**FOCUS: EMERGING INVESTIGATORS: APPLICATION NOTE**

Applying an Internal Standard to Improve the Repeatability of In-electrospray H/D Exchange of Carbohydrate-Metal Adducts

O. Tara Liyanage,[1] Chinthaka A. Seneviratne,[2] Elyssia S. Gallagher[1]

[1]Department of Chemistry and Biochemistry, Baylor University, One Bear Place #97348, Waco, TX 76798, USA
[2]Mass Spectrometry Center, Baylor University, One Bear Place #97046, Waco, TX 76798, USA
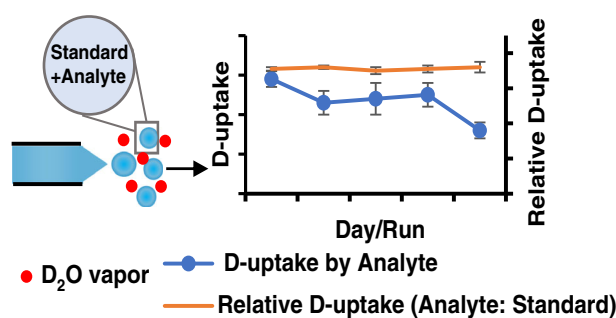
Abstract. In-electrospray (ESI) hydrogen/deuterium exchange–mass spectrometry (HDX-MS) has been used to characterize solvated carbohydrate structures. However, the rapid exchange rate of hydroxyls, as well as variations in source conditions and ambient humidity, alter the extent of forward and back exchange, resulting in poor repeatability when quantifying *D*-uptake on different days. Herein, we compare two internal standards, a peptide and derivatized carbohydrate, to improve the repeatability of in-ESI HDX of carbohydrate-metal adducts. Our results show that maltoheptaose, derivatized with Girard's T reagent, is a suitable internal standard for improving the repeatability of in-ESI HDX analyses of carbohydrates of varying size.

Keywords: Electrospray ionization, Hydrogen/deuterium exchange, Mass spectrometry, Carbohydrate, Internal standard

Hydrogen/deuterium exchange–mass spectrometry (HDX-MS) is a powerful technique for analyzing conformations and binding interactions of biomolecules [1, 2]. Recently, HDX-MS has been extended to characterize carbohydrates [3–8]. Of the currently available HDX techniques, in-electrospray (ESI) HDX provides a suitable time window for sampling the rapid exchange rate of carbohydrate hydroxyls and requires minimal instrument modification [8, 9]. We have previously shown that in-ESI HDX does not sample gas-phase conformations of carbohydrate-metal adducts, but rather analyzes solvated carbohydrates [8]. However, due to the rapid exchange rate of hydroxyls, variations in source conditions during ionization and changes in ambient humidity affect the amount of forward and back exchange, altering the measured *D*-uptake. Thus, this variability reduces measurement repeatability and limits the quantitative capabilities of this method.

One way to minimize measurement variability and improve accurate quantification of HDX is to utilize an internal standard that is subjected to the same conditions as the analyte during HDX. Peptides, including PPPI, bradykinin, angiotensin I, and leucine enkephalin, have been used as internal standards to account for variations in back exchange of backbone amides in bottom-up HDX-MS analyses of proteins [10]. PPPI has also been used as an internal standard to account for differences in instrument conditions that affect the gas-phase HDX kinetics of carbohydrate oxonium ions [6]. Herein, we compare the utility of two internal standards, a peptide and a derivatized carbohydrate, to improve the repeatability for quantifying HDX of rapidly exchanging hydroxyls in carbohydrates during in-ESI HDX.

Correspondence to: Elyssia Gallagher; *e-mail:* elyssia_gallagher@baylor.edu

Experimental

Melezitose, α-cyclodextrin, Girard's T reagent, maltoheptaose, and NaCl were purchased from Sigma Aldrich (St. Louis, MO). Maltotetraose was purchased from carbosynth (San Diego, CA). D_2O (99.96% purity) was purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA). Leucine enkephalin (YGGFL) was purchased from Waters (Milford, MA). KCl and all other materials were purchased from Fisher Scientific International, Inc. (Pittsburgh, PA). All chemicals were used without further purification. Nanopure water was acquired from a Purelab Flex 3 (Elga, Veolia Environment S. A., Paris, France).

