Journal of Chromatography A 1654 (2021) 462449

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Liquid chromatography setup-dependent artefactual methionine oxidation of peptides: The importance of an adapted quality control process

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ARTICLE INFO

Article history:
Received 1 March 2021
Revised 29 July 2021
Accepted 30 July 2021
Available online 5 August 2021

Keywords:
Methionine Oxidation
Liquid Chromatography
Peak Broadening
Mass Spectrometry
Complex Samples
Stationary Phases

ABSTRACT

In both biologics quality control experiments and protein post-translational modification studies, the analytical system used is not supposed to bring any artefactual modifications which could impair the results. In this work, we investigated oxidation of methionine-containing peptides during reversed-phase (RP) chromatographic separation. We first used a synthetic methionine-containing peptide to evaluate this artefactual phenomenon and then considered more complex samples (i.e., plasma and HeLa protein digests). The methionine oxidation levels of the peptides were systematically assessed and compared for the long-term use of the analytical column, the sample trapping time, the gradient length, the sample load and the nature of the stationary phase (HSS T3 from Waters, YMC Triart C18 from YMC Europe GmbH and BEH130 C18 from Waters). In addition to the oxidation of methionine in solution, we observed on the HSS T3 and the BEH130 stationary phases an additional broad peak corresponding to an on-column oxidized species. Considering the HSS T3 phase, our results highlight that the on-column oxidation level significantly increases with the age of the analytical column and the gradient length and reaches 56 % when a 1-year-old column set is used with a 180 min-long LC method. These levels go to 0 % and 18 % for the YMC Triart C18 and the BEH130 C18 phases respectively. Interestingly, the on-column oxidation proportion decreases as the injected sample load increases suggesting the presence of a discrete number of oxidation sites within the stationary phase of the analytical column. Those findings observed in different laboratories using distinct set of columns, albeit to varying degrees, strengthen the need for a standard of methionine-containing peptide that could be used as a quality control to appraise the status of the liquid chromatographic columns.

1. Introduction

The production of recombinant proteins for use as therapeutic agents and vaccine candidates has become a major market and is expected to continue its rapid expansion in the years to come [1,2]. In this context, considerable efforts are made on the quality control of the final product to carefully evaluate the properties of the proteins, including their structural integrity and their stability. These crucial aspects are of major concerns as they directly impact the efficacy and the safety of the therapeutic agents.

Liquid chromatography (LC) coupled with mass spectrometry (MS) is today the gold standard of analytical tools to confirm the protein primary sequence, and to identify and localize post-translational modifications (PTMs) [3]. To perform an exhaustive characterization and cover the whole protein sequence, different proteomics methods are available, ranging from top-down [4], middle-down [5] and bottom-up [6] strategies to combinatorial workflows [7]. As such, we recently developed a Multi-Enzymatic Limited Digestion (MELD) procedure that merges bottom-up and middle-down ideologies, resulting in the generation of overlapping peptides that enables refined and reliable characterization of proteins [8].

Nevertheless, a comprehensive protein characterization analysis relies on the adequate combination of both sample preparation and analytical methods to produce and detect signals free of artefactual modifications. Among the common protein modifications, methionine oxidation is frequently observed either as PTM directly

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https://doi.org/10.1016/j.chroma.2021.462449
0021-9673/

generated in the cell (*in vivo* modification) or as an artefactual modification induced by the sample preparation process (*in vitro* modification). Methionine can be oxidized to methionine sulfoxide (MetO) and, albeit less frequently observed, to methionine sulfone ($MetO_2$). Other amino acids can undergo *in vitro* oxidation but are less common.

Although *in vivo* modifications of methionine are relevant for biological purposes, artefactual *in vitro* modifications should be carefully minimized and evaluated in order to avoid misinterpretation of the results. Indeed, protein storage and purification, reduction and alkylation of cysteines, proteolysis, liquid chromatography separation as well as the ionization process [9–11] are all steps that can introduce undesired artefactual methionine oxidation and therefore impair the quality of the analytical results. This last decade, several new methodologies have been developed to reliably quantify or minimize methionine oxidation during the sample preparation process. Stable-isotope labelled reporter peptide and antioxidants that protect methionine residues from oxidation were used to monitor the artefactual modifications emerging during desalting procedures and the LC-MS separation [12]. Liu *et al.* uses stable-isotope labelling to generate completely oxidized methionine residues in proteins before the sample preparation [13]. Proteins with fully oxidized methionine residues are composed of a mix of ^{16}O and ^{18}O atoms, the ^{16}O atoms originally being associated with the *in vivo* oxidation level. Shipman *et al.* [14] enhanced this latter work by comparing theoretical and experimental MS spectra, eliminating the need for control samples and therefore reducing by half the protein consumption and analysis time. Rougemont *et al.* described the application of a triple calibration [15] with differently labelled standards to control the oxidation of the targeted peptide as well as the oxidation of the labelled standards. While several articles refer to the control of methionine oxidation on targeted peptides during sample preparation [11–22], only few of them take the LC separation in consideration as a potential source of oxidation [12,16] and none of these studies systematically evaluate the potential oxidation induced by the LC system.

Here, we focused on the methionine oxidation arising on our specific LC system (see materials and methods for more details). We first evidenced a significant oxidation phenomenon on methionine-containing peptides during the separation process and measured the evolution of this oxidation over a 1-year use of the column set. We then evaluated and compared the oxidation levels of methionine-containing peptides with different trapping times and gradient lengths which allows us to frame the exact localization of the LC separation bias. In addition, these results were also observed on publicly available data (PRIDE) where another RP column was used, indicating that this phenomenon is not limited to our setup. Subsequently, we showed that sample loads affect the oxidation response. Finally, we provide recommendations to evaluate, monitor and control this system-dependent artefact.

