Chromatographic behavior of peptides containing oxidized methionine residues in proteomic LC–MS experiments: Complex tale of a simple modification

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1. Introduction

Bottom-up proteomics is the leading tool in modern analytical chemistry of proteins [1]. Vast majority of such analyses include protein digestion, reversed-phase LC separation of the resulting peptide mixtures, and MS/MS analysis. The major obstacle encountered in these protocols is an inadequate depth of analysis due to a wide dynamic range of protein concentrations [2] and presence of post-translationally and chemically modified peptides. Modiﬁcations of biological systems [5,6]. All these issues make studying the behavior of affected peptides upon MS/MS fragmentation is critical to successful analysis. The application of a second identiﬁcation constraint such as predicted retention time would solidify all MS-based assignments and simplify the development of quantitative targeted assays.

Presently, about 500 modiﬁcations are known and reported [7]. Methionine oxidation is one of the most frequently observed post-translational modiﬁcations of peptide/proteins, and may constitute up to 10% of the total non-redundant peptide sequences [8] identiﬁed in LC and MS dimensions, leading to failure of a mass spectrometer to select the low-abundance peptides for MS/MS acquisition. Chemical instability of the analytes also complicates targeted (selected reaction monitoring, SRM) and data independent (SWATH) quantitative techniques. Peptides with potential modiﬁcation sites are typically excluded from SRM assays [3]. Assignment of modiﬁed peptides in SWATH datasets is often inﬂuenced by their modiﬁed counterparts, as they usually are detected within the same SWATH window [4]. On the other hand, monitoring in-vivo PTMs is an important part of modern systems biology studies. There is ample evidence that PTMs control the functions of biological systems [5,6]. All these issues make studying the behavior of modiﬁed peptides in LC–MS analysis extremely important. Knowing the exact mass shifts induced by these modiﬁcations, and the behavior of the affected peptides upon MS/MS fragmentation is critical to successful analysis. The application of a second identiﬁcation constraint such as predicted retention time would solidify all MS-based assignments and simplify the development of quantitative targeted assays.

On average, the oxidation of a single Met residue to Mso (methionine S-oxide, methionine sulfoxide) and Msn (methionine S,S-dioxide, methionine sulfone) decreases peptide retention in RP HPLC by 2.37 and 1.95 Hydrophobicity Index units (% acetonitrile), respectively. At the same time, the magnitude of the retention shift varies greatly (−9.1 to +0.4% acetonitrile for Mso) depending on peptide sequence. The latter effects are mostly associated with the stabilization of secondary structures upon peptide interaction with the hydrophobic stationary phase: when an oxidized residue is located in the hydrophobic face of an amphipathic helix, the decrease in retention is profound. The same amino acid positioning leads to complete or partial resolution of pairs of peptides containing diastereomeric Mso residues. Contrary to all previously reported observations, and the nature of this modiﬁcation, we also demonstrate for the ﬁrst time that methionine oxidation may increase peptide hydrophobicity. This behavior is characteristic for Met residues in the N3 position of an N-capping box stabilization motif prior to the amphipathic helix. All these ﬁndings indicate that the prediction of peptide secondary structures upon interaction with hydrophobic surfaces must become an integral part of peptide retention modeling in proteomic applications going forward.

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Systematic large-scale observations on chromatographic behavior of PTM peptides including Met oxidation appeared following rapid developments in LC–MS proteomics applications [8,10,20–22]. Literature indicates that peptides containing oxidized methionine are more hydrophilic under RP LC conditions than their unmodified analogs. Zybailov et al. [8] summarized the effects of 15 different PTMs on peptide elution times and found that retention increases in the order Mso < Met. Lengqvist et al. [10] investigated the effect of common modifications on retention and isoelectric point values and reached the same conclusion. Recently Moruz et al. [22] introduced application of their peptide retention model Elude to 5 different PTMs. The authors collected retention information from proteomic analysis of 12 different organisms and subsequently modified the peptide retention prediction model to incorporate PTMs. Some of these runs were performed using the COFRADIC approach, thus the authors encountered oxidation on both Mso and Msn. Model optimization was performed using ~1500 peptides for both Mso and Msn-containing peptides and showed increase in retention coefficients as follows: Mso < Msn < Met. Despite ample evidence of significant variation in retention shift upon oxidation provided by Blondelle et al. 20 years ago [13], these effects were never explored in detail. The assumption that the shift in peptide retention is independent of the modified residue’s position or secondary structure effects is sufficient for additive prediction models. The accuracy of such models reaches its potential maxima at 0.93–0.94 R2-value in prediction accuracy. Moving beyond these values was one of the major motivations for this study.