Leucine enkephalin (0.1 mg) was dissolved in 2 mL of 50.0:49.9:0.1 (v/v/v) acetonitrile: water: formic acid. Aliquots of this stock solution were diluted in methanol, yielding solutions of 100 nM leucine enkephalin.

Maltoheptaose was derivatized according to a previously published method [11]. Briefly, an excess of Girard's T reagent was reacted with maltoheptaose for 3 h at 75 °C in a solvent of 8.5:1.5 (v/v) methanol: acetic acid. Solvent was then removed by rotary evaporation and the sample was dried under vacuum. The reaction product was then reconstituted in 2 mL of methanol and spiked into each sample. Derivatized maltoheptaose from the same reaction was used as the internal standard for all analyses with the same oligosaccharide. The concentrations listed below assume that the reaction resulted in 100% yield of derivatized maltoheptaose.

Carbohydrates and chloride salts (Na^+ and K^+) were prepared as stock solutions in water. Mixtures were then prepared with 500 μM carbohydrate and Na^+ and K^+ at a 2:1:1 M ratio in a solvent containing 99:1 (v/v) methanol: water. These samples were spiked with internal standard and diluted in methanol to obtain optimum signal for the internal standard and metal-adducted carbohydrate. The final concentrations were 91 nM leucine enkephalin with 18 μM melezitose, 31 μM derivatized-maltoheptaose with 18 μM melezitose, and 58 μM derivatized-maltoheptaose with 33 μM maltotetraose or α-cyclodextrin.

In-ESI HDX-MS experiments were conducted with an Ion Max source coupled to an Orbitrap Discovery MS (Thermo-Fisher Scientific, Waltham, MA) using a previously published protocol [8]. Briefly, a 150-μL droplet of D_2O was placed on a plate in the source to generate an atmosphere saturated with D_2O vapors. To ensure a consistent D_2O environment, we averaged spectra collected between 4.0 min and 4.2 min [8], following positioning of the D_2O droplet in the source. After each run, the source was opened for 2 min to remove residual solvent vapors [7]. The following ESI parameters were used: spray voltage, 3.5 kV; sheath gas (N_2), 12 arbitrary units; auxiliary gas (N_2), 0 arbitrary units; sample flow rate, 20 μL/min; and capillary temperature, 300 °C. A declustering potential of 70 V [12] was used to decluster methanol-adducts of carbohydrates.

Equation (1) was employed to calculate weighted average masses (M values) of undeuterated and deuterated metal-adducted carbohydrates using experimental mass-to-charge values (m/z) and ion intensities (I).

$$M = \left[\frac{\sum (m/z) \cdot I}{\sum I} \right] \cdot z \tag{1}$$

As shown in Eq. (2), the D-uptake for carbohydrate-metal adducts was determined by calculating the difference between the weighted average masses of deuterated ($M_{Deuterated}$) and undeuterated ($M_{Undeuterated}$) species:

$$D-\text{uptake} = M_{Deuterated} - M_{Undeuterated} \tag{2}$$

Relative D-uptake values were calculated using the ratio of D-uptake for the carbohydrate-metal adduct and the internal standard using Eq. (3).

Relative D−uptake

$$= \frac{D-\text{uptake by the carbohydrate}-\text{metal adduct}}{D-\text{uptake by the internal standard}} \tag{3}$$

The D-uptake and relative D-uptake are presented as the average ± the standard deviation. Student's t test at the 95% confidence interval was used for statistical analyses.

Results and Discussion

When performing replicate in-ESI HDX analyses of melezitose, the D-uptake for the sodiated adduct ranged from 2.4 ± 0.2 to 4.2 ± 0.3 on different days (Table S1). The D-uptake was statistically different on day 2 (4.0 ± 0.5) and day 6 (4.2 ± 0.3) compared to all other days. Additionally, the D-uptake measured on day 1 (2.4 ± 0.2) differed from the D-uptake measured on day 4 (2.8 ± 0.1). This variability made it difficult to quantitatively compare the D-uptake measured on different days.

