Canadian Journal of Chemistry
Revue canadienne de chimie

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Journal:	<i>Canadian Journal of Chemistry</i>
Manuscript ID	cjc-2016-0281.R1
Manuscript Type:	Article
Date Submitted by the Author:	14-Jul-2016
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Keyword:	hydrogen-deuterium exchange, small organic molecule, high-resolution mass spectrometry, liquid chromatography, identification

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1 Post-column hydrogen-deuterium exchange technique to assist in the
2 identification of small organic molecules by mass spectrometry

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Draft

Abstract: In order to improve the certainty that a specific small organic molecule has been detected in a given sample by high-resolution mass spectrometry, other techniques that give conclusive evidence about the chemical structure of a compound like nuclear magnetic resonance (NMR) or complementary information on its composition such as hydrogen-deuterium exchange (HDX) are often necessary. This study presents a systematic investigation that aims to improve the applicability of post-column HDX for those purposes. Key parameters like mobile phase flow rates, volume percentage of H₂O in the mobile phase and D₂O addition flow rates were optimized in order to provide an isotopic pattern that allows the accurate determination of the number of exchangeable hydrogen atoms in small organic molecules. A loop injection setup was used to emulate chromatographic conditions in the optimization process, and trimethoprim, a widely-used anti-infective, was used as test compound for the experiments. As expected, results showed that deuteration percentage decreased with a higher mobile phase flow rate and increased with higher D₂O flow rate. The post-column HDX technique was then validated with extracts of samples of river water and plants separated by liquid chromatography in hydrophilic interaction or reversed phase modes. Mass spectra showed a completely visible isotopic pattern that allowed assessing correctly and unambiguously the number of exchangeable hydrogens in the compounds of interest. This study shows that post-column HDX can be used as complementary technique to identify unknown small organic molecules in complex matrices. The current paper proposes an efficient, cost-effective, versatile technique of HDX that is helpful to assign a unique structure to a given high-resolution mass spectrometry signal.

Keywords: hydrogen-deuterium exchange; HDX; small molecule; identification; post-column addition; organic contaminants; liquid chromatography; high-resolution mass spectrometry.

Résumé : Dans l'optique d'améliorer le niveau de certitude relatif à l'identité de petites molécules organiques détectées en spectrométrie de masse à haute résolution, plusieurs techniques additionnelles apportant des informations structurales supplémentaires comme la résonance magnétique nucléaire (RMN), qui amène des informations structurelles exhaustives, ou l'échange hydrogène-deutérium (HDX), qui est une méthode complémentaire, sont souvent nécessaires. La présente étude propose une investigation systématique afin d'améliorer l'applicabilité de la HDX post-colonne dans un contexte d'élucidation structurale. Des paramètres majeurs comme le débit des phases mobiles, la composition des phases mobiles en H₂O et le débit d'ajout de D₂O ont été optimisés afin de fournir un patron isotopique permettant une détermination précise du nombre d'hydrogènes échangeables dans une petite molécule organique donnée. Un montage avec une boucle d'injection a été utilisé afin d'imiter des conditions chromatographiques lors du procédé d'optimisation alors que le triméthoprim, un anti-infectieux très commun a été utilisé comme composé cobaye. Les résultats obtenus ont montré que le pourcentage de deutération décroissait avec un débit de phase mobile plus élevé et augmentait avec un débit d'ajout de D₂O plus élevé. La méthode a ensuite été validée en effectuant la HDX sur des extraits des échantillons de rivière et des plantes séparés par chromatographie à interactions hydrophiles (HILIC) et par chromatographie en phase inverse, respectivement. Les patrons isotopiques étaient complètement visibles dans les spectres de masses des composés analysés et fournissaient sans ambiguïté le nombre d'hydrogène échangeables dans les composés étudiés. La présente étude démontre que la HDX post-colonne peut être utilisée comme méthode complémentaire de manière rapide et économique dans un contexte d'élucidation structurale.

Mots clés : Échange hydrogène-deutérium, HDX, petites molécules, identification, dérivation post-colonne, chromatographie liquide, spectrométrie de masse, contaminants organiques.