The long-term goal of our research group is to construct an enhanced peptide retention prediction algorithm, which will encompass various chemical and biologically relevant post-translational modifications. Following development of our core Sequence Specific Retention Calculator (SSRCalc) model [23] we began systematic studies of modified peptides with N-terminal cyclization at Gln and Caboxamidomethyl-Cys [21], and with 5 different Cys-alkylating agents [24]. These studies highlighted the importance of amphipathic helicity in correct prediction of peptide retention times. Alpha-helices are one of the major elements of protein and peptide secondary structure. Yet this feature remains unincorporated in current peptide retention prediction models. The hydrophobic character of methionine, its abundance inside amphipathic helices, significant differentiation in retention shifts upon oxidation depending on secondary structure [13] make this modification a key to probing the effects of amphipathic helicity on RP LC peptide retention.

The goal of this study was to expand application of our Sequence Specific Retention Calculator model to process peptides with methionine oxidation. Priority was given to the ubiquitous Met oxide (Mso) containing peptides routinely observed in LC–MS experiments. We attempted analysis of the retention shifts for several thousands pairs of Met–Mso containing tryptic peptides observed in three 2D LC–MS runs. Retention behavior of Msn-containing peptides was investigated on a smaller scale by the 1D LC–MS analysis of tryptic digests oxidized with H2O2. Our observations were also supported by the analysis of designed synthetic peptides. The overarching objectives of this study included using Met oxidation as a probing tool to better understand effects of helicity on peptide interaction with hydrophobic surfaces – one of the key mechanisms determining peptide interaction in biological systems.

2. Materials and methods

2.1. Chemicals

All chemicals were analytical grade and sourced from Sigma Chemicals (St-Louis, MO), unless noted otherwise. Synthetic peptides were purchased from BioSynthesis Inc. (Lewisville, TX) and JPT (Berlin, Germany), in milligram quantities and in Spike Tide format (50 nmole per peptide), respectively. HPLC-grade acetonitrile and de-ionized water from Fisher Scientific (Toronto, ON) were used for the preparation of eluents. Sequencing-grade modified trypsin (Promega, Madison, WI) was used for digestion.
2.2. Protein digestion and sample preparation

Tryptic digests of whole cell lysates of Neurospora crassa, Thermoaeroebacter WC1, and Clostridium butyricum were prepared using the FASP protocol [25]. Digests were acidified with trifluoroacetic acid and purified by RP solid-phase extraction. Approximately 100 μg of each digest (according to NanoDrop 2000 (ThermoFisher Scientific) measurements) were used for 2D LC–MS analysis. Samples of synthetic peptides were prepared by dilution in Buffer A (0.1% formic acid in water) to provide injected amounts of ~200–400 fmole per injection. To produce samples with a higher degree of oxidation, and to generate Msn-containing species, the samples were subjected to oxidation with 1% H2O2. Depending on the desired oxidation state and degree of oxidation in original samples, we varied the hydrogen peroxide exposure time. The methionine-containing Spike Tide peptides from JPT usually exhibit a ~50–60% oxidation degree in Mso without any treatment. Synthetic peptides prepared using the conventional approach, and a tryptic digest of Thermoaeroebacter WC1 were each oxidized for up to 6 days to produce sufficient amounts of Msn-containing species. All individual samples and fractions collected in the first dimension of 2D LC procedure were spiked with a mixture of 6 standard peptides P1–P6 [26] for retention time alignment purposes (400–600 fmole per injection each).

2.3. Chromatographic conditions

The first dimension separation was performed using RP-HPLC at pH 10, using a method first described by Gilar et al. [27] and modified as described elsewhere [28]. Digest aliquots containing ~100 μg of peptide mixtures were fractionated. Forty 1-minute fractions were collected (roughly 2.5 μg per fraction); these fractions were then pair-wise concatenated to ensure optimal coverage space in the second separation dimension. One third or each fraction (~1.5–2 μg) was subjected to 1 h second dimension LC–MS analysis.

Both 1D and 2D analysis procedures employed identical nano-flow RP-HPLC system settings on the final stage of separation prior to MS acquisition. A splitless nano-flow 2D-Ultra system (Eksigent/ABSciex, Dublin, CA) with 10 μL sample injection via a 300 μm × 5 mm PepMap100 (Thermo Scientific) trap-column and a 100 μm × 200 mm analytical column packed with 5 μm Luna C18(2) (Phenomenex, Torrance, CA) was used. Both eluents A (water) and B (acetonitrile) contained 0.1% formic acid as the ion-pairing modifier. The linear gradients of 0.6–0.8% acetonitrile per minute (0.5–30% B) were used for LC–MS acquisitions in 2D analyses, and for the synthetic peptides. A 0.33% acetonitrile per minute linear gradient (0.5–30% B) was used for 1D LC/MS analyses of the digests. All gradient runs were completed with 5 min at 80% phase B and a 7 min equilibration with starting conditions of 0.5% B. Total LC–MS analysis time was ~1 h per fraction in 2D and ~2 h per sample in 1D runs, respectively.