Initially, we tested leucine enkephalin as an internal standard because this peptide is well characterized and used as a standard for many MS applications, including instrument tuning and testing fragmentation efficiency [13]. Following in-ESI HDX, melezitose was detected as sodiated (Figure 1a) or potassiated (Figure S1a) adducts in the presence of the internal standard, leucine enkephalin (Figure 1b and Figure S1b). For two runs collected on the same day, the average D-uptake for sodiated melezitose was 5.45 or 4.13 (Figure 1a). For the same two runs, the average D-uptake for leucine enkephalin was 2.68 or 2.47 (Figure 1b), respectively. The first run, which had a larger D-uptake for sodiated melezitose, did not yield a comparable increase in the D-uptake for leucine enkephalin. Further, it can be seen that the shift in the m/z isotopic envelope for sodiated melezitose did not result in a comparable shift in the m/z isotopic envelope of leucine enkephalin. The relative D-uptake values calculated for sodiated melezitose in the presence of leucine enkephalin were 2.15 ± 0.07, 1.8 ± 0.2, and 2.31 ± 0.03 on different days
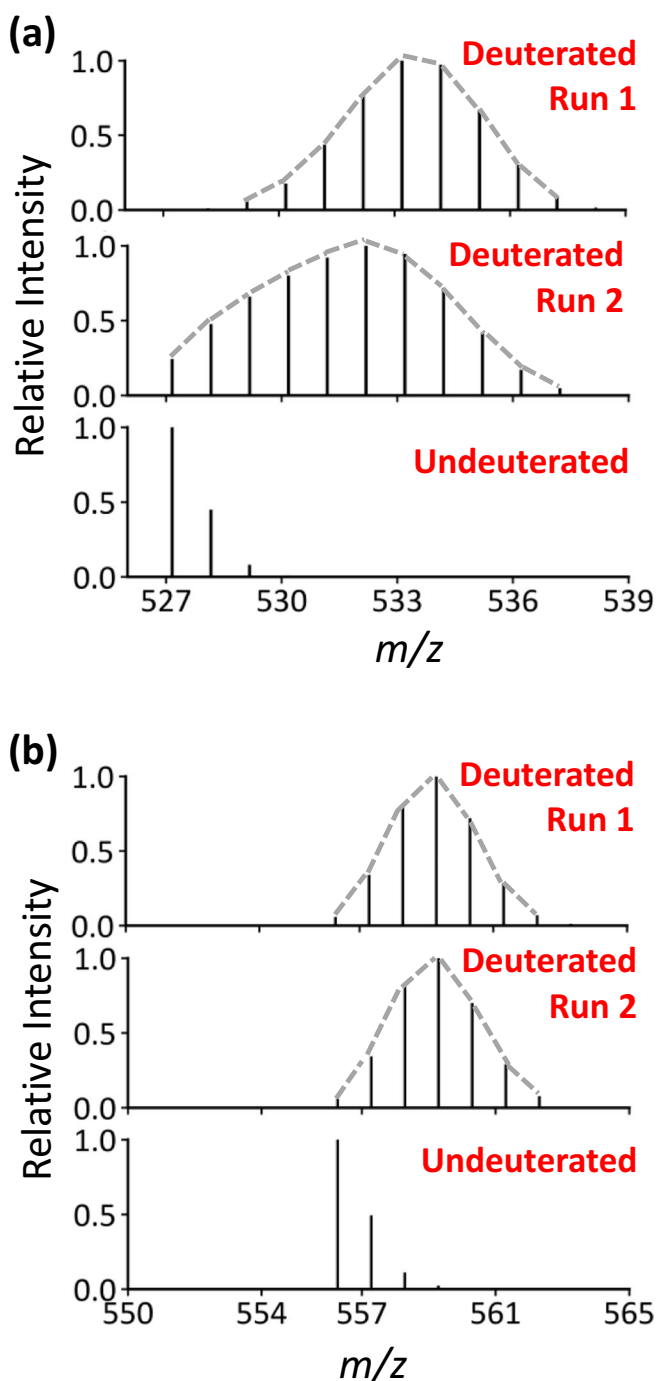
Leucine enkephalin contains four functional groups with labile hydrogens (Figure S2), including the rapidly exchanging tyrosine hydroxyl, N-terminal amine, and C-terminal carboxylic acid. The other labile hydrogens are present in backbone amides, which may not exchange rapidly enough to be detected during in-ESI HDX. Thus, the number of rapidly exchanging functional groups in leucine enkephalin does not provide a suitable dynamic range to normalize the D-uptake for sodiated

Figure 1. Mass spectral comparison of (a) sodiated melezitose and (b) internal standard, leucine enkephalin, before (undeuterated) or following in-ESI HDX (deuterated). Dashed lines illustrate isotopic distributions