2. Materials and methods

2.1. Chemicals

HeLa whole-cell extract was purchased from Antibodies-online (Aachen, Cologne, Germany). Standard of HeLa protein digest, MS grade trypsin protease >95% and C18 tips 10µL were obtained from Pierce (Thermo Fisher Scientific, Pittsburgh, PA, USA). Extra pure grade trifluoroacetic acid (TFA) 99.5% was purchased from Acros Organics (Thermo Fisher Scientific, Pittsburgh, PA, USA). Plasma sample from Belgian blood donors was obtained from the Belgian Red Cross. The reference methionine-containing peptide was synthesized by Kaneka Eurogentec (Seraing, Liège, Belgium). Acetonitrile (ACN) was obtained from Biosolve ("ULC-MS" grade) (Dieuze, Moselle, France). Water was obtained from a Milli-Q purification system from Millipore (Burlington, MA, USA). Ammonium bicarbonate (NH_4HCO_3) >99.5% and iodoacetamide (IAA) >99% were supplied by Sigma-Aldrich (St. Louis, MO, USA). Reducing agent and detergent compatible protein assay (RC-DC Protein Assay) was purchased from BIO-RAD (Hercules, CA, USA). 2D Clean-Up Kit was supplied by GE Healthcare (Chicago, IL, USA). Ultra-pure grade >99.5% dithiothreitol (DTT) was obtained from Affymetrix (Thermo Fisher Scientific, Pittsburgh, PA, USA).

2.2. Synthetic peptide EM(Mox)SGSPASGIPVK solution preparation

Stock solution of the synthetic methionine-containing peptide was prepared by resuspending the lyophilized powder in water/ACN 1:1 (v/v) to a final concentration of 2 mg/mL. The stock solution was separated into 10 µL aliquots and stored at -80°C. Before each LC-MS analysis, the aliquots were freshly thawed on ice and diluted to a final concentration of 55.5 nM with water/TFA 0.1 % (v/v). For each experiment, 9µL (500 fmoles, 0.63 ng) were injected on the column.

2.3. Tryptic digestion of human plasma samples

Lyophilized plasma sample was resuspended with 1mL of Milli-Q water and the total protein content was evaluated based on the RC DC protein assay kit from Bio-Rad. 20 µg of plasma sample were diluted to 1 mg/ml with 50 mM NH_4HCO_3 and further processed into peptides following a standardized procedure (reduction-alkylation, clean-up, overnight tryptic digestion, C18 tip purification). The purified sample was divided in 0.825 µg protein aliquots and stored at -80°C. Before each LC-MS analysis, the aliquots were freshly thawed on ice, diluted to a final concentration of 0.075 µg/µL with water/TFA 0.1 % (v/v) and 9µL (0.675 µg) were injected on the column.

2.4. Tryptic digestion of HeLa whole cell extract

The same digestion protocol was applied to the HeLa whole protein extract. Aliquots of in-house HeLa digests were stored at -80°C. Before each LC-MS analysis, the aliquots were freshly thawed on ice and diluted with water/TFA 0.1 % (v/v) to 0.056 µg/µl and 9 µL (0.5µg) were injected on the column.

2.5. Commercial standard of HeLa protein digest preparation

Commercial HeLa protein digests were used as standards and compared with our in-house HeLa protein digests. Before each LC-MS analysis, the aliquots were freshly thawed on ice and diluted with water/TFA 0.1 % (v/v) to 0.056 µg/µl and 9 µL (0.5µg) were injected on the column.

2.6. Liquid chromatography – mass spectrometry analysis

Samples were analysed on an ACQUITY UPLC M-Class System (Waters, Milford, MA, USA) hyphenated to a Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer equipped with a Nanospray Flex™ ion source (Thermo Fisher Scientific, Pittsburgh, PA, USA). The UPLC system is composed of two successive columns: a nanoEase M/Z Symmetry C18 Trap Column (100 Å, 5 µm, 180 µm × 20 mm) and a nanoEase M/Z HSS T3 Column (100 Å, 1.8 µm, 75 µm × 250 mm), both commercialized by Waters. The temperature of the LC column was set to 40°C (trap column is at room temperature). Two sets of trap and analytical columns were tested. The first set is composed of brand-new trap and analytical columns. The columns were first conditioned with two injections

of plasma sample and a quality control of the system was performed with a commercial HeLa protein digest standard to validate its performance thanks to a threshold in the number of proteins detected. The second set is composed of 1-year old trap and analytical columns used for proteomics analysis. The sample is first loaded in a 10 µL sample loop, sent to the trap column and desalted with an eluent composition of water/ACN 98:2 (v/v) with 0.1 % formic acid (FA) at a flow rate of 20 µL/min and subsequently eluted thanks to an acetonitrile gradient at a flow rate of 0.6µL/min. The trapping time was fixed to either 3 min or 15 min, while the length of the gradient elution was fixed to either 30 min or 150 min (supporting information, Table S1 and S2). Three combinations of trapping-gradient times were used: (1) a 60 min-long UPLC method made of 3 min trapping time, 30 min gradient elution and 27 min washing and re-equilibration (2) a 72 min-long UPLC method made of 15 min trapping time, 30 min gradient elution and 27 min washing and re-equilibration, and (3) a 180 min-long UPLC method made of 3 min trapping time, 150 min gradient elution and 27 min washing and re-equilibration. The eluted peptides were mass detected according to a data-dependent acquisition (DDA) method in positive electrospray ionization (ESI) mode. The mass spectrometer settings were set as follows: capillary voltage and temperature are 2.30 kV and 270°C respectively, S-lens RF level = 50.0, scan range was set from 400 to 1600 *m/z*. The MS scans were acquired with resolution = 70 000 (at 200 *m/z*), automatic gain control (AGC) target = 1×10^6, maximum injection time (IT) = 50 ms. The DDA acquisition was Top12 with resolution of 17 500 (at 200 *m/z*), AGC target = 1×10^5, maximum IT = 50 ms, isolation window = 2 *m/z*, a normalized collision energy NCE = 28 and a lock mass at 445.12003 *m/z*.

Analyses generated with two other instrumentations were investigated for comparison with the data created with our LC-MS setup: an Ekspert nanoLC 425 hyphenated to a TripleTOF 6600 system both from Sciex (Washington, D.C, USA) and a nanoACQUITY UPLC System from Waters coupled to a Q Exactive Plus™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer from Thermo Fisher Scientific (PRIDE data repository). The set of columns in use with the Sciex system is as follows: a YMC Triart C18 trap column (120 Å, 3 µm, 0.3 mm x 5mm) followed by a YMC Triart C18 column (120 Å, 3 µm, 0.3 mm x 150 mm) commercialized by YMC Europe GmbH (Dinslaken, Düsseldorf, Germany). The set employed with the Waters/Thermo system is a nanoEase M/Z Symmetry C18 trap column (100 Å, 5 µm, 180 µm x 20 mm) followed by a nanoEase M/Z BEH130 C18 column (130 Å, 1.7 µm, 75 µm x 250 mm) both from Waters.