2.4. Mass spectrometry

A TripleTOF5600 mass spectrometer (ABSciex, Concord, ON) was used in standard MS/MS data-dependent acquisition mode. Survey MS spectra (250 ms) were collected (m/z 400–1500) and followed by up to 20 MS/MS measurements on the most intense parent ions (300 counts/s threshold, +2–+4 charge state, m/z 100–1500 mass range for MS/MS, 100 ms each). Previously targeted parent ions were excluded from repetitive MS/MS acquisition for 12 s (50 mDa mass tolerance). Following search parameters for identification with X!Tandem were used: ±10 ppm and ±30 mDa mass tolerance for parent and fragment ions, constant modification of Cys with iodoacetamide; variable modifications +15.995 and +29.99 at Met; expectation value cut-off of Log(e) < −3 and < −1 for non-modified and oxidized peptides, respectively.

2.5. Hydrophobicity expression and calculations

Retention times for the standard peptides [14,26] and peptides from tryptic digests were assigned to each non-redundant identification as the intensity weighted time average for the two most intense MS/MS spectra. Peptide retention was expressed in Hydrophobicity Index units (HI, % acetonitrile) via a least squares linear regression fit using the HI values previously assigned to each member of the P1–P6 mixture [26]. Our SSRCalc algorithm was used for the HI prediction calculations. Hydrophobic moment values were calculated according to Eisenberg et al. [29].

3. Results

3.1. Impact of methionine oxidation on the output of LC–MS proteomic analyses

Table 1 shows the summary of protein and peptide identification for one- and two-dimensional LC–MS analyses of the digests used in this study, including the detection of peptides containing methionine sulfoxide. A coverage of 60–70% for the bacterial proteomes in 2 dimensional runs over ~20 h of MS acquisition was found typical for our Triple TOF5600 platform. The percentages of Msso-containing peptides in C. butyricum and Thermoaeroebacter WC1 digests typical for 1D and 2D analyses performed in our laboratory were ~3% and ~10%, respectively. As a comparison, Zybailov et al. [8] reported ~10% of peptides carrying methionine sulfoxide. Their 1D LC–MS runs, however, were performed for in-gel digested proteins, which usually results in additional methionine oxidation. The higher degree of oxidation in the N. crassa digest in our instance is likely due to differences in sample preparation. In all cases, the two-dimensional separation increases dynamic range of MS detection making lower-abundance species accessible; this results in better detection of Msso-containing peptides for all three samples. Additional sources of this increase include oxidation of peptides after collecting the fractions in the first dimension, as they were pair-wise concatenated and lyophilized.

It should be noted that we did not detect Msn containing peptides in the samples shown in Table 1. Zybailov et al. [8] found “Dioxidation (M) [+31.99]” modification, which correspond to oxidation of 2 methionines in the same peptide, rather than the formation of methionine sulfone.

3.2. Peak splitting for Msso-containing diastereomers: confirmation of literature data using synthetic peptides

The addition of an oxygen atom (Fig. 1), as Met is oxidized to Mso, results in increased polarity of the side chain leading to decreased RP HPLC retention of the modified peptide. According to Blondelle et al. [13] this decrease in retention is more profound when the modified residue is situated in the peptide’s preferred binding domain — the hydrophobic face of amphipathic helix. The same positioning leads to peak splitting for Msso-containing peptides. Addition of the second oxygen (Fig. 1) eliminates the stereogenic center, which should lead to the elimination of peak splitting. To verify this, we used oxidation with hydrogen peroxide to prepare mixtures containing sufficient amounts of all three versions (Met, Mso and Msn-containing analogs) of the synthetic amphipathic peptides ASGVSSVMSSVVK and ASGVSMVSSVVKG. Both peptides contain a comparable number of hydrophobic residues and exhibit well defined hydrophobic faces, as shown by their helical projections in Fig. 2. These two identical features were introduced to generate sequences with comparable hydrophobicity and to highlight the influence of Met positioning within the amphipathic structure. The following observations can
be made based on the corresponding extracted ion chromatogram (XIC) shown in Fig. 2.

a) Retention time (hydrophobicity of a side chain) increases in the following order: Mso < Msn < Met, which agrees with Moruz et al. [22] observations.

b) A much larger retention time shift is observed when the Mso was positioned in the hydrophobic face of the amphipathic alpha helical structure as compared to that on the hydrophilic face: −5.6% and −1.8% acetonitrile for ASGVSSVMSSVVGK and ASGVSMVVSSVVGK, respectively.

c) Peak splitting only occurs when Mso is positioned in the hydrophobic face of an amphipathic helix.

d) Further oxidation to methionine sulfone results in the elimination of peak splitting.

Manual inspection of the MS data from the proteomics runs in Table 1 confirms these findings (not shown) regarding the Mso–Met pair: larger negative retention shifts are associated with peak splitting. Due to the small difference in retention between pairs of peptides containing Mso diastereomers, these effects are largely ignored within large-scale proteomics runs. Both peptides have identical MS/MS spectra and are usually reported as a single detectable feature by peptide identification engines.

