditions of mass-spectrometric ionization, and warned of misinterpretation of this kind of results. Of particular importance this problem could become for ESI: during ionization the solvent undergoes slight electrolysis, which, in case of water, might lead to generation of trace amounts of hydroxyls.[25] The presence of base might induce exchanges of non-labile hydrogens, which are typical for base-catalyzed H/D exchange, e.g. due to keto-enol tautomerism.[3]

To demonstrate feasibility of in-ESI H/D exchange at the C-H sites capable of keto-enol transformations, we have relied on our previous studies with respect both to acid/base catalyzed

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HDX at non-labile sites in aqueous solution of the synthetic humic substances,[26] and to in ESI HDX at non-labile sites of 2-nitrophloroglucinol observed due to tautomeric transformations induced by heating of the desolvation capillary.[27] The last finding allowed us to suggest that in the presence of base in the sprayed solvent, heating of the capillary might facilitate conversion of the compounds capable of keto-enol tautomerism which would give rise to HDX at these specific non-labile sites. If this is the case, a simple and robust technique could be developed for conducting selective H/D exchange directly in the mass-spectrometer.

The objective of this study was to demonstrate feasibility of in-ESI H/D exchange at the C-H sites capable of keto-enol transformations, which can be induced by an increase in desolvation temperature as shown in Fig 1.

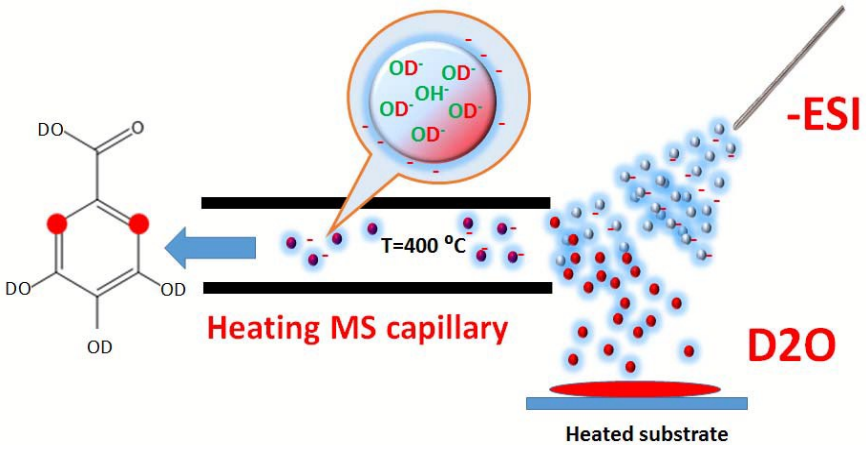

Fig. 1 The design of the in-ESI source H/D exchange experiment. The charged droplets are produced by the ESI source; they pass through the D2O-saturated region, enter heated capillary and evaporate producing the gas phase ions. During this transport the charged droplets interact with D_2O containing trace amounts of hydroxyls, and exchange protium against deuterium . Red dots designate C-H sites capable of keto-enol tautomerism, which have undergone labeling.

To avoid H/D back exchange, all in ESI HDX experiments were run in the D_2O-saturated atmosphere which was created by placing a D_2O droplet right underneath the ESI cone as

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described previously.[28,29] A model set of hydroxybenzoic acids and aromatic amino acids was used for this purpose, which possesses different non-labile sites capable of keto-enolic tautomerism. The obtained MS results were compared to the base-catalyzed HDX experiments in solution, which were quantified using ^{2}H NMR spectroscopy.

Experimental

Materials. All reagents used in this study are commercially available. Methanol, ethyl acetate and ammonium hydroxide were of analytical grade. The enrichment of D_2O (Merck) was 99.9%.

All model compounds were purchased from Sigma-Aldrich with purity of 99% and included: 2,5-dihydroxybenzoic acid, 2,3- dihydroxybenzoic acid, 2,4- dihydroxybenzoic acid, 3,4,5,-trihydroxybenzoic (gallic) acid, tyrosine, 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenethylamine, 5-acetylsalicylic acid, and 2,2-diphenylacetic acid.

Sample preparation for FT ICRMS measurements. The samples were prepared in methanol and in its mixture with distilled water (1:1 V/V). All concentrations were 0.4 mg·mL^{-1}.

H/D exchange in deuterated solvents in solution.

1-amino-2-(3,4-dihydroxy-2,5,6 trideuteropheny1)propanoic acid (Dopa-$^2H_3$). Deuteration of DOPA was conducted according to the modified procedure.[30,31] In brief, a weight of DOPA (200 mg, or 1 mmol) was placed into glass tube, added with 660 μL of 4M NaOD, sealed, and heated for 40 h at 120˚C. Then, the solution was acidified with conc. HCl; the precipitate was collected, washed by cold distilled water and dried. The yield of DOPA-$^2H_3$ was 193 mg (95%).

2,4-dihydroxybenzoic acid (DHB-2,4) and 5-acetylsalycilic acid.

A weight of 150 mg of DHB-2,4 or 5-acetylsalycilic acid was added with 660 μL of 4M NaOD, heated in a sealed tube for 40 h at 120 ˚C, acidified with conc. HCl until pH 2, and extracted with

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ethylacetate. The organic phase was dried using anhydrous Na_2SO_4, and rotor-evaporated. The yield was 130 mg (86%).