(Table S2). Both the average D-uptake for sodiated melezitose and the relative D-uptake were statistically different on day 2 compared to days 1 and 3. Similar results were observed for the potassiated-melezitose adduct in the presence of leucine enkephalin (Table S2). Thus, this peptide was not able to normalize variations in D-uptake and was not a suitable internal standard for improving the repeatability of in-ESI HDX.
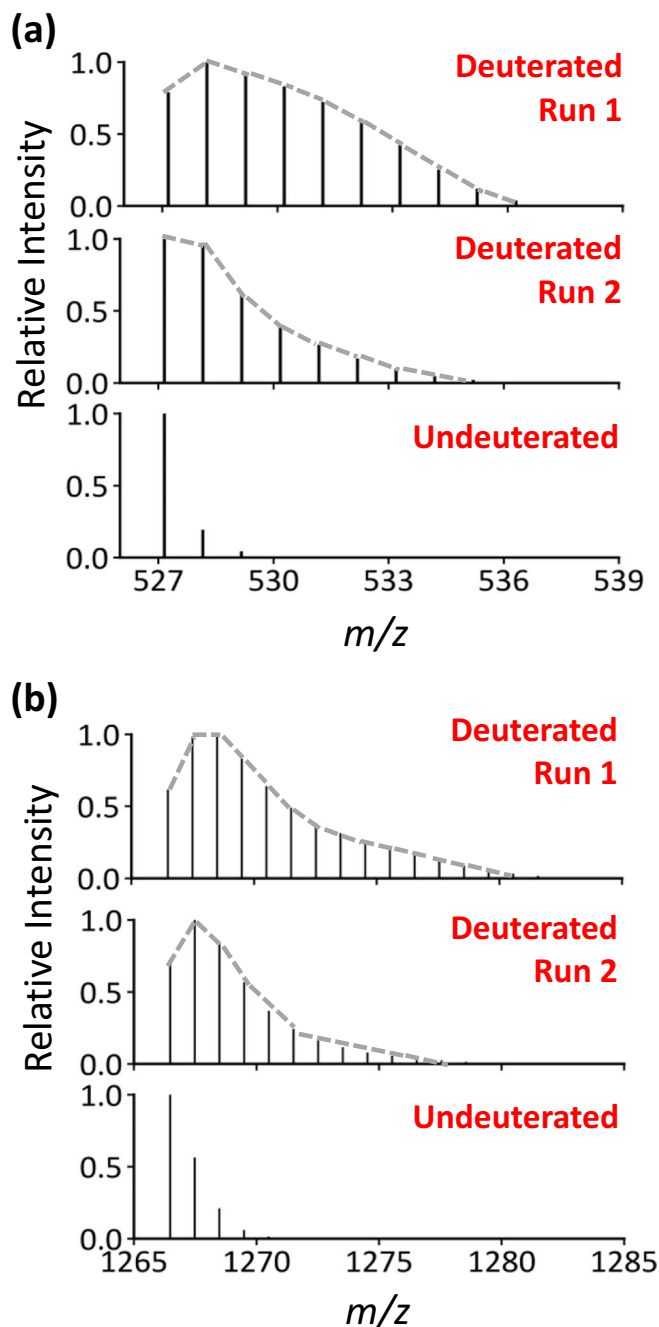

Figure 2. Mass spectral comparison of (a) sodiated melezitose and (b) internal standard, derivatized maltoheptaose, before (undeuterated) or following in-ESI HDX (deuterated). Dashed lines illustrate isotopic distributions

melezitose, which contains 11 labile hydrogens in hydroxyl functional groups.