Introduction

Identification of unknown organic compounds and characterization of transformation products of known organic compounds by mass spectrometry is rapidly expanding in the environmental and natural product fields because of the increasing access to tandem mass spectrometers with accurate mass and high resolution capabilities ¹. However, compound identification based on accurate mass measurements and tandem mass spectra is often difficult due to the possibility of numerous isomers. Therefore, techniques giving conclusive evidence such as nuclear magnetic resonance (NMR), or complementary techniques like chemical derivatization or hydrogen-deuterium exchange (HDX) are often used to correctly identify the compounds of interest ². The latter is an established technique in protein analysis ³ and can also be used as a mean to improve the level of confidence in the structural identification of small organic molecules of interest, especially when available amounts rule out NMR. HDX is based on the capacity of labile H atoms present in organic molecules, generally bound to heteroatoms such as N, O or S, to exchange with D atoms from enriched media (usually D₂O) ⁴. The result of such exchange is the increase in the *m/z* ratio by *n* mass units depending in the number *n* of labile hydrogens on the compound.

According to a recent publication ⁵, the identification of small molecules by mass spectrometry can be classed in five different levels of decreasing confidence: from confirmed structure (level 1) to exact mass (level 5). High resolution-mass spectrometry yields the lowest level of confidence, level 5 data. The level of confidence in the assignment of a unique structure to a

given high resolution mass spectrometry signal can be improved to probable structure (level 2) by obtaining additional information on the compounds of interest and deducing the structure by eliminating other candidates. HDX can be used for such purposes.

So far, four different approaches have been used to apply HDX for characterization of small organic molecules: offline ⁶, in-source ⁷, online ⁸ and post-column HDX ⁹. Each of these techniques has clear advantages as well as disadvantages that may limit their routine application in some laboratories. In offline HDX, purified compounds previously dissolved in deuterated solvents are directly infused into the mass spectrometer. This technique was recently used by Bourgin *et al.* ⁶ to characterize ozonation by-products of estrone-sulfate, a potential endocrine disruptor. While this approach is the easiest to implement, its major drawback is that compounds have to be previously purified before offline HDX, since no separation is performed before introduction of the samples into the mass spectrometer. In the in-source technique, a pump delivers D₂O to a second atmospheric pressure ionization source, creating a D₂O-rich atmosphere inside the ionization source in order to achieve HDX before the analytes are introduced in the mass spectrometer. This approach was applied by Wolff and Laures ⁷ to the analysis of antiulceratives and anthelmintics and can be used to determine the number of exchangeable H atoms of an analyte after visual inspection of its isotopic pattern. However, the application of this technique to the identification of unknowns is difficult since it is limited by low exchange levels, in some cases <50%, which makes difficult the determination of the correct number of labile H atoms in a compound. Additionally, a dual-spray source may not be available for many spectrometers and is usually used for internal mass calibration in high resolution mass spectrometers. High deuteration levels are obtained in the online technique in which a deuterium-enriched mobile phase is used to induce HDX during the liquid chromatography separation

process. Online HDX has been successfully applied for the identification of metabolites, pharmaceutical compounds as well as other small molecules^{2,8}. As an example, online HDX was shown helpful in reducing the number of potential isomers during the characterization of sulfadiazine metabolites present in pig manure¹⁰. A significant reduction of deuterated solvents consumption can be achieved using online HDX with hydrophilic interaction liquid chromatography (HILIC), as the composition of the mobile phase in that technique is generally 50 to 95% organic¹¹. HDX in the HILIC mode was applied recently to the separation of 4-aminomethylpyridine from its degradation products¹². The major drawbacks of this approach are the high cost of deuterated solvents and shifts in retention time observed for deuterated analytes, in some cases, up to 21% longer compared to non-deuterated solvents⁸. The post-column HDX technique introduced by Tolonen *et al.*⁹ is an interesting cost savings alternative to online HDX since D₂O is added to the chromatographic column effluent using a syringe pump before entering the ionization source. However, a detailed study on the key parameters affecting the performance and applicability of post-column HDX has not yet been done.

The objective of the present work is to study the effect of mobile phase composition, mobile phase flow rate and the type of mixing device on the deuteration percentage in the post-column HDX technique. Such study will improve the application of this technique to assist on identification of unknown small organic molecules separated by liquid chromatography.

Experimental

Reagents

Water, acetonitrile and 0.1% formic acid in acetonitrile were all LC-MS Optima grade and were obtained from Fisher Scientific. D₂O (99.9%) was obtained from Cambridge Isotope Laboratories, while trimethoprim (purity $\geq 98\%$) was purchased from Sigma Aldrich as were caffeine (99%) and theophylline (99%).