2.7. Database search

PEAKS Studio software v.10.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) was used for database searches on the HeLa protein digests and the plasma samples both with a human database containing 20 365 sequences (Uniprot, Swiss-Prot reviewed, Homo Sapiens 9606, downloaded on 22/10/2019). The tolerance on the precursor and fragment masses were fixed to 5.0 ppm and 0.015 Da respectively. Carbamidomethylation of cysteines was set as a fixed modification, whereas deamidation of asparagine and glutamine and oxidation of methionine were set as variable modifications. A maximum of 3 variable post-translational modifications per peptide were allowed. Trypsin was selected according to a specific digest mode with a maximum of 2 missed cleavages allowed. A false discovery rate (FDR) < 0.1 % was applied to MS/MS peptide identification.

Due to the complexity of the chromatographic profiles of methionine containing peptides, the data were reprocessed in the Skyline software v.20.1.0.76 [23] (MacCoss Lab Software, University of Washington, WA, USA) following their identification via the PEAKS Studio database search. This step allows to manually select and integrate multi-peaks chromatographic profiles associated with a given methionine-containing identified peptide. Peptides identified in the 180 min-long UPLC run of both HeLa protein digest and plasma samples were selected to generate two starting lists of peptides (generated from the Peaks analysis, one from the HeLa protein digest sample and the other one from the plasma sample) as this method showed the highest number of identified proteins/peptides. These lists were then filtered to exclusively keep peptides that are characterized by a single methionine residue and imported with their respective PEAKS results. Out of all the manually integrated peptides in the 180 min-long UPLC run, the ten most intense peptides were considered for integration in the other elution time conditions. The peak areas associated with the synthetic peptide EM(M_{ox})SGSPASGIPVK (peptide injected alone) were only integrated using the Skyline software.

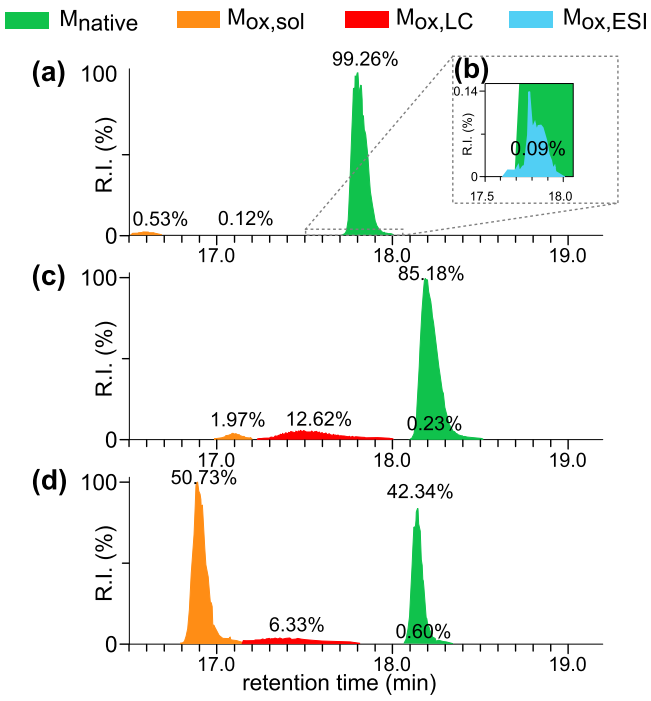

Fig. 1. Extracted ion chromatograms corresponding to the methionine-containing peptides EMSGSPASGIPVK and EM_{ox}SGSPASGIPVK analysed with a 60 min-long UPLC method. The peptide EMSGSPASGIPVK was analysed on (a) a brand-new set of trap and analytical columns and (c) a 1-year old trap and analytical columns set. (b) At the same retention time of M_{native}, small contribution of the in-source oxidized peptide $M_{ox,ESI}$ with an increment of 15.9949 m/z. (d) Separation of EMSGSPASGIPVK spiked with EM_{ox}SGSPASGIPVK on a 1-year old trap and analytical columns set.

3. Results and discussion

3.1. Oxidation of synthetic peptide

Based on a 13 residue-long synthetic peptide, EMSGSPASGIPVK, we first evaluated the level of methionine oxidation in solution $M_{ox,sol}$ by performing a direct infusion of this standard in positive ionization mode on a Q Exactive (Thermo Fisher Scientific, Pittsburgh, PA, USA) mass spectrometer (supporting information, figure S1). Based on the *m/z* intensity ratio of the oxidized (+15.9949 *m/z*) to the native forms of the standard, we evaluated the oxidation level to about 2 % of the sum of the two forms. The behaviour of the standard peptide when subjected to different LC-MS analysis setups was then probed. Otherwise stated, results are reported and discussed for the HSS T3 column. Fig. 1 shows the