To increase the dynamic range of the internal standard, we chose to utilize a carbohydrate that also contained many rapidly exchanging functional groups, e.g., maltoheptaose with 23 hydroxyls. We derivatized the carbohydrate internal standard using Girard's T reagent to add
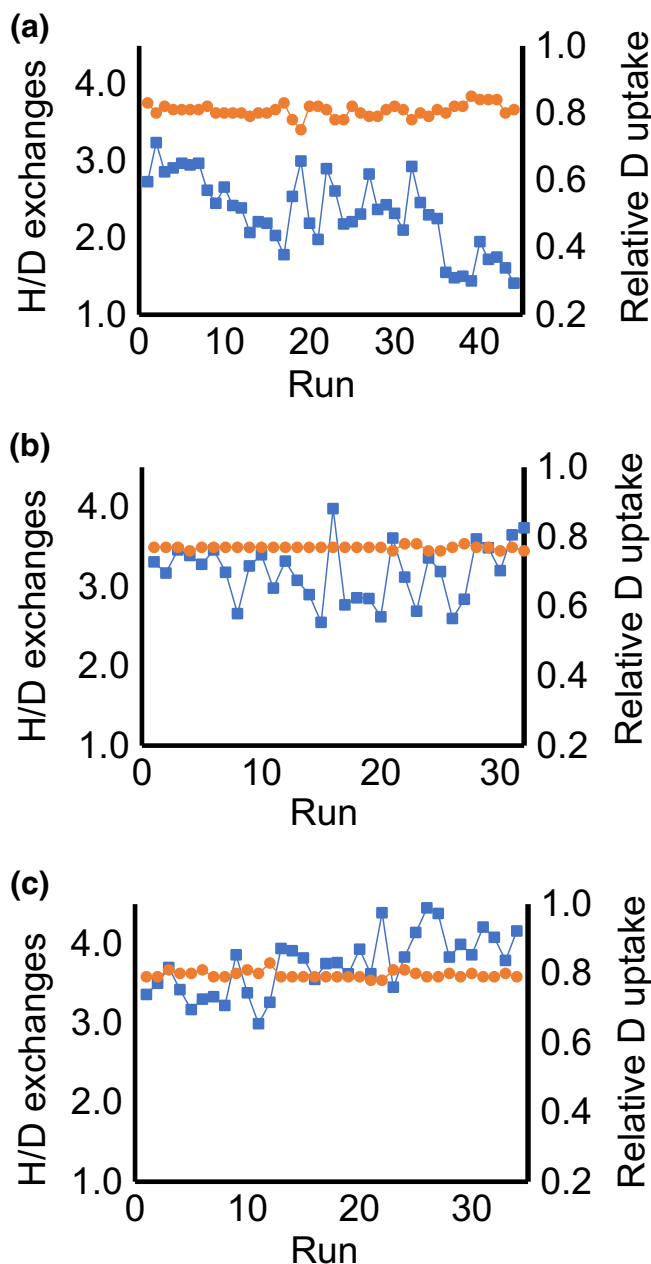
(a)

(b)

(c)

Figure 3. In the presence of an internal standard (derivatized maltoheptaose), the variability in measuring D-uptake is reduced. Plots illustrate D-uptake (blue squares) and relative D-uptake (orange circles) for (a) sodiated melezitose, (b) sodiated maltotetraose, and (c) sodiated α-cyclodextrin analyzed in the presence of derivatized maltoheptaose. Data shows replicate measurements recorded over 5 days with a minimum of six runs per day

a positive charge to ensure that detection of the analyte and the internal standard would not compete in forming metal-ion adducts. Once again, melezitose was detected as sodiated (Figure 2a) or potassiated (Figure S3a) adducts. For two runs collected on different days, the average D-uptake for sodiated melezitose was 2.73 and 1.55 (Figure 2a). For the same two runs, the average D-uptake for derivatized maltoheptaose was 3.31 and 1.95 (Figure 2b). The increase in D-uptake for sodiated melezitose was comparable to the increase in D-uptake for derivatized maltoheptaose. Additionally, the shift in the m/z isotopic envelope by sodiated melezitose was comparable to the shift in the m/z isotopic envelope by derivatized maltoheptaose. Further, the relative D-uptake calculated for sodiated melezitose on five different days varied from 0.80 ± 0.01 to 0.82 ± 0.01 (Figure 3 and Table S3). Though the magnitude of D-uptake for sodiated melezitose on day 1 was statistically different than the D-uptake measured on day 2 and day 5; the relative D-uptake values were within the measurement error for all 5 days. Similar trends were observed for the potassiated melezitose in the presence of derivatized-maltoheptaose (Figure S3 and Table S4). Taken together, this work illustrates the utility of this derivatized carbohydrate as an internal standard for correcting for daily variations in D-uptake.