To rationalize the observations in Fig. 2, it is important to recognize that depending on the peptide sequence, the hydrophobic Met could play different roles upon interaction with the hydrophobic C18 surface. When placed in a preferred binding domain (the hydrophobic face of an amphipathic helix) it provides additional helix stabilization due to the hydrophobic character of its side chain. Consequently, when Met is oxidized to Mso, the essentially more hydrophilic side chain disrupts this stabilization (Fig. 2 A,B). Oxidation of a Met residue outside the preferred binding domain has less effect on the peptide retention time (Fig. 2 C,D). Due to the close “intimate” [13] contact of the preferred binding domain with the hydrophobic surface, even a small alteration in peptide chemistry in this domain substantially impacts the interaction energy. In case of the Mso diastereomeric pair, this results in a chiral separation on an appropriate chromatographic system.

### Table 1

Detection of methionine oxidation in typical 1D and 2D LC–MS bottom-up proteomic experiments.

<table>
<thead>
<tr>
<th>Sample</th>
<th># of spectra/# of identified peptides</th>
<th>Identified proteins</th>
<th># of unique non-modified peptides</th>
<th># of unique Met-containing peptides</th>
<th># of unique Mso-containing peptides</th>
<th>Oxidation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1D LC–MS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>C. butyricum</td>
<td>34182/20001</td>
<td>912</td>
<td>6565</td>
<td>2204</td>
<td>222</td>
<td>3.4%</td>
</tr>
<tr>
<td>N. crassa</td>
<td>15398/10712</td>
<td>1153</td>
<td>5979</td>
<td>1419</td>
<td>745</td>
<td>12.5%</td>
</tr>
<tr>
<td><em>Thermoanaeobacter</em></td>
<td>35886/20939</td>
<td>1101</td>
<td>6969</td>
<td>1957</td>
<td>224</td>
<td>3.2%</td>
</tr>
<tr>
<td><strong>2D LC–MS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. butyricum</td>
<td>301661/164756</td>
<td>2174</td>
<td>25027</td>
<td>8800</td>
<td>2880</td>
<td>11.5%</td>
</tr>
<tr>
<td>N. crassa</td>
<td>306150/180347</td>
<td>4288</td>
<td>41504</td>
<td>11693</td>
<td>7441</td>
<td>17.9%</td>
</tr>
<tr>
<td><em>Thermoanaeobacter</em></td>
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<td>1998</td>
<td>26633</td>
<td>8567</td>
<td>2733</td>
<td>10.3%</td>
</tr>
</tbody>
</table>

* a) Expectation value cut-off Log(e) < −3 for non-modified peptides and Log(e) < −1 for Mso-containing peptides.

* b) Expectation value cut-off of Log(e) < −3.

* c) Oxidation rate: ratio of Mso-containing peptides to total number of unique peptides.

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**Fig. 2.** Retention time shifts upon oxidation of Met residue to Mso and Msn depending on residue’s location within amphipathic helical structure. A, B — oxidation of Met in hydrophobic face of amphipathic helix (ASGVSSVMSSVVGK) leads to significant decrease in retention, and separation of peptides containing Mso diastereomers; C, D — oxidation of Met in hydrophilic face of amphipathic helix (ASGVSMVVSSVVGK). Oxidation with H2O2 was used to generate sample containing comparable amounts of peptides in all oxidation states. Slope of gradient for these separations was 0.78% acetonitrile per minute. Therefore retention time shift of 7.2 min (A) is equivalent to 5.6% acetonitrile.
3.3. Impact of methionine oxidation on peptide RP-LC retention: positional and secondary structure effects

We used 2D LC–MS identifications from Table 1 to compile retention data sets of 6421 pairs of non-modified peptides and their counterparts containing one Mso residue (Supplementary Table 1). This allows for the uniform comparison of retention shifts, which might reveal additional features required for proper algorithm implementation.

Fig. 3 shows the dependence of retention time shifts expressed in HI units from peptide molecular weight (a) and hydrophobicity (b). Three trends are worth noting from these dependencies:

a) Retention shifts caused by Met oxidation range from +0.3 to −8.1% acetonitrile, or −2.37% on average. In most cases, oxidation of Met to Mso reduces peptide hydrophobicity (retention in RP HPLC). However we found five instances of positive retention shifts.

b) Met oxidation shows smaller effects for large peptides (Fig. 3A), which is consistent with our previous observation for other modifications [21,24]: the modification of single residues induces smaller changes in larger peptides.

c) Extremely large retention time shifts (5–8% acetonitrile) were found only for relatively hydrophobic peptides (HI > 10% ACN in Fig. 3B).