Deuterium (^{2}H) NMR spectroscopy. ^{2}H NMR spectra (61.397 MHz) were acquired using NMR spectrometer Agilent 400MR allowing the use of lock channel for observation of the deuterium-proton decoupled spectra and equipped with a 10 mm deuterium selective probe.

Duration of 90º pulse for ^{2}H nuclei was 25 μs. The acquisition time of the free induction decay was at least 4 s and the relaxation delay between pulses was 1 s. The spectral width was 1100 Hz. Chemical shifts were measured with a reference to the solvent (D_2O – 4.72 ppm, DMSO – 2.47 ppm). Integral intensities of signals were determined by an iteration analysis of the total line shape taking into account the residual field inhomogeneity and phase distortions using the INTSPECT2 program.[32]

MS analysis. All experiments were performed using a LTQ FT Ultra (Thermo Electron Corp., Bremen, Germany) mass-spectrometer equipped with a 7T superconducting magnet. Ions were generated by an IonMax Electrospray ion source (Thermo Electron Corp., Bremen, Germany) in both negative and positive ESI modes. The temperature of the desolvating capillary varied from 200 ^{o}C to 400 ^{o}C. The length of the desolvating capillary was 105 mm and its inner diameter was 0.5 mm. The infusion rate of the sample was 1 μL/min and the needle voltage was 3 kV. Full-scan MS spectra (m/z 200–2000) were acquired in the FTICR with a resolution R = 400 000 at m/z 400.

In-ESI source H/D exchange. The experimental setup for performing the H/D exchange was based on our previous developments described in.[22,28] The current design is shown in Fig.1. The charged droplets are produced by the ESI source; they pass through the heated capillary and evaporate producing the gas phase ions. During this transport, the ions interact with D_2O vapors

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and exchange labile protium against deuterium. To create an atmosphere saturated with D_2O vapor between the ESI needle and the MS entrance capillary, 400 μL D_2O were placed on a copper plate positioned approximately 7 mm underneath the ESI needle. All experiments were run at normal and high desolvation temperatures, which accounted for 200°C and 400°C, respectively. A choice of the high desolvation temperature was based on preliminary HDX experiments on 2,4- dihydroxybenzoic acid (2,4-DHB), which were run at 300°C, 400°C, and 450°C. The obtained results showed that a raise in the capillary temperature from 200°C up to 400°C was accompanied by a substantial growth in the intensity of the additional peak related to the non-labile HDX, whereas the further heating up to 450°C did not bring about any significant change in this peak intensity. The corresponding data are shown in Fig. S1 in the Supporting Information. As a result, all HDX experiments at the elevated desolvation temperature were run at 400°C.

Results and discussion

In-ESI source H/D exchange. To explore if an increase in the desolvation temperature may facilitate exchange of the non-labile protons, we have run H/D exchange reactions at normal and high desolvation temperatures (200°C and 400°C, respectively) for all model compounds shown in Table 1. The model set was composed of hydrozybenzoic acids and aromatic amino acids which can be considered as constituents of natural complex systems such as humic substances (HS), proteins, and others.[33] To account for a strong –M effect of carboxylic group on electronic density of the ring, we included into a model set three dihydoxybenzoic acids (DHBs) and gallic acid as representatives of the aromatic compounds with carboxyl group as a ring substituent (compounds from ***1*** to ***4***), whereas two amino acids were used as compounds with carboxyl

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group in the side chain (***5, 6***). We also used aromatic amine (compound ***7***) which does not possess any carboxyls, and 5-acetylsalicylic and 2,2-diphenylacetic acids (***8*** and ***9***, respectively) as containing α-carbons. A use of this model set was to allow us for in-depth exploration of the relationship between the structure and reactivity governing the H/D exchange reactions. The formulas designations and amounts of the H/D exchanges observed at the two desolvation temperatures for each compound are summarized in Table 1.

Table 1 Extent of Hydrogen-Deuterium exchange of model compounds with D_2O at normal (200 ^oC) and high (400 ^oC) desolvation temperatures.

Neutral compound producing ion	no. of labile H atoms in anion or cation*	Maximum no. of H/D exchanges observed at 200 ^oC	Maximum no. of H/D exchanges observed at 400 ^oC
2,4-OH[C6H3]COOH ***(1)***	2	2	4
2,5- OH[C6H3]COOH ***(2)***	2	2	2
2,3- OH[C6H3]COOH ***(3)***	2	2	3
3,4,5-OH[C6H2]COOH ***(4)***	3	3	5
4-OH[C6H4]CH2CH(NH2)COOH ***(5)***	3	3	3
3,4-OH[C6H3]CH2CH(NH2)COOH ***(6)***	4 or 6*	4 or 6*	7 or 9*
3,4-OH[C6H3]CH2CH2NH2 ***(7)***	5*	5*	8*
2-OH,5-CH3CO[C6H3]COOH ***(8)***	1	1	4
2,2-C6H5[CH]COOH ***(9)***	0	0	1

* cations in positive-ion mode

** mixture water-methanol (1:1 V/V) was used as a solvent

For desolvation temperature of 200^0C, a number of isotopic exchanges observed for all model compounds is equal to the theoretically expected from the number of labile H-atoms present in

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the anion or cation under study. However, a very different situation is observed when the desolvation temperature is set to 400^0C.