The applicability of the derivatized-maltoheptaose internal standard was validated for two additional oligosaccharides, maltotetraose and α-cyclodextrin. The concentrations of maltotetraose and α-cyclodextrin were selected to optimize the ion signal, and thus, were different from the concentration used for melezitose. The D-uptake values and relative D-uptake values for the different carbohydrates are therefore not comparable for different oligosaccharides since the concentrations differ, which has been shown to affect D-uptake [8]. Figure 3 and Table S3 show that the D-uptake was statistically different on different days; however, in the presence of the derivatized-maltoheptaose internal standard, the relative D-uptake was consistent on multiple days. Therefore, the D-uptake by the derivatized maltoheptaose provided a suitable dynamic range for normalizing D-uptake for carbohydrates of varying sizes.

Conclusions

An internal standard should be chemically similar to the analyte of interest and should not react or compete with the analyte for detection. Here, we show that derivatized maltoheptaose, which contains many rapidly exchanging hydroxyls, is a suitable internal standard for in-ESI HDX analyses of carbohydrates of varying size. The improvement in measurement repeatability, when using this internal standard, will allow for quantification of D-uptake following in-ESI HDX, providing opportunities to apply this method to characterize solvated carbohydrate conformations.

Acknowledgements

References

1. Engen, J.R.: Analysis of protein conformation and dynamics by hydrogen/deuterium exchange MS. Anal. Chem. **81**, 7870–7875 (2009)
2. Gallagher, E.S., Hudgens, J.W.: Mapping protein–ligand interactions with proteolytic fragmentation, hydrogen/deuterium exchange-mass spectrometry. In: Kelman, Z. (ed.) Methods in enzymology, vol. 566, pp. 357–404. Academic Press, Cambridge (2016)
3. Price, N.P.J.: Oligosaccharide structures studied by hydrogen–deuterium exchange and MALDI-TOF mass spectrometry. Anal. Chem. **78**, 5302–5308 (2006)
4. Kostyukevich, Y., Kononikhin, A., Popov, I., Nikolaev, E.: In-ESI source hydrogen/deuterium exchange of carbohydrate ions. Anal. Chem. **86**, 2595–2600 (2014)
5. Kostyukevich, Y., Kononikhin, A., Popov, I., Nikolaev, E.: Conformations of cationized linear oligosaccharides revealed by FTMS combined with in-ESI H/D exchange. J. Mass Spectrom. **50**, 1150–1156 (2015)
6. Uppal, S.S., Beasley, S.E., Scian, M., Guttman, M.: Gas-phase hydrogen/deuterium exchange for distinguishing isomeric carbohydrate ions. Anal. Chem. **89**, 4737–4742 (2017)
7. Kim, H.J., Liyanage, O.T., Mulenos, M.R., Gallagher, E.S.: Mass spectral detection of forward- and reverse-hydrogen/deuterium exchange resulting from residual solvent vapors in electrospray sources. J. Am. Soc. Mass Spectrom. **29**, 2030–2040 (2018)
8. Liyanage, O.T., Brantley, M.R., Calixte, E.I., Solouki, T., Shuford, K.L., Gallagher, E.S.: Characterization of electrospray ionization (ESI) parameters on in-ESI hydrogen/deuterium exchange of carbohydrate-metal ion adducts. J. Am. Soc. Mass Spectrom. **30**, 235–247 (2019)
9. Kostyukevich, Y., Kononikhin, A., Popov, I., Nikolaev, E.: Simple atmospheric hydrogen/deuterium exchange method for enumeration of labile hydrogens by electrospray ionization mass spectrometry. Anal. Chem. **85**, 5330–5334 (2013)
10. Zhang, Z., Zhang, A., Xiao, G.: Improved protein hydrogen/deuterium exchange mass spectrometry platform with fully automated data processing. Anal. Chem. **84**, 4942–4949 (2012)
11. Naven, T.J.P., Harvey, D.J.: Cationic derivatization of oligosaccharides with Girard's T reagent for improved performance in matrix-assisted laser desorption/ionization and electrospray mass spectrometry. Rapid Commun. Mass Spectrom. **10**, 829–834 (1996)
12. Kim, J.-S., Monroe, M.E., Camp, D.G., Smith, R.D., Qian, W.-J.: In-source fragmentation and the sources of partially tryptic peptides in shotgun proteomics. J. Proteome Res. **12**, 910–916 (2013)
13. Sztáray, J., Memboeuf, A., Drahos, L., Vékey, K.: Leucine enkephalin—a mass spectrometry standard. Mass Spectrom. Rev. **30**, 298–320 (2011)