retention time (RT) profiles of the synthetic peptide on both a brand-new set of trap and analytical columns (Fig. 1a) and a 1-year old columns set (Fig. 1c). Fig. 1a essentially presents a single chromatographic peak (RT = 17.8 min) corresponding to the native form of the methionine-containing peptide M_{native}. Two smaller contributions at M_{native}+15.9949 *m/z* are observed (relative abundance < 1 %) with RRT = 0.93 and RRT = 1 (RRT = relative retention time with the native form taken as reference). The latter species (Fig. 1b) is characterized by the exact same elution time as M_{native} and corresponds to in-source oxidation $M_{ox,ESI}$ occurring during the electrospray process [9]. The second contribution corresponds to $M_{ox,sol}$. As expected, on RP columns, the more hydrophilic side chain of the oxidized methionine makes this modified peptide less retained compared to M_{native}. Similar experiments performed on an old columns set (Fig. 1c) highlight an additional broad chromatographic contribution (RRT = 0.96) lying in between the retention times of $M_{ox,sol}$ and M_{native} and corresponding to an increment of 15.9949 *m/z*. To identify the origin of this contribution, we postulated that the physical location of the oxidation process may differ from both $M_{ox,sol}$ and $M_{ox,ESI}$. First, the retention time lies between RT($M_{ox,sol}$) and RT($M_{ox,ESI}$) and suggests that the oxidation occurs after the injection of the sample and before the ESI process, therefore into the LC system. Second, the chromatographic peak is broader (elution time difference ΔRT = 50 sec) than the peaks associated with $M_{ox,sol}$ and M_{native} (ΔRT = 15-25 sec) which is compatible with a modification arising at the stage of the chromatographic analysis. Altogether, both observations are consistent with an on-column oxidation of methionine residues $M_{ox,LC}$ taking place along the chromatographic separation. To further evidence this phenomenon, we exploited the oxidized synthetic counterpart (EM_{ox}SGSPASGIPVK) of our standard peptide by spiking it with the unoxidized synthetic peptide at a 1:2 (w:w) ratio before the UPLC separation (Fig. 1d). We observed a 25-fold increase in the intensity of $M_{ox,sol}$, while the intensity of the broad chromatographic peak is barely affected by the addition of the oxidized standard. This suggests that, as EM_{ox}SGSPASGIPVK is oxidized before entering the LC system, only little extra methionine oxidation can happen within the LC system. These observations support our initial hypothesis stating that the additional artefactual oxidation of methionine residue is located within the LC system.

3.2. Oxidation of complex samples

Methionine residues present in cells are referred as oxidative stress protectors [24] being easily oxidized in the cells. Specific biological molecules, including catalases and superoxide dismutases protect methionine residues by scavenging the free radicals responsible for oxidation [22]. In plasma, the methionine sulfoxide reductases a and b are reported to inhibit oxidation phenomenon by reducing oxidized methionine back to methionine [18,25–27]. In parallel, previous works have demonstrated that metal-containing proteins such as haemoglobin are well-known protagonists of methionine oxidation [27–30]. In biological samples, oxidative degradation also occurs via reactive oxygen species such as free radicals or peroxides [24,31,32]. In contrast to pure peptide samples, complex matrix environments may therefore present antioxidant properties that can be exploited to potentially prevent oxidation phenomenon during both the sample preparation steps and, to a lesser extent, the chromatographic separation. Such behaviours have to be considered and assessed when evaluating the methionine oxidation level of $M_{ox,sol}$ in more complex samples.

Fig. 2 shows the proportions of M_{native}, $M_{ox,sol}$, $M_{ox,LC}$ and $M_{ox,ESI}$ for the 10 most abundant methionine-containing peptides of a plasma sample (Fig. 2a), an in-house HeLa protein digest (Fig. 2b) and the synthetic methionine-containing peptide EMSGSPASGIPVK as a reference point (Fig. 2c). The retention time profiles of the selected methionine-containing peptides are similar to the synthetic peptide presented in the previous section and display a clear broad chromatographic peak intermediate to RT($M_{ox,sol}$) and RT($M_{ox,ESI}$). We observed similar levels of $M_{ox,sol}$ and $M_{ox,LC}$ in the plasma ($M_{ox,sol}$ = 9.7 ± 5.7 %, $M_{ox,LC}$ = 55.7 ± 16.5 %) and the HeLa ($M_{ox,sol}$ = 12.8% ± 27.1 %, $M_{ox,LC}$ = 57.9 ± 26.61 %) samples. In similar LC conditions, the synthetic peptide has a $M_{ox,LC}$ of 51.4 ± 1.3 % and a low formation of $M_{ox,sol}$ as this peptide is not subjected to digestion. Moreover, the peptides presented in figure 2a and 2b are sorted according to increasing retention time which shows no particular trend in $M_{ox,LC}$ either in the plasma or the HeLa samples. These similar levels of $M_{ox,sol}$ and $M_{ox,LC}$ in the complex samples and for the synthetic peptide unequivocally implies that no potential molecules naturally present in the complex samples inhibits the methionine oxidation phenomenon. We observed that while the so-called protector effect of a complex matrix plays a role *in vivo*, it does not provide oxidation protection *in vitro*.

Comparing the in-house HeLa digest samples with the commercial HeLa protein digest standard (supporting information, figure S2), we observed that levels of $M_{ox,sol}$ and $M_{ox,LC}$ are higher in the former samples than in the later. In particular, a significantly higher level of $M_{ox,LC}$ is observed for the in-house HeLa digest samples which may be attributed to our sample preparation protocol that involves 19 h tryptic digestion. As a matter of fact, the digestion incubation time has been proven to have a significant impact on the methionine oxidation rate [11,20,33]. The commercial HeLa protein digest standard was digested with LysC and trypsin to ensure less than 10 % methionine oxidation in solution according to the manufacturer. Although the digestion time was not mentioned, we expect a shorter digestion time than in our protocol. As for the significant difference of $M_{ox,LC}$, it is not excluded that, to reach a peptide quality with less than 10 % methionine oxidation, some oxidant scavengers are used in the HeLa protein digest formulation. Scavengers which potentially protect methionine residues from important on-column oxidation. Nevertheless, the levels of $M_{ox,LC}$ drops to zero in both complex matrices when the LC separation is performed on a brand-new set of trap and analytical columns (supporting information, figure S3).

3.3. How do the oxidation levels evolve with the age of the columns set?

To evidence the substantial differences on the measured oxidation levels when using either a brand-new set of columns or a 1-year old one, we studied the evolution of M_{native}, $M_{ox,sol}$, $M_{ox,LC}$ and $M_{ox,ESI}$ throughout the age of the columns set by routinely injecting a commercial HeLa protein digest standard during a whole year on the exact same set of columns. Fig. 3 shows the evolution of the different forms of DLTDYLMK peptide with time. Additional trends are provided in supporting information figure S4. We observed that $M_{ox,sol}$ remains constant over a year of routine injection (2.8 ± 0.6 %), while $M_{ox,LC}$ increases progressively from 0 % at day-0 up to 15 % at day-365. After 10 months of injections, $M_{ox,LC}$ reaches a plateau value. These results demonstrate that the use of periodic control samples to assess the quality of the chromatographic separation should also allow the measurement of the oxidation levels in order to operate in optimal conditions. One can therefore systematically inject a QC sample which should include a methionine-containing peptide as an oxidation sensor.