Study post-column HDX parameters using loop injections

In a loop injection setup, the mobile phase is used to transport the analyte of interest from an injection loop placed on a divert valve to the ionization source of the mass spectrometer. Loop injections were used to reproduce chromatographic conditions in real time and to study three key parameters that have a major impact on the post-column HDX: water volume percent in the mobile phase (0, 5, 10, 15, 20, 25, 30 % v/v), mobile phase flow rate (150, 200, 250 and 300 $\mu\text{L min}^{-1}$) as well as D₂O addition flow rate (30, 50, 70 $\mu\text{L min}^{-1}$). Composition of the mobile phase was tested to measure the impact of the presence of exchangeable H atoms in H₂O. The organic solvents used in the mobile phase had to be void of exchangeable hydrogens since otherwise they could compete with exchangeable H atoms of the target molecules, thus significantly reducing the deuteration percentage. Acetonitrile (ACN) is a widely used aprotic solvent and one of the most common organic solvents in liquid chromatography-mass spectrometry (LC-MS). Therefore it was used with or without formic acid (FA) 0.1% v/v or ammonium formate as additive in this work. Trimethoprim was selected as a test compound because of its 4 exchangeable amine hydrogens present in its molecular structure and therefore its isotopic pattern in cases of incomplete deuteration is larger and can provide more information. Furthermore, this polar compound is a frequently detected environmental contaminant ¹³. In the loop injection

experiments, trimethoprim was introduced in a 20 μL stainless steel loop through an injector port placed in the mass spectrometer's divert valve using a 500 μL Hamilton syringe. D_2O was added to the mobile phase with a syringe mounted on a syringe pump using a mixing tee connector. Targeted parameters and their effects were measured at least three times to evaluate signal variation. The effects of different mixing devices such as a tee connector (IDEX, part number P-727, swept volume 0.57 μL) and a HPLC mixer (Waters, Acquity BSM zirconia mixer, internal volume 50 μL) were compared to a mixing tee (IDEX, part number U-466, swept volume 2.2 μL) on the deuteration percentage of trimethoprim.

Collection and preparation of samples

River water samples

Water was sampled from the Magog River (Sherbrooke, Quebec, Canada) on May 14th, 2015 in amber HDPE bottles and conserved in an ice cooler until arrival to the laboratory, where it was immediately stored at -20°C . Upon extraction, samples were thawed at room temperature and extracted using a previously published method¹⁴. In summary, 250 mL of the water was acidified with a solution of orthophosphoric acid to a pH of 2.8 and extracted by solid-phase extraction (SPE) using Phenomenex Strata-X polymeric reversed phase cartridges (bed mass 200 mg, volume 6 mL, particle size 33 μm). Cartridges were conditioned sequentially with 5 mL of methanol and 5 mL of H_2O , and then loaded with the river water samples at a flow rate of $\approx 8 \text{ mL min}^{-1}$ using a SPE manifold connected to a vacuum diaphragm pump. Cartridges were eluted with $2 \times 3 \text{ mL}$ of a 1:1 acetonitrile: methanol mixture. Samples were then reconstituted in 10 mL

162 acetonitrile and spiked with 4 $\mu\text{g L}^{-1}$ of the trimethoprim, caffeine, and theophylline standards.
163 Samples were then injected analysis using a HILIC-ESI(+)-QqTOFMS method.

164
165 **Fig. 1.** Molecular structure of trimethoprim showing the presence of 4 exchangeable hydrogen
166 atoms. In ESI+, a mass shift of up to 5 mass units can be observed after HDX of trimethoprim
167 because of the formation of a D^+ adduct $[\text{M}(-4\text{H}+4\text{D})+\text{D}]^+$.

169 *Plant samples*

170 *Sorghum bicolor*, commonly referred as sweet sorghum, was first ground and sieved to particle
171 sizes between 40 and 60 mesh (250-425 μm). It was then put in a steam explosion reactor. A
172 soxhlet extraction was then used on the remaining biomass with toluene and ethanol as solvents
173 in a 1:1 ratio. The solution was then dried with a nitrogen flow apparatus and reconstituted in
174 methanol. The methanol solution was then centrifuged at 3000 g for 5 minutes and filtered on a
175 0.45 μm PVDF filter before being injected for analysis using a RPLC- ESI(+)-QqTOFMS
176 method.