Figure 2 shows mass-spectra of H/D series for the three DHBs used in our study. At 200^0C, the length of isotopic exchange series is equal to the amount of labile H atoms (highlighted with blue color in the corresponding spectra and formulas). At 400 ^{0}C, the spectrum does not change for compound **2**, but one and two additional peaks can be seen for the compounds **1** and **3**, respectively (highlighted with red color). Given stability of the signals of deuteriated analyte ions over the time demonstrated in our previous studies,[13] we interpreted the observed impact of the capillary temperature on the H/D exchange of the DHBs as additional H/D exchanges occurring at the non-labile sites, which become reactive only under conditions of keto-enol tautomerism (highlighted in red in molecular structures shown in Fig. 2). Those sites are 3,5 for 2,4-DHB (***1*)**, 3 for 2,5-DHB (***2*)** and 5 for 2,3-DHB (***3*).** The reason is +M effect of electron-donating OH-substituent and neutral effect of COOH-group to meta-positions in the aromatic ring. However, in the case of 2,5-DHB (*2*), additional exchanges at C-H sites were not detected at the both temperatures tested, whereas a significant labeling was observed for 2,3-DHB, and 2,4-DHB at the elevated temperature.

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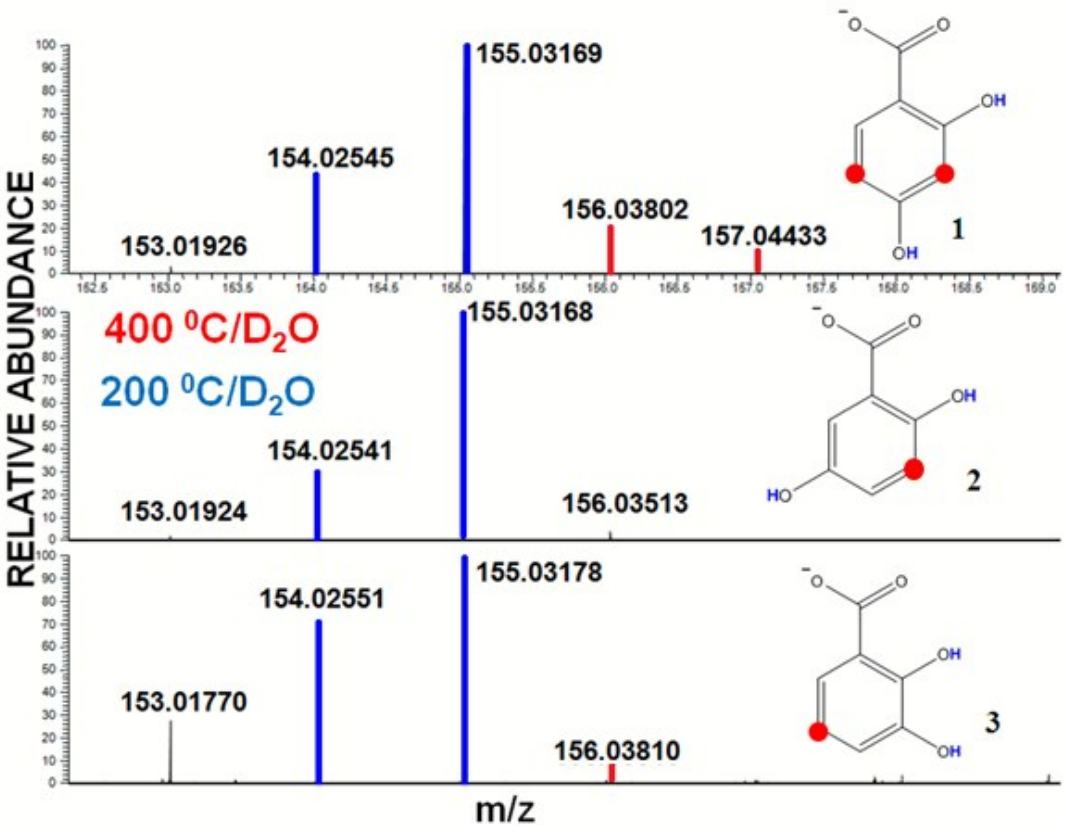

Fig. 2 In-ESI source H/D exchange series of 2,4-DHB, 2,5-DHB, and 2,3-DHB at 200 ^{0}C (blue lines) and 400 ^{0}C (additional peaks are colored in red). Red dots in the structural formulas designate the feasible sites of deuteration in accordance with keto-enol tautomerism and mesomeric substituent effects in the aromatic ring.

To explain these differences we have taken into account that the keto-form of phenol loses its proton to base generated in the solvent in the ESI source[25] to produce carbanion intermediate, which undergoes HDX. In the absence of a base, the proton abstraction becomes a rate-limiting reaction[34,35] and in case of MS analysis it should impact the detected results. To increase the rate of H elimination, we used 10% NH_3 solution in D_2O that resulted in appearance of additional OH-anion in the reaction mixture, which is a strong base. We performed H/D exchange of the gallic acid in the presence and absence of NH_3. The obtained mass-spectra are shown in Fig. 3. Gallic acid undergoes exchanges of all labile sites at 200 ^{0}C. An increase in the

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temperature leads to incorporation of two additional deuterons, which is indicative of HDX at both C-H sites. An addition of NH_3 to D_2O significantly magnifies enrichment of C-H sites with deuterium at the elevated temperature. We can assume that addition of the base catalyzes the HDX reaction similar to base-catalyzed reactions in solution, and the proton elimination is the limiting stage.