The last observation provides additional insight into the effect of peptide amphipathic helicity on peptide RP HPLC behavior. The presence of methionine in the hydrophobic face of an amphipathic helix is a prerequisite for a significant retention time shift. Therefore these peptides must exhibit a well-defined hydrophobic face, e.g. contain several hydrophobic residues in their sequences. Because of that, relatively hydrophilic species (with HI < 10) do not fit this criterion as they simply contain a relatively low number of hydrophobic amino acids. Fig. 3a also shows the average values of the Eisenberg's hydrophobic moment [29] for groups of peptides, depending on their retention shifts (6–7, 5–6, 4–5% ACN, etc.). Clearly, the amphipathicity increases for peptides with large negative retention shifts.

To further elucidate the positional and structural effects of methionine oxidation we analyzed retention shifts for five different groups of peptides, examples of which are shown in Table 2. The variation in retention time shifts was explained based on our current understanding of separation mechanisms according to the SSRCalc model, and new findings associated with effects of secondary structure:

a) Small negative retention shifts (or small positive on two occasions) are often associated with Met being in an N-terminal position such as MAGSHTDITER from N. crassa (green circles in Fig. 3C). This effect is caused by the ion-pairing mechanism in peptide RP HPLC. The positively charged N-terminal amino group interacts with hydrophilic formate counter ions, which provide a “shielding” effect of neighbor amino acids [30]. This leads to a significant reduction of the apparent hydrophobicity of the residues at the peptide N-terminus and as such in smaller difference between Met (hydrophobic) and Mso (hydrophilic) containing peptide analogs.

b) Similar ion-pairing nearest-neighbor effects [23] are responsible for the relatively low decrease in retention times for methionines neighboring positively charged residues (Lys, Arg, His).

![Fig. 3. Retention time shifts (ΔHI) due to oxidation of single methionine into Mso depending on bulk properties of peptides. A — dependence on peptide size. Note increase of average peptide amphipathicity (average hydrophobic moment μ) for peptides with large decrease in retention; B — ΔHI vs. hydrophobicity of non-modified peptides; C — ΔHI vs. hydrophobicity for subsets of peptides with specific properties (detailed in Table 2). Green circles — peptides with oxidation at N-terminal methionine residue; red circles — amphipathic peptides with Met located in hydrophobic face of helix.](image-url)
c) Extremely large negative retention shifts are always associated with Met residues that are part of the hydrophobic face of an amphipathic helix. We confirmed this effect by selecting a group of peptides with extremely high hydrophobic moment values ($\mu > 3$), which contain Met in the hydrophobic face of the helix (red circles in Fig. 3 C).

d) Relatively small negative retention shifts are observed for Met residues located in the hydrophilic face of the amphipathic helix (similar to Fig. 2 C, D). The example of EAITMVNDMELLSAVTK (C. butyricum) in Table 2 with two Met additionally highlights the difference between groups c and d. Met (5) is located in the hydrophilic and Met (10) in the hydrophobic face of the amphipathic helix. Retention shifts upon oxidation of the same parent peptide were found to be $-1.3$ and $-4.2\%$ for these two positions, respectively. This example indicates that the chromatographic behavior clearly discriminates between peptides with different position of the modified residue.

e) Three out of five peptides exhibiting small positive retention shifts contain a Met residue in the N3 position of the N-capping box stabilization motif prior to the amphipathic helix. The number of instances found in naturally occurring peptides from our biological samples is very small: 3 cases from 6421 pairs. However, they highlight an extreme importance of the structural features for understanding effects of peptide's helicity. We further elucidated this unexpected behavior using designed synthetic sequences (see the following section).

Detailed inspection of oxidized/non-oxidized peptide pairs may reveal additional features related to the effects of secondary structures. For example, the amphipathic GMFQQMQQDKQR carrying three Met residues was found with all three potential locations of the Mso residue. All methionines are located in the hydrophobic face of the helix, however exhibiting different shifts upon oxidation: $-3.4$, $-5.0$ and $-3.7\%$ for positions 2, 6, and 10, respectively. This indicates that residues located in the middle of helical stretch (Met 6) are more involved in stabilization of the molecules upon interaction with hydrophobic surface compared to the terminal ones.

3.4. Positive retention time shift upon oxidation of Met in the N3 position of N-cap box motif: confirmation using synthetic peptides

Out of five peptides with positive retention shifts, two contain N-terminal Met and three (SLEMELESQCNARV, TLQMLTILSPDTR, TLEMLADTSEQYK) are amphipathic and contain the common sequence motif X-Leu-Z-Met, where X is represented by Ser or Thr and Z by Gln or Glu. Recently we demonstrated that similar to helices in proteins, amphipathic helical structures upon interaction with hydrophobic C18 phase are stabilized by N-cap box motifs [31]. This motif consists of four residues with each position showing different preferences for individual residues. The first (N-cap, according to Richardson and Richardson's terminology [32]) position is preferable for Asn, Asp, Ser, Thr, and Gly. Second (N1) and third (N2) positions are favoring hydrophobic residues [31]. Forth (N3) position is predominantly occupied by Gln, Glu and Asp.