177 **Instruments and methods**

178 Post-column HDX was tested with different instruments in order to evaluate the variability
179 between electrospray ionization (ESI) sources. In all cases, ionization was performed in positive
180 mode. Study of the effect of the HDX parameters on the deuteration percentage was done in triple

quadrupole mass spectrometer (QqQMS) and a linear ion trap mass spectrometer (LITMS). Analysis of river samples and plants extracts were done with UHPLC coupled to a quadrupole-time-of-flight mass spectrometer (QqTOFMS).

ESI(+)-QqQMS

The liquid chromatography-tandem mass spectrometry system used for this work composed was an Acquity Ultra Performance LC from Waters coupled to a Waters Quattro Premier XE triple quadrupole mass spectrometer. Source parameters were as follows: capillary voltage was 3.2 kV, cone voltage was 35 V, extractor voltage was 5 V, source temperature was 120 °C, desolvation temperature was 450 °C, desolvation gas flow was 700 L h⁻¹, cone gas flow was 50 L h⁻¹. The QqQMS parameters were the following: mass range was m/z 200 to 400; scan duration was 1 s. Data analysis was performed with Waters' MassLynx V4.1 SCN805.

ESI(+)-LITMS

HDX parameters were optimized using an Accela liquid chromatograph from Thermo Scientific coupled to a LTQ XL linear ion trap mass spectrometer (LITMS) also from Thermo Scientific and equipped with an electrospray ionization (ESI) source. Ionization was performed in the positive mode, capillary temperature was 275 °C, the sheath gas flow was 35 and the auxiliary gas flow was 20. Spray voltage was 3 kV, the source current was 100 µA, capillary voltage was 25 V and the tube lens voltage was 57 V. Mass range was m/z 290 to 300, scanning was in the zoom mode. The data analysis software was Thermo's Xcalibur (version 2.2) for both the LC and MS systems.

HILIC-ESI(+)-QqTOFMS

The liquid chromatography-high resolution mass spectrometry system was a Shimadzu Nexera LC-30AD for the pump module, a SIL-30AC for the autosampler module, a CTO-30A for the column oven module and a SPD-M20A Prominence diode array detector. The mass spectrometer was Maxis quadrupole-time-of-flight (QqTOF) from Bruker. Data analysis was performed with Bruker's Compass for otof Series 1.7 patch 2 and was used in conjunction with Bruker's DataAnalysis Version 4.3 (Build 110.102.1532). Analysis of water samples was done in the HILIC mode using an XBridge Amide column (100 × 2.1 mm, 3.5 µm) from Waters. Solvent A was 10 mM ammonium formate in H₂O with 0.05% FA and solvent B was ACN. The chromatographic gradient was the following (% of A): 0 min, 5%; 4 min, 5%; 12 min, 40%; 16 min, 40%, 16.01 min, 5%; 26 min, 5%. Mobile phase flow rate was 300 µL min⁻¹, injection volume was 1 µL and column temperature was 30 °C.

Source parameters were as follows: capillary voltage was 2200 V, end plate offset voltage was 500 V, nebulizer pressure was 4 bar, dry heater was 200 °C, dry gas flow rate was 10.0 L min⁻¹. QqTOFMS parameters were the following: scan range was from *m/z* 50 to 1200, funnel RF was at 250 Vpp, transfer time was 35 µs and the pre-pulse storage time was 5 µs. The TOF calibrant used was sodium formate.

RPLC-ESI(+)-QqTOFMS

The LC and MS systems and software used for the analysis of water samples were also employed for the analysis of sweet sorghum extracts. The column used for these experiments was an Acquity UPLC HSS T3 column (50mm×2.1 mm, 1.8 µm). Phase A was H₂O with 0.1% FA and phase B was ACN with 0.1% FA. Chromatographic method for triclin deuterium exchange was as follows: at initial time, 10% of B; at 2.50 minutes, 10% of B; at 20 minutes, 50% of B; at

21 minutes, 100% of B; at 27 minutes, 100% of B; at 27.10 minutes, 10% of B; at 31.10 minutes, 10% of B. Mobile phase total flow rate was maintained at $400 \mu\text{L min}^{-1}$. Column temperature was 30°C . Method run time was 31.10 minutes. MS parameters were identical than those used for HILIC chromatography except: capillary voltage was 2000 V, ion cooler RF was 30-200 Vpp and transfer time was 30-60 μs .