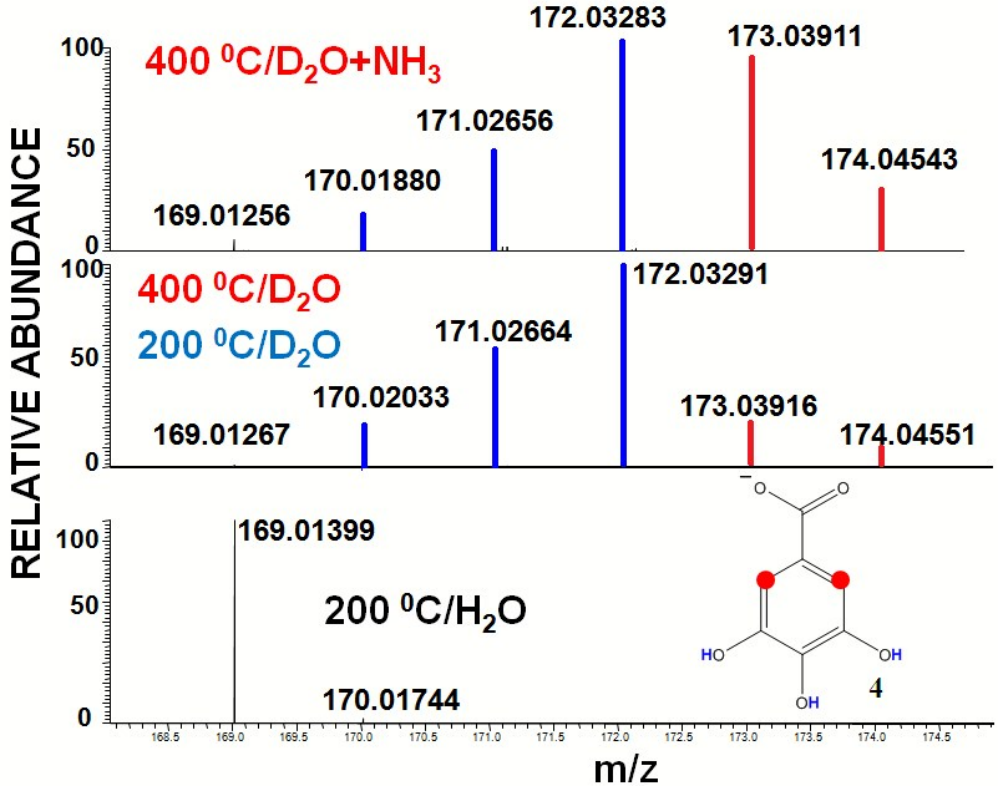

Fig. 3 In-ESI source H/D exchange series of gallic acid (**4**) in the absence and presence of ammonia. The peaks corresponding to labile and backbone deuterium are highlighted with blue and red color, respectively. Red dots in the structural formulas designate the feasible sites for deuteration in accordance with keto-enol tautomerism.

To evaluate an impact of carboxyl substituent, which is a strong electron acceptor, on H/D exchange in the aromatic ring, we conducted labeling of tyrosine (**5**) and DOPA (**6**). The major structural difference of **5** and **6** is the presence of one and two hydroxyl groups,

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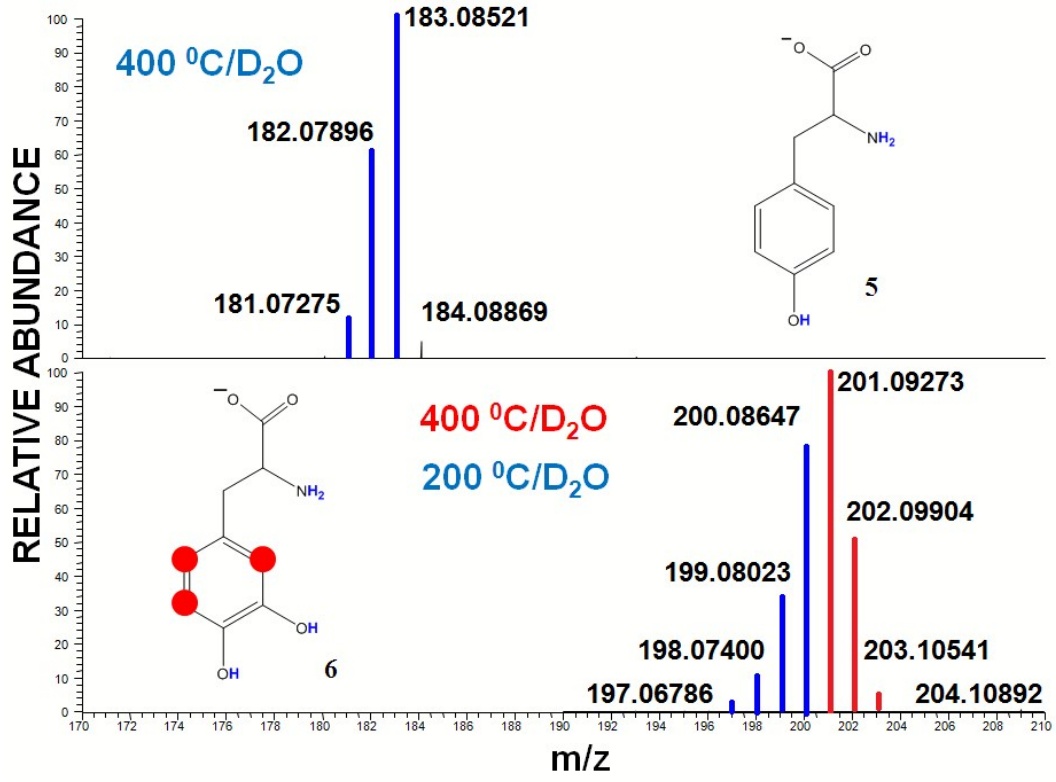
respectively. Figure 4 (a) shows 3 and 4 exchanges related to all labile protons in case of tyrosine and DOPA, respectively. At the elevated desolvation temperature substantial differences can be seen for HDX of tyrosine and DOPA: the former undergoes no additional exchanges, while the latter undergoes 3 additional exchanges at 400 ^{0}C.