We found that N-cap box motifs prior to amphipathic helical structure significantly increase chromatographic retention due to helix stabilization. This stabilization occurs due to reciprocal hydrogen bonding interactions of the N-cap residue’s side chain with the backbone C = O group of the N3 residue [33], and vice-versa side chain of N3 residue with the —NH backbone group of the N-cap amino acid.

Three peptides with positive retention shifts start with typical N-cap residues (Ser, Thr), followed by hydrophobic Leu (N1) and have Met residue in position #4 (N3): SLEM, TLQM, and TLEM. We believe that the positive retention time shift upon Met oxidation is observed due to better ability of the Mso residue to form hydrogen bond with the N-cap — NH group compared to a non-modified Met, and thus to stabilize the amphipathic helix.

We confirmed this finding by analyzing the retention behavior of oxidized/non-oxidized pairs for four synthetic peptides carrying predicted N-cap motif with the Met residue placed in the N-cap (hydrophilic face), N1 (hydrophobic face), N3 (hydrophilic face) and in the middle of the amphipathic helical stretch (hydrophobic face of the helix). The GSIVMEIEEVIGEGER peptide with optimal composition of N-cap box motif (SIVE) prior to two turns amphipathic sequence (IVEEIEV) was chosen as a template. To additionally increase the helical interactions we used amino acids with small compact aliphatic chains to construct the hydrophobic face of the helix (Ile and Val). Fig. 4 shows the respective extracted ion chromatograms and axial helical projections for these sequences.

GSIVMEIEEVIGEGER has the Met residue in the N-cap position, placed in the hydrophilic face of the helix (Fig. 4 A). This explains the relatively low ($-1.6\%$ acetonitrile) decrease in retention upon oxidation to Mso and no peak splitting for Mso diastereomers.

GSVMIEIEEVIGEGER has the Met residue shifted into the next position compared to the previous molecule. However it was enough to move it into the hydrophobic face of the helix (Fig. 4B), causing a diastereomers’ peak splitting and a significantly larger decrease in peptide hydrophobicity upon oxidation ($-5.5\%$ acetonitrile).

GSIVMEIEEVIGEGER contains the Met residue in the N3 position of the N-cap box motif (Fig. 4 C). The hydrophobicity of the oxidized peptide is higher ($+0.4\%$), which confirms our findings made based on the analysis of real tryptic digests in Table 2.

![Fig. 4. Retention time shifts upon oxidation of Met residue to Mso depending on position within an N-capping box motif (underlined) prior to amphipathic helix: A – GSIVMEIEEVIGEGER: Met residue in N-cap position, hydrophilic face; B – GSVMIEIEEVIGEGER: at N1 position in hydrophobic face of the amphipathic helix; C – GSVMIEIEEVIGEGER: at N3 position in N-cap box motif, hydrophilic face; D – GSIVMEIEEVIGEGER: Met residue in hydrophobic face and in middle position within a 2-turns helical stretch.](image-url)
GSIVEEMEVEIGER features the Met residue in the middle of both the hydrophilic face in the axial helical projection and the linear sequence of the amphipathic stretch (IVEEMEEVI). This position plays the most important role in the stabilization of the amphipathic helix on the C18 surface. Replacement of Met for more hydrophilic Mso decreases the retention time dramatically (−9.1% acetonitrile) and gives baseline resolution of Mso diastereomers (Fig. 4 D).

These four examples illustrate that knowing the rules of helices’ interaction with the hydrophilic C18 surface has allowed us to design peptides exhibiting extreme values of retention shifts, both positive and negative.

3.5. Retention prediction for peptides carrying oxidized Met residues

Incorporation of new (modified residues) into peptide retention modeling should reflect all major mechanisms contributing to peptide retention. In a first approximation these include hydrophobic, electrostatic, ion-pairing interactions and effects related to stabilization of amphipathic helical structures. The latter by itself is determined by different components, as the helical stabilization depends on hydrophobic interactions of side chains with the C18 phase, hydrogen bonds between backbone C = O and N–H groups, and electrostatic interactions between basic and acidic residues in hydrophilic face of the helix. It should be noted that our algorithm for the correct assignment of the helical stretch and computation of its contribution into the peptide retention value is still under development, and does not currently incorporate the effect of N-cap stabilization. As described previously [23], our retention prediction model was optimized across the parameter space to maximize the R² value between predicted and observed retention values for our training set. In this study the training set included both modified and non-modified peptides, with the latter providing an “anchor” for mapping to HI values, as their predicted values remained constant within the optimization process. The following alterations were made to the SSRCalc model to incorporate methionine oxidation (both Mso and Msn):