In the following sections, we focus our work on the methionine oxidation process taking place within the chromatographic system and more precisely investigate how the trapping time, the elution time, the sample load and the nature of the RP stationary phase influence the oxidation level during a standard chromatographic analysis.

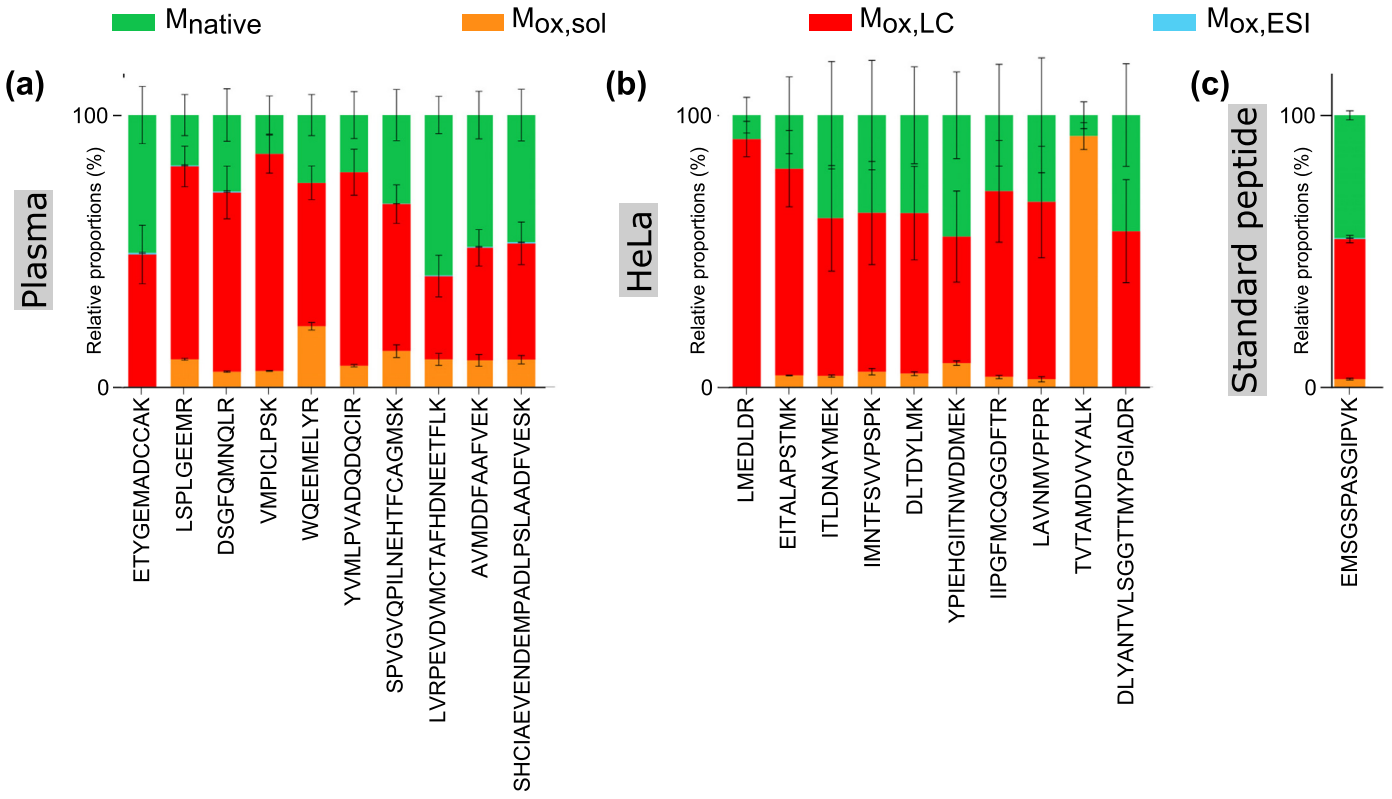

Fig. 2. Relative proportions of M_{native}, $M_{ox,sol}$, $M_{ox,LC}$ and $M_{ox,ESI}$ for the 10 most intense methionine-containing peptides identified in (a) plasma samples, (b) in-house HeLa protein digest and (c) the synthetic methionine-containing peptide EMSGSPASGIPVK following a 180 min-long UPLC method performed on a 1 year-old columns set with the HSS T3 phase. Contribution from $M_{ox,ESI}$ is minor (< 1 %). Peptides are sorted according to increasing retention time and error bars are estimated based on technical triplicates. Methionine-containing peptides issued from complex matrix are significantly oxidized along the chromatographic separation process (red contribution).

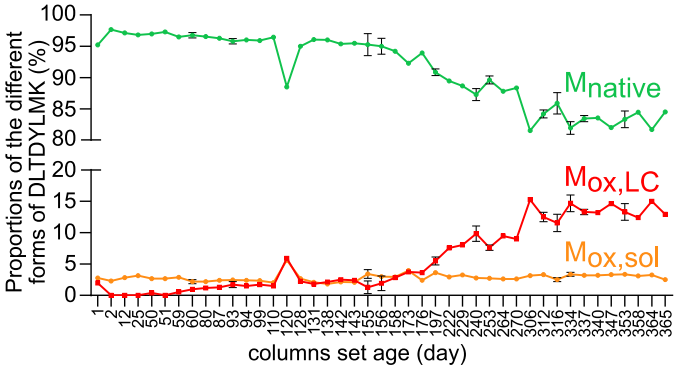

Fig. 3. Evolution of the percentage of M_{native}, $M_{ox,sol}$ and $M_{ox,LC}$ of a HeLa protein digest peptide (DLTDYLMK) over a 1-year UPLC use. Brand-new trap and analytical columns set (HSS T3 phase) were fitted on an ACQUITY UPLC M-Class system routinely used for proteomics analysis on day-0 and were removed on day-365. Error bars are given when several HeLa were injected the same day. $M_{ox,ESI}$ was not detected for this peptide. $M_{ox,sol}$ remains constant over the year, while $M_{ox,LC}$ progressively increases with the age of the column.