a)

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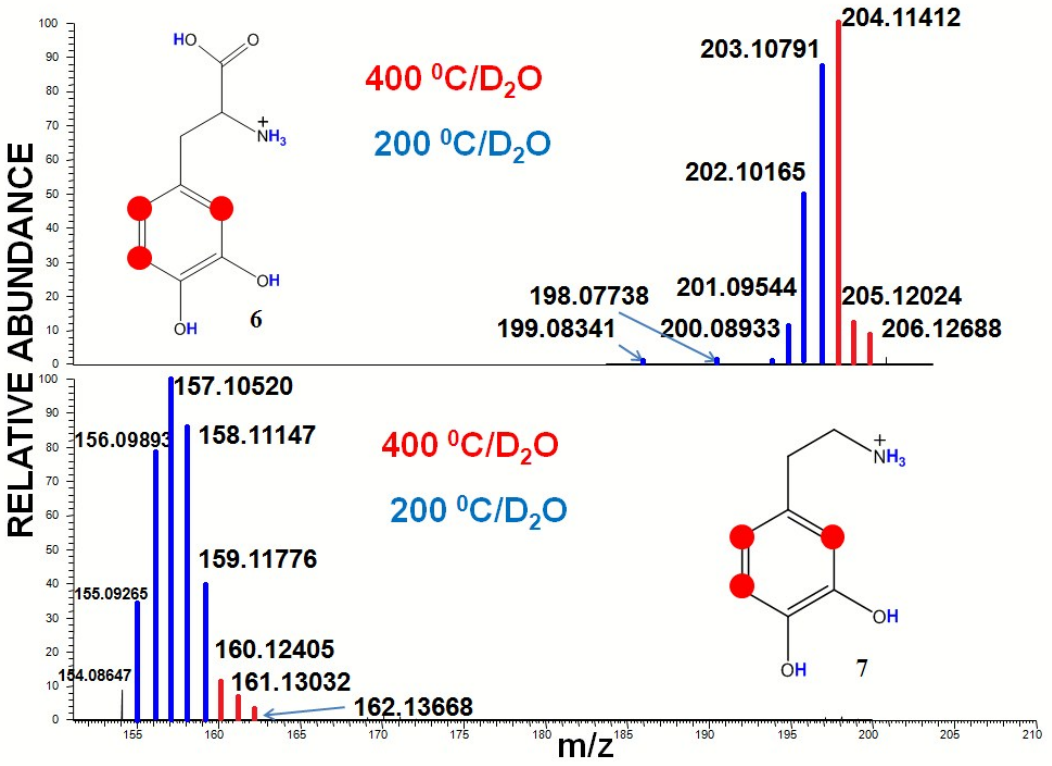

b)

Fig. 4 In-ESI source H/D exchange series at 400 °C of tyrosine (**5**) and DOPA (**6**) in negative mode (a) and of DOPA (**6**) and dopamine (**7**) in positive mode (b). The peaks corresponding to labile and backbone deuterium are colored in blue and red, respectively. Red dots in the structural formulas designate the feasible sites of deuteration in accordance with keto-enol tautomerism and mesomeric substituent effects in the aromatic ring.

The observed phenomenon is in line with the similar trend for DHBs, while compounds with hydroxyl groups attached to adjacent carbon atoms underwent extended isotopic exchanges. At the same time, neither compound **2** nor **3** were labeled at all sites. This implies that the extent of deuteration is significantly influenced by the –M effect of the carboxyl group.

To evaluate the influence of ionization mode on the labeling reactions, we conducted the HDX experiments with DOPA (**6**) and Dopamine (**7**) in positive mode. The corresponding spectra are shown in Fig. 4 (b). We observed 6 and 5 H/D exchanges for DOPA and Dopamine,

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respectively, at 200^{0}C (Fig. 4b, blue lines), which equaled amount of mobile protons, but at 400 ^{0}C, both compounds have undergone 3 more exchanges (Fig. 4b, red lines). At the same time the peak intensity of these ions was significantly smaller than in the case of negative ESI mode. In the positive mode of ESI almost a full charge is provided by H_3O^+ ions, which possesses a low catalytic activity for aromatic H/D exchange reactions[3]. However, given that reactions in the charged droplets of electro-spray can occur at higher rates than in the bulk solution[36], we believe that in this case H/D exchange proceeds through electrophilic substitution.