A split valve setup was necessary for the HDX of the plant extracts. The water percentage in the mobile phase being of roughly 40% at $400 \mu\text{L min}^{-1}$ at the time the target compounds were eluting was too high for an effective exchange to take place. The split valve setup was placed before the mixing tee as most of the flow was sent to an UV-DAD and a fraction was sent to the QqTOFMS for the HDX. This allowed to more than double the deuteration percentage of tricetin, an O-methylated flavonoid on which HDX was performed.

The deuteration percentage was determined using McCloskey's method ⁴ in which the contribution of naturally occurring stable isotopes is subtracted from the relative intensities of all peaks of the isotopic pattern of the compound in order to provide corrected relative intensities. Those latter values are then used to calculate molar deuteration percentages, which then are employed to determine the total deuteration percentage in the compound relative to the maximum possible value. A detailed example of this calculation for trimethoprim is shown in the Supplementary material. A theoretical spectrum can be generated with the calculated deuteration percentage using a binomial distribution where the number of possible configurations is the number of coefficients in a row in the Pascal's triangle. A correction with the naturally occurring isotope is then added. An example is given in the Supplementary material (Fig. S1).

Results and Discussion

*Study of the post-column HDX parameters using loop injections on the deuteration**percentage*

Since high deuteration percentages are necessary to correctly identify the total number of exchangeable hydrogens in a given compound, the effect of three key parameters for post-column HDX (H_2O volume percentage in the mobile phase, mobile phase flow rate and D_2O addition flow rate) was studied using loop-injections and mixing tee setup with an ESI-QqQMS system.

First, the effect of the H_2O volume percentage in the mobile phase on the deuteration percentage was measured using 0.1% v/v FA in H_2O (as solvent A) and two different solvents: ACN without additive or 0.1% v/v FA in ACN as solvent B. Fig. 2 shows that the deuteration percentage of trimethoprim decreases from 77.7 ± 0.6 to 46.1 ± 0.1 and from 60.4 ± 0.6 to 50.4 ± 0.6 when using ACN as compared to a mixture of 0.1% FA in ACN, respectively, when the volume percentage of H_2O in the mobile phase increases from 0 to 30%. As expected, this is caused by an increasing competition between hydrogens of H_2O , FA and trimethoprim for the D atoms of D_2O . The presence of FA in ACN had a significant impact on the deuteration percentage at 0% of water in the mobile phase. This was explained as the result of the competition between FA and the target compound for available D atoms during the optimization. At higher percentages of H_2O in the mobile phase, the effect of FA is much less important since H_2O is present at increasingly higher concentrations than FA.

Fig. 2. Effect of H_2O volume percent in the mobile phase on the deuteration percentage of trimethoprim using the post-column HDX technique in a QqQMS. Mobile phase flow rate was

268 300 $\mu\text{L min}^{-1}$, D_2O addition flow rate was 50 $\mu\text{L min}^{-1}$ and the mixing device was a mixing tee.

269 Error bars represent ± 1 standard deviation.

270

271 As presented in Fig. 2, HDX is never complete but remains fairly stable when using 5 to 30%
272 v/v of H_2O . This could be explained by the weak mixing due to diffusion in the capillary tubing,
273 which limits the dilution of the sample volume injected in the loop with mobile phase. Therefore,
274 H_2O content in the mobile phase higher than 5% has reduced effect on the deuteration percentage
275 since the mobile phase mainly “pushes” the sample through the capillary and has little interaction
276 with it before the mixing tee. From the results obtained in this experiment it was observed that in
277 a low resolution mass analyzer such as a QqQMS deuteration percentages $\geq 45\%$ are enough to
278 identify the correct number of exchangeable H atoms in trimethoprim, 4 in total. Application of
279 the McCloskey method to the isotopic pattern of trimethoprim after post-column HDX showed
280 that the corrected relative intensity of the peak m/z 296 was $> 1\%$. That peak corresponds to $[\text{M}(-$

281 $4\text{H} + 4\text{D}) + \text{D}]^+$), a trimethoprim molecule in which 4 hydrogen atoms were replaced by 4 D
282 atoms plus a deuterium adduct formed during ESI+ (Fig. S2, Supplementary material).