3.4. Where does the oxidation take place within the chromatographic system?

Once injected to the chromatographic system, a sample successively flows along a sampling needle, an injection port, a sample loop, a trap column and an analytical column before reaching the ESI stage, each part being coupled together by various capillary connections. All these components may potentially contribute to oxidation during the chromatographic analysis of a sample. Capillary connections are made of polyetheretherketone (PEEK), an inert material known to not react with biological samples, therefore ruling out these parts as significant oxidation places. The needle, the injection port and the sample loop are all made of stainless steel, a material that has been proposed as a source of oxidation for methionine-containing peptides in the LC system [16]. Only one single injection per vial was performed with a strong needle/injection port wash (ACN with FA 0.1%) between each injection to avoid the introduction of iron into the LC system. Although the trap column has been previously suggested as a potential place for methionine oxidation [12], little is known on how the residence times in both the trap and the analytical columns influence the methionine oxidation level $M_{ox,LC}$ during the chromatographic separation.

Fig. 4a and 4b respectively highlight the impact of the trapping time and the gradient length on the relative proportions of M_{native}, $M_{ox,sol}$ and $M_{ox,LC}$ measured on the HSS T3 1-year old columns set. Data are presented for the synthetic methionine-containing peptide (EMSGSPASGIPVK), together with two arbitrarily selected methionine-containing peptides from plasma and in-house HeLa protein digests. Additional profiles are reported in supporting information figure S5. The trapping time was varied from 3 minutes to 15 minutes with a constant 57-min long UPLC analytical run (Fig. 4a). We observed that the different oxidation levels of all the methionine-containing peptides are barely affected by an increase in the trapping time, with $M_{ox,LC}$ of about 7 ± 2 %, 19 ± 8 % and 12 ± 1 % for respectively the plasma peptide, the HeLa peptide and the standard peptide. The gradient length was varied from 30 min to 150 min with a fixed trapping time of 3 min (Fig. 4b). Longer UPLC gradients significantly affect the on-column oxidation level with a 5-fold increase in $M_{ox,LC}$ for the plasma peptide (43 ± 8 %), a 3 fold-increase for the HeLa peptide (59 ± 17 %) and up to 4-fold for the standard peptide (51 ± 1 %). Although there is a clear

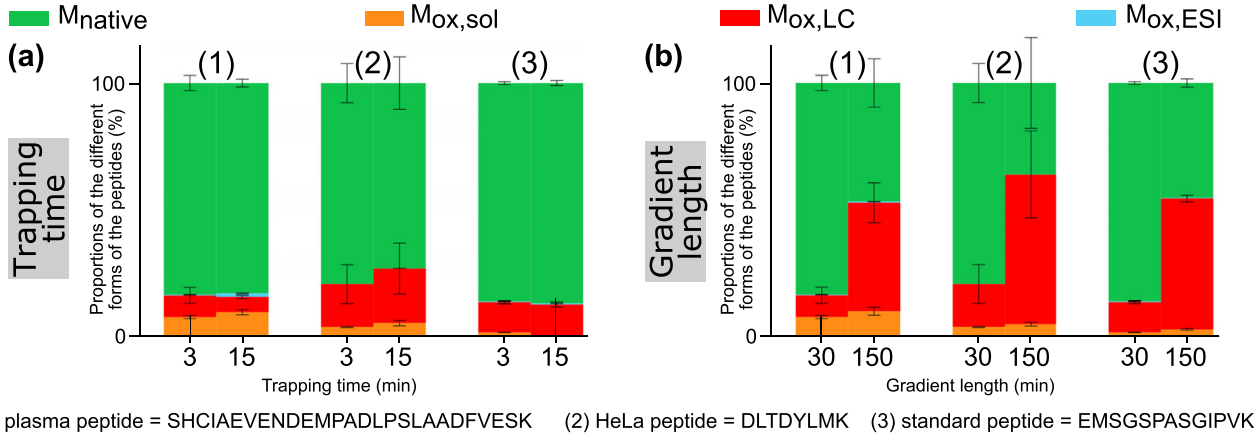

Fig. 4. Effect of (a) the trapping time (3 or 15 min on a 30 min-long UPLC gradient) and (b) the gradient length (30 or 150 min with a 3 min trapping time) on the relative proportions of M_{native}, $M_{ox,sol}$ and $M_{ox,LC}$ associated with (1) a plasma peptide (SHCIAEVENDEMPADLPSLAADFVESK), (2) an in-house HeLa protein digest peptide (DLTDYLMK), and (3) the synthetic methionine-containing peptide (EMSGSPASGIPVK). The chromatographic analysis was performed on a HSS T3 1-year old columns set. An increase in the trapping time does not induce significant changes in M_{native}, $M_{ox,sol}$ and $M_{ox,LC}$ proportions, while an increase in the gradient length drastically enhanced the on-column oxidation $M_{ox,LC}$.

tendency to favour the on-column oxidation with longer UPLC gradient, we observe no significant correlation between the retention time *RT* and $M_{ox,LC}$ (see additional profiles sorted by RT in the supporting information figure S5). An identical procedure was applied to a brand-new set of trap and analytical columns for sake of comparison. The results are reported in supporting information figure S6. In contrast to aging columns, we observed that neither the trapping time nor the UPLC gradient length affect $M_{ox,sol}$ and $M_{ox,LC}$.

Altogether, these results highlight that the trap column is not the place of methionine oxidation, while significant oxidation of methionine-containing peptides is observed on the 1-year old analytical column. Long UPLC gradients enhance the methionine oxidation level whereas short UPLC gradients minimize $M_{ox,LC}$. Such observations may be impeded to the nature of the chemical function used for the stationary phase of the column. The bonding technology of the column is called High Strength Silica (HSS) T3 and is derived from C18 ligands and end-capping. Although the exact surface structure of the column has not been released by the manufacturer, we may consider that the methionine residues react with free oxidant sites within the column and that the availability of these sites increases with the column aging. Additionally, free silanols, present in all silica columns, are the place of hydrogen-bonding interactions as well as ion-exchange interactions [34] that may play a role in methionine oxidation inside the analytical column. In the following section, we compare the HSS T3 stationary phase with other available technologies in order to better understand the origin of the on-column oxidation process.