Collectively, the data obtained could be interpreted as a substantial contribution of keto-enol tautomerism into H/D exchange at the non-labile sites at the elevated desolvation temperature. It is indicative of HDX reactions taking place rather in the charged droplets than in the gas phase. This looks feasible given specific experimental conditions used in our study, where an atmosphere saturated with D_2O vapor was created between the ESI needle and the MS entrance capillary. In this case, the capillary heating in the presence of base might lead to two-step dissociation of the DHBs bringing about formation of phenoxide anions. This is facilitated by substantial temperature dependence of the acidity of hydroxyl group, which is not the case for carboxylic group: phenols possess dpK_a/dT values of about −0.1 to −0.2 units per 10 K.[37]As a result, the ambident phenoxide anions form carbanions both at ortho- and para-positions.[38] These positions may undergo HDX according to the relay mechanism proposed by Ghan and Enke[39] for the gas phase HDX at the non-labile sites of aryl-compounds. This mechanism consists in formation of six-membered-ring intermediate between localized negative charge site on the aromatic ring and D_2O followed by the relocation of the charge site to the adjacent center.[39] Still, another mechanism of skeletal HDX in the gas phase is possible: intramolecular D-transfer proposed for anions produced by negative ESI.[40,41] This mechanism is based on thermochemical

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calculations conducted by Tian et al[41] to explain additional HDX which was observed for the aromatic hydrogen atom located between two carboxylic groups. It was explained by formation of hydrogen bonds with deuterated solvent, which stabilize the aryl anion, followed by intramolecular D transfer on the localized negative charge site.

It should be noted that both relay and D-transfer mechanisms alone (both of them are developed for the gas phase) did not explain the HDX results observed in our study. So, in accord with D transfer mechanism we would expect 0 skeletal HDX for 2,3-DHB, 1 HDX for 2,4-DHB, and 1 significant HDX – for 2,5-DHB (the latter contains hydrogen atom located between carboxylic and hydroxyl groups). However, the experimental results contradict these suggestions: we observed 1 HDX for 2,3-DHB, 2 HDX for 2,4-DHB, and 0 HDX- for 2,5 DHB. This could be explained by the occurrence of HDX reactions in the charged micro droplets rather than for gas-phase reactions into vacuum zone of the mass-spectrometer. Indeed, if the observed HDX reactions would occur in gas-phase, then a use of other non-deuterated solvent (e.g. methanol) would not impact HDX results. We conducted this experiment by dissolving model compounds in methanol, and none of them has undergone extradeuteration in this case. Moreover, it is known that ionization of DHBs in methanol occurs via dissociation of phenolic OH which is more acidic than carboxyl under conditions of ESI[42]. In case of gas phase HDX, this would lead to an intermediate favorable to the D transfer mechanism implementation, which was not observed in our experiments. The major difference is composition of the spray droplets in case of water compared to methanol. Trace amounts of hydroxyls in the droplets which are generated in water are not produced in methanol.[43] This highlights the particular importance of the presence of base during the in-source H/D exchange and indicates that for our system the reactions occur in the charged micro droplets rather than in gas phase as shown in Fig 1.[44,45]

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Taken the above considerations into account we propose the following HDX reactions at the non-labile sites of the DHBs which account for contribution of keto-enol transformations taking place in the charged droplets at the elevated capillary temperature in the negative ESI mode (Fig. 3).