a) Retention coefficients (Rc) were optimized for both Msn and Mso depending on residue position relative to peptide ends [23]. These included retention coefficients for N-terminal (RcN1, RcN2, ..., RcN6), C-terminal (RcC1, RcC2, ..., RcC6) and the internal locations of the residues (Rc) (Supplementary Table 2). The differences in hydrophobicity values for N-terminal positions are caused by ion-pairing interactions involving the positively charged N-terminal amino group. As expected (Fig. 2), the retention coefficients for our PTMs increases in the order Mso < Msn < Met. The Rt values for Mso and Msn were found to be very close to that of Ala—a weakly hydrophobic residue. Optimization set for Msn-containing peptides included 273 molecules identified in 1D LC–MS analysis of oxidized Thermanaerobacter WC1 digest (Supplementary Table 1).

b) Due to significantly higher hydrophilicity of Mso and Msn residues, the effect of ion-pairing at adjacent Arg, Lys and His residues in the sequence is not as profound. Msn and Mso were assigned to the group of hydrophilic residues for which these corrections do not apply.

c) The helical contribution is computed by summing energies of i; i + 3 and i + 4 interactions between hydrophobic residues within the assigned hydrophobic face of amphipathic helices. Interaction energies were optimized for each pair of interacting residues; this optimization was performed against a training set of ~10,000 amphipathic helical peptides (manuscript under preparation). We did not perform a helicity model parameter optimization for Mso residues: there are not enough amphipathic molecules in our 6421 peptide study set. The number Met–Msn pairs used for the optimization of the retention coefficients was even lower, and could result in “overfitting” of the model. Instead, following from (a) we simply assigned helical interaction energies for both Mso and Msn to be equivalent to the Ala values by a substitution on the model’s sequence input (Supplementary Table 2). A more sophisticated general approach for incorporating PTMs into helicity prediction is under development.

Variation of the helical contribution depending on position of the Mso residue can be illustrated using example of QMFOQMQDMQMR discussed above. Three i; i + 3 and i + 4 interactions were assigned to hydrophobic face of this peptide: M - - - M, F - - M and M - - M. Oxidation of terminal Met (2 and 10) will affect only one interaction. At the same time oxidation of Met (6) will affect all three, thus reducing helical stabilization in larger extent.

Fig. 5 A, B shows SSRCalc HI prediction for 6421 Met containing (a) and single Mso containing (b) peptides. The latter shows worse correlation and a −2.48 HI units intercept, which is very close to the average contribution of single oxidation of −2.37% acetonitrile. Due to reduced hydrophobicity of Mso, the application of a non-modified SSRCalc model yields a poor correlation of −0.90 when applied to combined set of modified and unmodified peptides (Fig. 5 C). Our modifications to the SSRCalc model brought accuracy of retention prediction back to a normal (−0.96) value with identical prediction errors for both modified and non-modified species (Fig. 5 D). Note that dependence in Fig. 5 D includes Mso and Msn containing peptides as well as peptides with multiple oxidations in the sequence.

4. Discussion

Better understanding the behavior of oxidized peptides in RP LC benefits many areas of applied proteomics. Establishing the range of retention time variation and rules governing them would help in optimizing Met-COFRADIC protocols, improve the accuracy of SWATH quantitation and confidence of peptide identification in shotgun analyses. We compared our observations and previously reported data [8,13,17,22] on retention shifts by converting retention values in minutes into HI units (% acetonitrile) using the provided experimental conditions (gradient slopes % ACN/min). The largest negative retention shift was reported by Blondelle et al. [13] using the Met substitution in synthetic “perfect” amphipathic helical peptide Ac−LKLKKLLKLLKKLLKKLLK−NH₂ featuring nine Leu and nine Lys in a 3.6 residue periodicity. Retention time shifts were measured between −9.3 and −0.5 min, which corresponded to −9.3 and −0.5% ACN for 1% per minute gradient. Gevaert et al. [17] used −7 to 1 min (−4.9 to −0.7% ACN) retention shift window for fraction collection in COFRADIC experiments. Zybalov et al. [8] reported average retention shift of −3.4% ACN with variation between −5.0 and −1.8%. Retention shifts in Moruz et al. [22] data were estimated between −8.3 and −0.4% ACN. Our results (−9.1 and −0.4) confirmed the largest retention shifts values observed by others, and showed for the first time that oxidation may increase peptide hydrophobicity.

We also demonstrate for the first time a number of peptides’ compositional and sequence features, which affect retention upon Met oxidation. For example, small negative (or even positive) retention shift could be the result of an N-terminal location of Met or its N3 position in an N-capping box motif. Different mechanisms in these two instances result in similar chromatographic behavior. It is interesting to note that both these cases may represent a complicated case for SWATH analysis [4]. Many peptides from these two categories will generate oxidation products with m/z values in the same 25 Da SWATH window, and similar retention times and y-ions patterns. Distinguishing between them may require additional efforts.