3.5. Does the on-column oxidation occur on other RP columns?

Two additional RP columns, relying on different technologies, were tested to evaluate the exclusiveness of the artefactual oxidation of methionine to our 1-year old HSS T3 C18 column and UPLC systems. Similarly to the previous experiments performed on a 1-year old HSS T3 C18 column, we injected a plasma sample in triplicates on a 1-year old YMC Triart C18 column with a 180 min-long method. Alongside this analysis, we considered another dataset accessible on the Proteomics Identification Database (dataset PXD006882 from PRIDE archive https://www.ebi.ac.uk/pride/archive/). It consists of skeletal muscle biopsy samples injected on a BEH130 C18 column following a 175-min long method, the age of the analytical column is not reported. These data are biological replicates from three different donors and not technical replicates as the others (for more details, see [35]). Fig. 5 displays the proportions of M_{native}, $M_{ox,sol}$, $M_{ox,LC}$ and $M_{ox,ESI}$ for the 10 most abundant methionine-containing peptides of the samples injected on a HSS T3 C18 column (figure 5a), a YMC Triart C18 column (figure 5b) and a BEH130 C18 column (figure 5c). We observe similar broad chromatographic peaks corresponding to $M_{ox,LC}$ for peptides separated on a HSS T3 C18 column ($M_{ox,LC}$ = 55.7 ± 16.5 %) and a BEH130 C18 column ($M_{ox,LC}$ = 18.1 ± 11.4 %), whereas no broad peak is identified on the YMC Triart column ($M_{ox,LC}$ = 0.0 ± 0.0 %). Although the age of the BEH130 C18 column is unknown, we expect that the column is not brand new in light of the $M_{ox,LC}$ levels. The three RP columns are based on trifunctionally C18 alkyl phase bonding and end-capping, the T3 bonding of the HSS T3 column being an enhancement of the trifunctional bonding characterized by a lower carbon coverage than a normal C18 trifunctional column. They however differentiate by their particle technology: ethylene bridged hybrid for the BEH column, high strength silica for the HSS T3 and silica-organic hybrid stationary phase for the YMC Triart. This similarity of bonding phase for the latter two may explain their analogous chromatographic profiles pattern. Furthermore, the YMC Triart columns are part of a so-called biocompatible system in which all the components that may come into contact with the samples or the solvents are made of PEEK. This is different from the two other columns where, as described before for the HSS T3, samples and solvent are in contact with some stainless-steel parts. Even if the age and wear is unknown for the BEH column, the chromatographic profile anomaly is definitely present. This phenomenon observed in two different laboratories using two distinct instrumentation setups could therefore be witnessed on other setups.

3.6. Does the oxidation depend on the sample load?

To assess the impact of the sample load on $M_{ox,LC}$, four different quantity of commercial HeLa protein digests were injected in technical triplicate on the 1-year old columns set: 0.10 µg/9 µL, 0.25 µg/9 µL, 0.50 µg/9 µL and 1.00 µg/9 µL. Fig. 6 shows the evolution of $M_{ox,LC}$ for a selected methionine-containing peptide (see supporting information figure S7 for more peptides). We observed that $M_{ox,LC}$ decreases as the injected protein load increases. Consequently, as $M_{ox,sol}$ stays constant, the evolution of M_{native} increases as the injected protein load increases. The data are fitted with a one-phase exponential decay model exhibiting a R^2 of 0.9746 and 0.9752 for $M_{ox,LC}$ and M_{native} respectively. Based on these results,

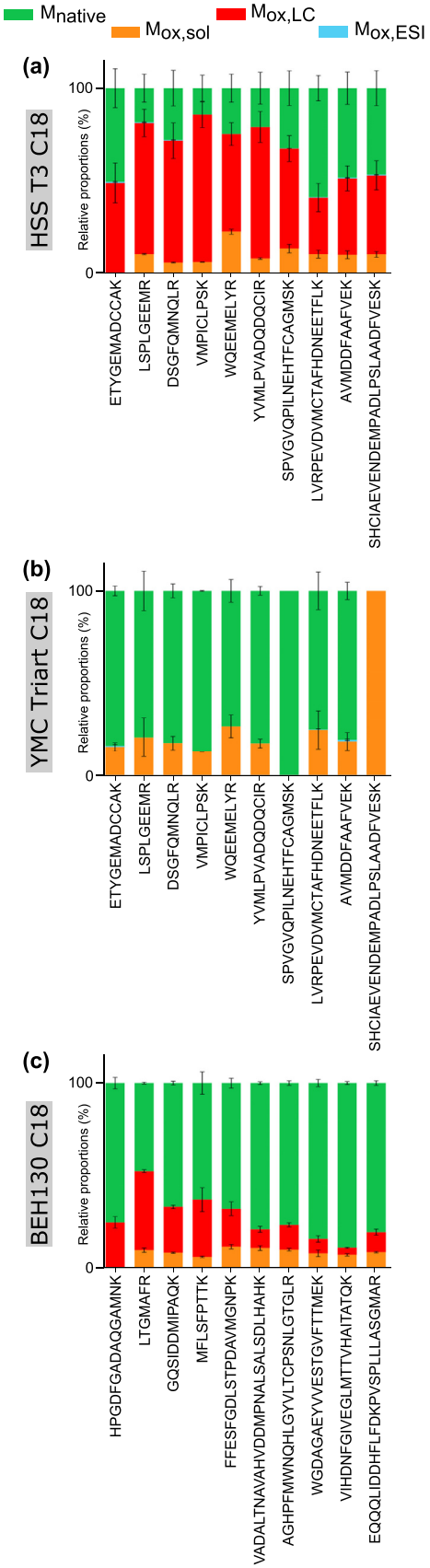

Fig. 5. Relative proportions of M_{native}, $M_{ox,sol}$, $M_{ox,LC}$ and $M_{ox,ESI}$ for the 10 most intense methionine-containing peptides identified in complex matrices using three different RP columns: (a) plasma sample injected on a HSS T3 C18 column following a 180 min-long method, (b) the same plasma sample injected on a YMC Triart C18 column following a 180 min-long method, and (c) skeletal muscle biopsy samples injected on a BEH130 C18 following a 175 min-long method. Peptides are sorted according to increasing retention time and error bars are estimated based on technical triplicates for (a) and (b) and biological triplicates for (c).