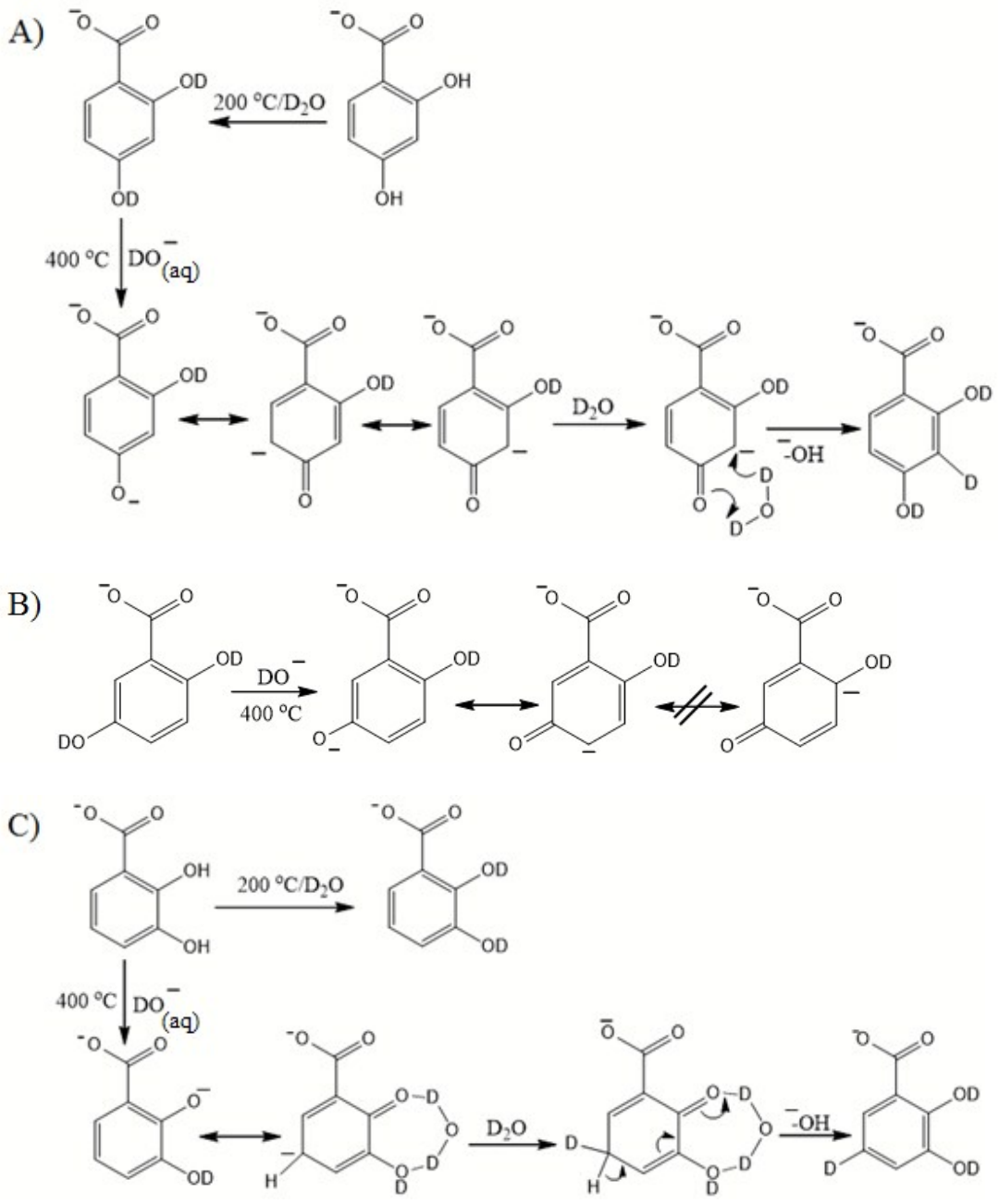

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Fig. 5 The proposed reactions of H/D exchange of the aryl protons at the elevated desolvation temperature (400^{o}C) in the charged microdroplets of the saturated atmosphere of D_2O favoring keto-enolic transformations of the DHBs in the negative ionization mode: A) 2,4-DHB, B) 2,5-DHB, C) 2,3-DHB. (The second non-labile proton of 2,4-DHB (A) is exchanged via similar pathway which is realized for the first resonance structure as well).

In the case of 2,4-DHB an intermediate carbanion possesses two resonance structures, while two other DHBs possess only one. This is because the structures with formal negative charge adjacent to substituents are energetically unfavorable. Therefore, the tautomeric form of 2,4-DHB is relatively stabilized which results in significant H/D exchange of both C-H protons at 400 ^{o}C. The feasible reaction pathway is shown in Fig. 5A. The high capillary temperature leads to phenol dissociation, causes keno-enol transformation of phenoxide anion followed by deuteration of the carbanion by D_2O molecule oriented by carbonyl oxygen and proton elimination resulting in a stable aromatic structure formation. in terms of substituent effects. The corresponding HDX pathways for 2,3-DHB and 2,5-DHB are shown in Figs. 5B and 5C, respectively. Despite 2,3- and 2,5-DHBs have similar substituent patterns, 2,3-DHB undergoes 3 H/D exchanges, whereas 2,5-DHB – only two. This is indicative of one additional H/D exchange in case of 2,3 DHB. It might result from stabilization of short-living ketone by hydrogen bonds between two adjacent groups in accord with relay mechanism facilitating exchange at non-labile site.[46] The similar stabilization cannot be implemented in case of 2,5-DHB which does not show exchanges at the non-labile sites.

To confirm the suggestion that keto-enol tautomerism is the essential part of skeletal HDX, we carried out the MS labeling reactions with 5-acetylsalicylic (**8**) and diphenylacetic (**9**) acids, which are characterized by inactive aromatic protons and active α-protons. We anticipated to observe 3 extra H/D exchanges in the case of acetylsalicylic acid related to CH_3-group of a

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side chain, and only one exchange in the case of diphenylacetic acids. The corresponding results are shown in Fig. 6. The heating of the capillary to 400 ^{0}C gave rise to 3 and 1 additional H/D exchanges for compounds 8 and 9, respectively. These observations are in accordance with our suggestion about the significant contribution of tautomerism into H/D exchange under conditions studied.