Amphipathic helicity was found to be the major contributor to diverse effects of Met oxidation on chromatographic properties. This was previously demonstrated using substitution with Met in a model synthetic peptide [13]. Our results expand these observations into real tryptic peptides observed in proteomics experiments. The incorporation of helical corrections will be a major improvement in further developing peptide retention models. Our preliminary attempts for incorporating
helix into the SSRCalc model demonstrate that we can achieve identical prediction accuracy for both modified and non-modified peptides of $-0.96 R^2$ correlation.

Understanding separation mechanisms and the effect of molecular structure on chromatographic behavior of biopolymers has served as a central theme of the research for many separation scientists. Following original developments in various protein separation techniques, Regnier noted that most of them are surface-mediated separation methods [34], therefore the structural features of proteins will determine their chromatographic behavior. The reversed-phase separation mechanism was described as a multi-point interaction of the hydrophobic sites on a protein surface and the hydrophobic stationary phase [35], where retention is a function of contact surface area with elution occurring due to a stoichiometric displacement between solute and solvent molecules [36]. Karger and co-workers showed that the hydrophobic interactions with alkyl-silica surface in RP LC changes the structure of polypeptides and may induce protein unfolding [37,38]. Similarly, for shorter peptides, hydrophobic chromatographic packing may induce helical structures in potentially helical molecules [39]. Therefore one can envision three different types of peptide binding to a hydrophobic surface: i) where the conformation of the peptide plays no role; ii) a peptide helical conformation is induced to maximize the contact area; and iii) a peptide already contains an amphipathic helix and binds with little change to its conformation. Multiple studies in the 1990’s demonstrated such behavior using designed model peptides [39–41] and led to establishing separate hydrophobicity scales for random coil and helical conformations of peptides upon interaction with RP sorbents [42]. The arrival of proteomic techniques provides chromatographers with access to thousands of peptides with secondary structures that providing a significant contribution to their RP LC retention [31]. These studies will continue the search for a better understanding the underlying mechanisms, and ultimately, to more accurate prediction of peptide retention properties.

5. Conclusions

We used the outputs of large-scale LC–MS/MS analyses to systematically study the RP-HPLC behavior of peptides containing oxidized methionine residues. In addition to confirming the commonly accepted phenomenon of lowered hydrophobicity upon Met side chain oxidation, we focused on the retention mechanism and the effects of peptide secondary structure. Oxidation to methionine S-oxide (Mso, +15.995 Da) is the most typical modification resulting in a negative ($-2.37\%$) acetonitrile retention shift on average. Further oxidation to methionine S,S-dioxide (Msn, +31.99 Da) increases the retention slightly ($-1.95\%$ compared to Met). The former represents the most typical modification observed in bottom-up proteomics, and was the major focus of this study. The latter is relatively rare, and is typically observed when oxidation is applied explicitly in sample preparation protocols.

A detailed overview of the chromatographic behavior of peptides carrying oxidized Met revealed an extremely complex picture in which multiple interactions (hydrophobic, electrostatic, hydrogen bond) impact peptide hydrophobicity. The amplitude of retention shift may vary dramatically: we found extreme cases of this variation from $-9.1$ to $+0.4\%$ acetonitrile. Stabilization of amphipathic helical structures upon interaction with hydrophobic C18 surface (i.e. secondary structure effect) is the main reason for such variations. Methionine is a moderately hydrophobic residue, which may be placed in the hydrophobic face of the amphipathic helix — the preferred binding to C18 surface domain for amphipathic helical molecules. Any alteration (Met oxidation in this case) in this domain causes a significant change in peptide retention. Due to the presence of the stereogenic center in the

![Graphs showing retention prediction for modified and non-modified peptides using SSRCalc algorithm. A – Retention prediction for set of 6421 non-modified peptides used in this study (Fig. 3); B – retention prediction for set of their 6421 oxidized counterparts carrying single Mso residue; C – retention prediction for combined set A and B; D – modified SSRCalc model provides identical accuracies for modified and non-modified species. This plot includes 273 Msn containing peptides and 336 peptides carrying multiple Mso residues.](image-url)
Mso structure, this also leads to partial or complete resolution of peaks corresponding to the Mso diasteromers. Moreover, for the first time we demonstrate that methionine oxidation may increase peptide retention. This phenomenon is also related to amphipathic helicity. When located at the N3 position of an N-cap box motif, the side chain of Mso serves as better partner for hydrogen-bond interaction, which additionally stabilizes the helical structure.

Specific properties of methionine (hydrophobic, prone to oxidation) make it a key in the understanding of retention mechanisms and in developing better peptide retention prediction models. Collection of representative retention datasets has been one of the major problems in this field. Peptide selection based on retention shift upon oxidation will direct data collection and subsequent model development for expanded sets of amphipathic helical peptides, thus contributing to solving this fundamental problem in peptide chromatography.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2015.05.018.

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