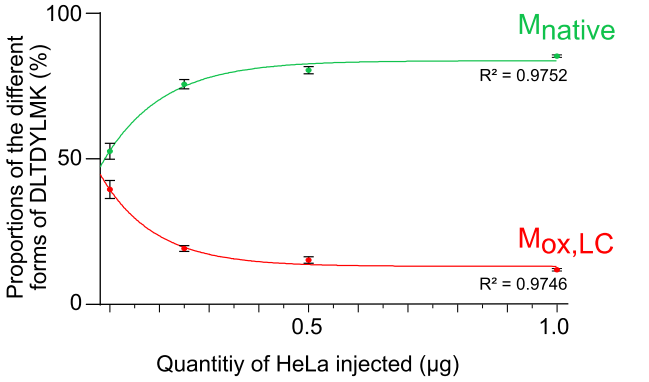

Fig. 6. Evolution of $M_{ox,LC}$ and M_{native} for DLTDYLMK peptide as function of the quantity of HeLa protein digest injected in the LC system. Four different quantities 0.10 µg, 0.25 µg, 0.50 µg and 1.00 µg were injected in triplicates on a 1-year old columns set using a 180 min UPLC method (3 min of trapping time and gradient length of 150 min). The data points are fitted by a one-phase exponential decay model with a R^2 of 0.9746 and 0.9752 for $M_{ox,LC}$ and M_{native} respectively. The oxidation level reaches in the analytical column increases as the sample load decreases evidencing the presence of limited available oxidation sites within the analytical column.

we hypothesized that there is a limited number of available oxidation sites embodied in the stationary phase of the analytical column and that may be related to the increase in free silanols availability with the lifetime of the column. As such, a low number of methionine-containing peptides, associated with low sample load, results in a complete oxidation of these peptides as there are enough oxidation sites available within the analytical column to oxidize the entire population of these peptides, subsequently leading to higher $M_{ox,LC}$. On the contrary, a high number of methionine residues, associated with high sample load, cannot be entirely oxidized as the oxidation sites within the analytical column are saturated. This leads to a high percentage of methionine residues in their native form and, consequently, to the decrease of the proportion of oxidized residues.

4. Conclusions

In this work, we focused our research on the identification and the understanding of the parameters that influence the artefactual oxidation of methionine residues during the analytical separation of biomolecules and complex samples on reversed-phase chromatographic systems. We observed that, in addition to solution and electrospray oxidations, methionine-containing peptides can be significantly oxidized inside the analytical column, along the chromatographic separation. Such phenomenon is identified based on the retention time profile of the analytes: methionine-oxidized peptides are systematically characterized by a broad chromatographic peak having an intermediate retention time between methionine-containing peptide oxidized in solution and their native counterpart. Similar observation was noticed on another setup from another laboratory based on publicly available data. With our instrumental setup, we observed a progressive increase in on-column oxidation proportion with the age of the columns set, the gradient length and a reduced sample load. With regards to the bonding technology of the column (HSS T3), the observations were rationalized by the presence of a limited number of oxidation sites within the column stationary phase, being more and more exposed to the mobile phase and to the sample over time. Residual metal ions were pointed out as a source of variability in methionine oxidation measurement. Traces of metal ions could indeed potentially catalyse the oxidation of methionine residues in the chromatography. However, the fact that discrepancies are observed between old and new columns undermined this assumption. The difference

between both configurations exclusively lies in the age of the trap and the analytical columns, while the buffers and the injection system were kept identical. Nevertheless, an accumulation of metal ions in the analytical column is not excluded and could damage the stationary phase by creating available oxidant sites in the column. The complete on-column oxidation suppression observed on the YMC Triart C18 column, being part of a biocompatible system, reinforces the metal ions accumulation hypothesis and suggests that the nature of the stationary phase may not be the issue. In the future, this assumption will be evaluated by using HSS T3 and BEH130 columns on biocompatible LC system over a year of routine injections.

Altogether, this work highlights the necessity to implement a procedure to follow the liquid chromatography instrumental setup status which induces undesired artefactual oxidation both in routine analysis and in general proteomics studies. At this stage, general guidelines can be drawn:

(1) A periodic monitoring of the on-column oxidation level based on standard methionine-containing peptides should help to diagnose LC system failures or an early disruption of the analytical column.

(2) Fast digestion workflows and short gradient lengths should be favoured in order to reduce undesired methionine oxidation in solution and during the LC separation respectively.

(3) For targeted therapeutic proteins studies, labelled internal standard will allow to correct for LC induced bias.

These guidelines are of particular interests for quantification studies in which database searches expect single chromatographic peak for oxidized methionine peptides. As demonstrated, several peaks are associated with a given oxidized analyte, such multiple chromatographic signatures can possibly lead to erroneous assignment and quantification.

Finally, complementary studies dedicated to the stabilization of the oxidation during the chromatographic separation will help to prevent and to further understand the origin of on-column methionine oxidation. Indeed, with the emergence of downscaling proteomics, analysis of peptide modifications when low quantity is injected need to be rigorously monitored and controlled. Other experiments will be conducted to draw conclusions with regards to the bonding technology of the stationary phase and the structure of the surface of the RP columns.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

France Baumans: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Emeline Hanozin:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Dominique Baiwir:** Conceptualization, Methodology, Writing – review & editing. **Corentin Decroo:** Investigation. **Ruddy Wattiez:** Conceptualization, Writing – review & editing. **Edwin De Pauw:** Conceptualization, Writing – review & editing. **Gauthier Eppe:** Resources, Project administration. **Gabriel Mazzucchelli:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgements

The authors thank the Belgian Red Cross for providing the plasma sample. They also acknowledge Kaneka Eurogentec S.A. for synthesizing the reference methionine-containing peptide. Thank you to Baptiste Leroy from the Proteomics and Microbiology Laboratory (University of Mons) for the plasma samples dataset acquired on the Ekspert nanoLC 425 coupled to the TripleTOF 6600 System. This study was supported by the European Regional Development Fund (grant number 2.1.2/988).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462449.

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