283 Deuteration percentages $\geq 45\%$ were obtained with H_2O volume percentages in the mobile
284 phase $\leq 30\%$, therefore H_2O volume percentage experiments indicate that post-column HDX may
285 not yield sufficiently high deuteration percentages to identify correctly the total amount of
286 exchangeable hydrogen atoms in highly polar compounds separated by reversed phase liquid
287 chromatography (RPLC). Polar organic compounds are weakly retained by the stationary phase
288 in RPLC and are eluted at mobile phase compositions that are usually between 90 to 95%
289 aqueous. However, the post-column technique could be used with less polar and more retained
290 compounds in RPLC or with polar compounds separated by hydrophilic interaction liquid
291 chromatography (HILIC), since maximum volume percentage of H_2O in that mode of
292 chromatography is usually around 40 to 50%.

293 The effects of various mobile phase flow rates and post-column D_2O addition flow rates on the
294 deuteration percentage of trimethoprim were then tested with the same previous loop-injection
295 setup but using 100% ACN as mobile phase. Deuteration percentage was estimated to be
296 inversely proportional to the mobile phase flow rate since high flows should reduce the contact
297 time between analytes and D_2O . Such behavior would reduce the possibility for the exchange
298 reaction to take place while increasing the competing HDX reactions that cause back-exchange
299 with water. Furthermore, deuteration percentage should increase with D_2O addition flow rate
300 since a higher amount of D atoms are available for exchange with the target compound. These
301 hypotheses were validated by the results illustrated in Fig. 3. It can be seen that deuteration
302 percentages (up to 96.2 ± 0.1) are achieved at a low mobile phase flow rate ($150 \mu\text{L min}^{-1}$) when
303 using at a D_2O addition flow rate of $70 \mu\text{L min}^{-1}$ while the deuteration percentage decreased to

75.4 $\pm$ 0.5 at the highest mobile phase flow rate tested (300 $\mu\text{L min}^{-1}$) and at a low D_2O addition rate (30 $\mu\text{L min}^{-1}$). These results show that the present post-column HDX technique would perform better with capillary microbore columns (0.15 to 0.8 mm ID) than with narrow bore columns (1 to 2 mm ID), since the former are generally used at flow rates of 2 to 20 $\mu\text{L min}^{-1}$ while the latter are used generally at flow rates of up to 300 $\mu\text{L min}^{-1}$ ¹⁵. Nonetheless, as discussed earlier, the performance of the present technique is sufficient for identification purposes in common HILIC-MS methods which usually employ narrow bore columns and flow rates between 200 and 300 $\mu\text{L min}^{-1}$.

Fig. 3. Effect of the mobile phase flow rate and of the D_2O addition flow rate on the deuteration rate of trimethoprim using the post-column HDX technique in a QqQMS. Mobile phase composition was 100 % ACN and the mixing device was a mixing tee. Error bars represent ± 1 standard deviation. The three series in the insert indicate the D_2O addition flow rate.

317

318 *Effect of mixing devices on the deuteration percentage*

319 A mixing tee, an HPLC solvent mixer, and a regular tee in two different configurations (Fig.
320 S3, Supplementary material) were employed to study the effect of the type of post-column
321 mixing device on the deuteration percentage, since an optimal mixing between the target
322 compound and D₂O is necessary to attain high deuteration percentages. Results showed that the
323 best deuteration percentage was achieved using the mixing tee (52.4 ± 0.3), while with the
324 regular tee in 90° and 180° configurations deuteration percentages were not significantly different
325 (49 ± 1 and 48 ± 1 , respectively). The lowest deuteration percentage (39.2 ± 0.4) was obtained
326 with the HPLC mixer, which is used to improve solvent mixing. However, when comparing the
327 internal volume of this type of mixer (50 µL) with the internal volume of the optimal mixing
328 device according to this experiment (the mixing tee of 2.2 µL), it becomes clear that a good
329 blending of the target compound and D₂O is not the only factor affecting deuteration percentage.
330 A high internal volume has a negative effect on the deuteration percentage since it increases
331 mixing of D₂O with the mobile phase, thus decreasing the effectiveness of HDX with the targeted
332 compound. Therefore, a mixing tee with a low internal volume and a 10 µm porosity frit to aid
333 mixing, such as the one used in the work, is ideal to reduce mobile phase-D₂O blending and
334 maximize deuteration percentage of target compounds.

