Research Article

Methionine oxidation of proteins analyzed by affinity capillary electrophoresis in presence of silver(I) and gold(III) ions

Oxidative damage of biopharmaceuticals during manufacturing and storage is a key concern throughout pharmaceutical development. However, few simple and robust analytical methods are available for the determination of oxidation sites. Here, the potential of affinity capillary electrophoresis (ACE) in the separation of proteins with oxidized methionine (Met) residues is shown. Silver(I) and gold(III) ions have the attribute to selectively form complexes with thiethers over sulfoxides. The addition of these ions to the BGE leads to a selective complexation of Met residues and, thus, to a change of charge allowing separation of species according to the different oxidation states of Met. The mechanisms of these interactions are discussed and binding constants for peptides containing Met with silver(I) are calculated. Additionally, the proposed method can be used as an indicator of oxidative stress in large proteins. The presented technique is easily accessible, economical, and has rapid analysis times, adding new approaches to the analytical toolbox of Met sulfoxide detection.

Keywords:
Affinity capillary electrophoresis / Gold / Methionine sulfoxide / Monoclonal antibody / Silver

Additional supporting information may be found online in the Supporting Information section at the end of the article.

1 Introduction

Damage to proteins caused by oxidation is a commonly observed problem in biopharmaceutical development, mainly affecting methionine (Met), cysteine (Cys), histidine (His), and tryptophan [1, 2]. Unlike the side chains of the branched chain amino acids, the Met side chain is rather flexible but possesses a similar hydrophobicity [3, 4]. This makes Met unique among the proteinogenic amino acids, allowing it to adapt its shape to nonpolar structures. Met oxidation caused by reactive oxygen species leads to the formation of the polar methionine sulfoxide (MetO). In vivo, this modification is altering protein structures and accelerating age-dependent diseases [5, 6]. For proteins stored in vitro, oxidation of Met residues to MetO causes loss of potency and aggregation [7–10]. Monoclonal antibodies (mAbs), which are of special interest to the pharmaceutical industry, tend to have altered pharmacokinetic properties, due to Met oxidation [11]. The effects caused by MetO are regarded as critical quality attributes. Hence, special techniques for the detection of this posttranslational modification are required.

As an antioxidant defense, cells use methionine sulfoxide reductases, which catalyze the reduction of MetO using thioredoxin. For the monitoring of MetO in vivo, recombinant methionine sulfoxide reductases labeled with a fluorescent marker can be used [12]. For chromatographic detection, the chemical attributes of MetO can be exploited: It is more polar, bulkier, and less flexible than Met [13–15]. This change of attributes may lead to a small increase in polarity if Met is located on the exterior of the protein. However, the polarity difference is typically too small for a direct separation with HPLC. Only if the oxidation takes place at a critical Met residue and, thus, leads to a conformational change, does the resulting species have a completely changed hydrophobicity. Oxidation variants can then be separated in RP-HPLC or
hydrophobic interaction chromatography [16, 17]. In the case of a reduced affinity of a protein to its target caused by critical residue Met oxidation, an affinity chromatography can be used to separate a protein [11]. Nevertheless, these approaches can just work for certain classes of proteins and are not applicable in a generic way. For an overview of all Met residues typically a proteolytic digest is performed. In the resulting peptides, the increased polarity of an oxidized residue is of a greater significance than in a protein, allowing separation with RP-HPLC. Coupling of LC with MS was already introduced in the early 90’s and helps to identify all oxidized Met in a protein [18]. With the development of high throughput proteomics, the identification of MetO is now possible for whole cell extracts [19]. However, this procedure is relatively time consuming and complex.

Changes in polarity by Met oxidation do not typically have a great impact on the electrophoretic mobility in CE. However, Nashabeh and colleagues [20] successfully separated Met oxidation variants of the 70 amino acid long insulin-like growth factor 1 (IGF-I). In a polyacrylamide-coated capillary with an acidic buffer composed of β-alanine, citric acid, zwitterionic N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (DAPS) and acetonitrile, hydrophobic interactions between DAPS and the protein separated the Met and MetO variants of IGF-I. In this study, acetonitrile inhibited micelle formation in the BGE leading to a RP-HPLC-like separation mode.

The addition of metal ions to the BGE has been shown in a wide range of studies to be an appropriate method for studying interactions of biomolecules including DNA and proteins [21–24]. Here, we have performed a feasibility study on identification and quantification of Met oxidation variants by using ACE with metal ions as complexing reagent for nonoxidized Met. In our study, we choose to focus on silver and gold salts. As soft acids, silver and gold tend to strongly bind to soft bases such as unreduced sulfur. This interaction is known to be several orders of magnitude higher than to nitrogen and oxygen containing compounds [25]. Since the 1940s, thiol concentrations in amino acid and protein containing solutions were determined via amperometric titration in the presence of silver compounds [26–28]. Gold interactions with Met were mostly studied in the context of developing new (anticancer) drugs [29]. To best of our knowledge, the usage of both ions for the detection and measurement of Met oxidation by ACE has not yet been reported. We describe the reaction mechanisms between peptides containing Met and Ag/Au ions that are proposed to take place during electrophoretic separation. Binding constants between these peptides and Ag are calculated and the utility of the method for an estimation of protein oxidation is demonstrated.