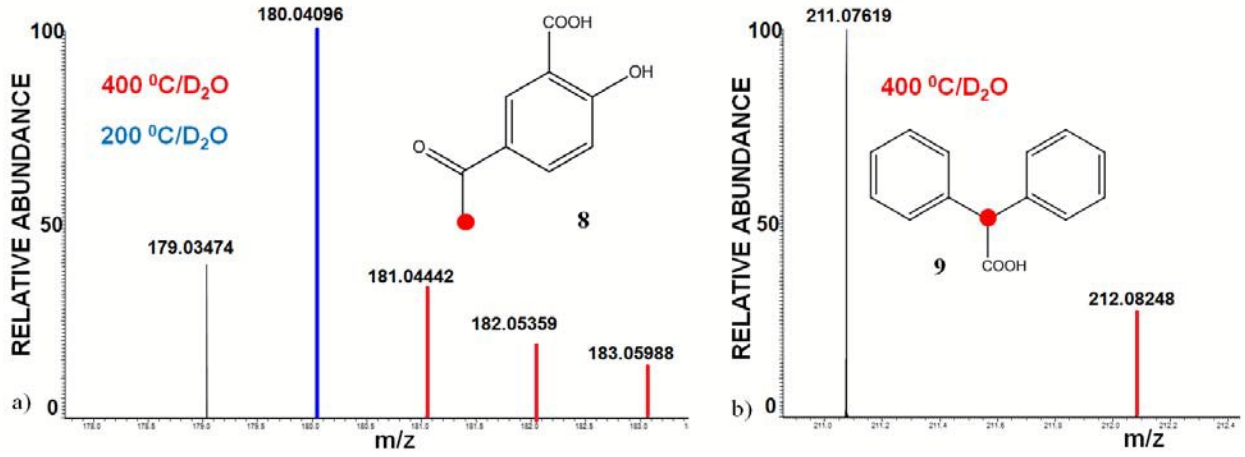

Fig. 6 In-ESI source H/D exchange series of a) diphenylacetic acid (**8**) and b) 5-acetylsalysilic acid (**9**). Red dots indicate α-carbons.

Therefore, we concluded that additional H/D exchanges could occur via keto-enol tautomerism at the most reactive non-labile sites similar to the reactions in the solution phase HDX followed by conventional relay mechanism leading to deuterated products.

H/D exchange in the liquid phase. To confirm that H/D exchange during ESI under conditions studied occurs rather in the charged droplet than in the gas phase, our next goal was to validate sites capable of H/D exchange due to keto-enolic transformations in the liquid phase by running the same reactions in deuterated water and analyzing them with a use of ^{2}H NMR spectroscopy. For this purpose, we used three model compounds, which showed the most intensive exchanges in the MS experiments: DOPA (**6**), 2,4-DHB (**3**), and 5-acetylsalycilic acid (**8**). The corresponding ^{2}H NMR spectra are shown in Figs. 2S-4S in the Electronic Supplementary

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Information. For DOPA (Figure 2S) we observed a large wide peak at 6.71 ppm corresponding to overlapped signals of all aromatic deuteriums and negligibly small signals assigned to α-deuterium at 3.76 ppm. The ^{2}H NMR spectrum of 2,4-DHB (Figure 3S) shows a large peak at 6.13 ppm, which corresponds to deuterium in the positions 3 and 5, and a very small signal at 6.92 ppm related to deuterium at position 6. ^{2}H NMR spectrum of the 5-acetylsalicylic acid shows a peak at 2.42 ppm (Figure 4S) assigned to α-protons which is in line with our expectations. ESI FT ICR MS spectra of the same labelled compounds obtained from the proitic solvent are shown in Fig. 5S. The lengths of exchanging series (3 for both DOPA and 5-acetylsalicilic acid) are consistent with the results of ^{2}H NMR spectroscopy.

^{2}H NMR spectroscopy enables evaluation of not only the number of exchanges, but also of a degree of deuteration. This is because NMR 2H-{1H} provides high accuracy estimation of the integral intensity of signals even then measured at a natural abundance level of deuterium using precision methods of integration.[32,47] In case of DHB-2,4 the ratio of total deuterium integrals at 3 and 5 positions to the integral of deuterium at the position 6 was 215. Further, the relative integral intensity of the methyl group of acetylsalicylic acid was 100% and there were no signals observed within an aromatic region. The calculated integral intensities are in agreement with the results of MS experiments, which show 2 and 3 additional exchanges, respectively.

Therefore, the analysis of base-catalyzed H/D exchange reactions in liquid state indicates the site specificity, which proves the proposed selectivity of deuteration during in ESI-source exchange.

CONCLUSIONS

In this paper we demonstrate that in-ESI source H/D exchange under atmospheric pressure leads to site-specific exchange of non-labile protons at high desolvation temperatures. This might

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indicate that capillary heating facilitates keto-enol transformations taking place in the charged droplets followed by HDX at non-labile sites in accord with relay mechanism. This allowed us to conclude that negative ionization mode can be considered as an analogue of the base-catalyzed conditions in the liquid state and an increase in desolvation temperature shifts equilibrium between tautomeric forms. Of importance is that a switch to positive mode reduces efficacy of H/D exchange. A use of ^{2}H NMR spectroscopy allowed us to examine selectivity of base-catalyzed labeling reactions qualitatively and quantitavely. The obtained results proved the crucial role of the compound structure in H/D exchange at non-labile sites and revealed an influence of acceptor group (e.g. carboxyl) on reaction selectivity. We believe that the results obtained can be of use for structure elucidation using ESI-HDX mass-spectrometric experiments for proteins and natural organic matter. In addition, the observed phenomena could shed light on driving force of back exchange of labelled compounds caused by capillary heating during ESI MS analysis.

Electronic Supplementary Information (ESI) Available:

ESI includes FT ICR MS spectra of labeled DHB-2,4 at different capillary temperature, ^{2}H NMR spectra and FT ICR MS of labeled compounds **3**, **6** and **8**.

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AUTHOR CONTRIBUTIONS

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