335 An additional experiment was performed on a LITMS with the same loop injections setup
336 except for the sample loop volume, which was 5 µL (Fig. 4). As it can be observed, deuteration
337 percentages are significantly lower ($\approx 20\%$) than those observed with the QqQMS, which could
338 be explained by the differences in source design, a known factor affecting HDX⁴. Collisions

between deuterated species and ion source surface containing layers of adsorbed water and organic non-deuterated material can produce back-exchange, *i.e.* exchange of deuterium incorporated in the analyte of interest with protons from other compounds⁴. Source design also affects back-exchange caused by inadequate desolvation conditions in the ion source¹⁶. Nonetheless, LITMS results confirmed that a systematic improvement in deuteration percentages was obtained with a mixing tee compared to regular tee connector.

Fig. 4. Effect of mixing device type on the deuteration percentage of trimethoprim using the post-column HDX technique in a LITMS. Mobile phase composition was 0.1% FA v/v in ACN and D₂O addition flow rate was 70 $\mu\text{L min}^{-1}$. Error bars represent ± 1 standard deviation.

Post-column HDX with river water and plants extracts

Post-column HDX experiments were performed in a QqTOFMS with two types of samples: SPE extracts of water samples collected in a local river and spiked with the target compounds and extracts of sweet sorghum obtained after steam explosion and soxhlet extraction. For river water samples separated by HILIC, deuteration percentages after post-column HDX of the $[M+H]^+$ ions was 51 ± 1 for trimethoprim (Figure 5), 56 ± 1 for theophylline (Fig. S4, Supplementary material) and 81 ± 4 for caffeine (Fig. S5, Supplementary material). These results allowed unambiguous identification of the number of exchangeable hydrogens in each molecule in the presence of a complex matrix.

Fig. 5. Extracted ion chromatogram and experimental and theoretical mass spectra of trimethoprim after post-column HDX of a SPE extract of spiked river water.

For plant extracts separated by RPLC, post-column HDX was used to further assess the identity of m/z 331.0810 (neutral formula $C_{17}H_{14}O_7$, $\Delta m=0.2$ mmu) a compound that was suspected to be the O-methylated flavone triclin or the mycotoxin aflatoxin G₂ (Figure 6). A split valve was used to reduce the column flow rate before post-column HDX which helped obtain an acceptable level of deuterium exchange (40 %).

Fig. 6. Extracted ion chromatogram and experimental and theoretical mass spectra after post-column HDX of a compound of formula $C_{17}H_{14}O_7$ observed in a sweet sorghum extract. The split ratio was about 1:10 and the D₂O addition flow rate was 30 $\mu\text{L min}^{-1}$. The experimental mass spectra for compound $C_{17}H_{14}O_7$ was obtained in the MS² mode.

The mass spectra confirmed that the compound in question had 3 exchangeable hydrogens. This information, along with tandem mass spectrometry spectra, allowed increasing the

confidence level in the identification of tricin from exact mass (level 5) to probable structure (level 2). Ion m/z 335 in the experimental spectrum indicates the presence of 4 exchangeable hydrogens; therefore aflatoxin G2 was rejected as a possible structure. In this case, the experimental and theoretical spectra were very similar as can be seen in Fig. S6 (Supplementary material).

Conclusion

The effects of mobile phase composition, mobile phase flow rate and the type of mixing device on the deuteration percentage achieved by post-column HDX were studied in order to use this technique to identify accurately the number of exchangeable hydrogens in small organic molecules. Results showed that the studied parameters can be controlled to obtain acceptable deuteration percentages that allow the accurate identification of the number of exchangeable hydrogens in small organic molecules. In the proposed post-column HDX technique, only about 100 μL of D_2O were used per injection. Compared to the online HDX technique, this represents significant savings in operating costs. The incomplete exchange in post-column HDX is a minor inconvenience as the contribution of naturally occurring stable isotopes, ^{13}C and ^{15}N can be corrected using the method reported by McCloskey *et al*⁴. The proposed technique can be helpful to improve the level of confidence in the assignment of a unique structure to a given compound present in complex matrix and detected by high-resolution mass spectrometry.

Supplementary material

Supplementary material is available with the article through the journal Web site.

Acknowledgment

Financial support was provided by Mitacs, CÉROM Centre de Recherche sur le Grain, the Faculty of Sciences of Université de Sherbrooke and Natural Sciences and Engineering Research Council of Canada (NSERC). We would also like to thank René Gagnon and Philippe Venne for their technical assistance and Thermo Scientific for their help with the LITMS.

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