2 Theory of ACE

ACE is a highly efficient tool for binding studies. For the complex formation [PM] between a peptide analyte P with a metal ion M, a simple 1:1 equilibrium (Eq. 1) and the corresponding mass action law can be formulated (Eq. 2):

\[ P + M \xrightleftharpoons[\kff]{\ksw} PM, \]

\[ K_0 = \frac{1}{K_D} = \frac{k_{sw}}{k_{ff}} = \frac{[PM]}{[P][M]}, \]

where \( k_{sw}, k_{ff} \): rate interaction constants of the complex formation and dissociation, respectively. \( K_0 \): binding constant of the complex; \( K_D \): dissociation constant of the complex; and \([PM], [P], [M]\): equilibrium concentrations of PM, P, and M.

Most systems with weak to moderate interactions show fast kinetic reactions. In CZE, a stable equilibrium between analyte and ligand can be reached by adding the ligand to the BGE. If the ligand changes the charge of the analyte, as can be expected for a metal ion, the analyte migration time will be shifted according to the concentration of the ligand in BGE.

The proportion of the complex in the total analyte population is given by its mole fraction \( \chi_{PM} \) (Eq. 3):

\[ \chi_{PM} = \frac{[PM]}{[P] + [PM]} \]

The observed mobility of the analyte \( \mu \) is a result of the mobility of the complex \( \mu_{PM} \) and the mobility of the free analyte \( \mu_P \) and their mole fraction in equilibrium [30] (Eq. 4):

\[ \mu = (1 - \chi_{PM}) \mu_P + \chi_{PM} \mu_{PM}. \]

Insertion of Eqs. (2) and (3) in Eq. (4) yields Eq. (5):

\[ \mu = \frac{\mu_P + K_D[M] \mu_{PM}}{1 + K_D[M]}. \]

Since \( \mu_P \) and \([M] \) are known, while \( \mu_{PM} \) can be determined by the saturation plateau of the curve, the resulting binding curve can be used to estimate \( K_D \) via nonlinear regression. For a more sophisticated stepwise 2:1 metal to peptide stoichiometry, the following equilibrium can be formulated (Eq. 6):

\[ P + _2^+^+ M \xrightarrow{\kff_1} PM + _2^+^+ M, \]

\[ P + _2^+^+ M \xrightarrow{\kff_2} PM_2, \]

In this case, Eq. (5) is extended to Eq. (7):

\[ \mu = \frac{\mu_P + K_{B1} [M] \mu_{PM} + K_{B1} K_{B2} [M]^2 \mu_{PM_2}}{1 + K_{B1} [M] + K_{B1} K_{B2} [M]^2}, \]

where \( \mu_{PM_2} \): mobility of the double bound complex; \( K_{B1} \): binding constant of the single bound complex; and \( K_{B2} \): binding constant of the double bound complex.

In order to estimate the electrophoretic mobility from raw data, one can use Eq. (8):

\[ \mu = \frac{L_t L_d}{U} \left( \frac{1}{t_A} - \frac{1}{t_{EOF}} \right), \]

where \( L_t \): total capillary length; \( L_d \): length to detector; \( U \): System voltage; \( t_A \), \( t_{EOF} \): migration time of analyte and EOF, respectively.
3 Materials and methods

3.1 Reagents

NaOH (Cat. no. 28 244.295) and orthophosphoric acid (Cat. no. 20 626.292) were from VWR Chemicals (Radnor, USA). HCl (Cat. no. 387 800 010) was purchased from Acros organics (Geel, Belgium) and thiourea/HCl cleaning solution (Cat. no. 51 350 102) from Mettler Toledo (Columbus, USA). The following chemicals were from Sigma-Aldrich/Merck KGaA (Darmstadt, Germany): AgF (Cat. no. 226 858), AuCl₃ (Cat. no. 379 948), catalase from bovine liver (Cat. no. C9322), CuCl₂ (Cat. no. 203 149), e-aminocaproic acid (EACA) (Cat. no. A2504), hydrogen peroxide (Cat. no. 16 911), histidine (Cat. no. 53 319), hydroxypropyl methylcellulose (HPMC) (Cat. no. H7509), NiCl₂ (Cat. no. 451 193), PdCl₂ (Cat. no. 205 885), PtCl₃ (Cat. no. 79 840), sodium phosphate dibasic (Cat. no. S0876), and triethylenetetramine (TETA) (Cat. no. 90 460). Water of HPLC grade was prepared in a Milli-Q-Station (Merck Millipore/Merck KGaA). Solutions were filtered through 0.2 μm membrane filters (Corning, New York, USA).

Peptide variants with the base sequence YAMAAMKA were synthesized by JPT Peptide Technologies (Berlin, Germany). This nonoxidized base sequence is referred to as MOMO. Single oxidized species YAM(ox)AAMKA and YAMAAM(ox)KA are called M-(3)-O and M-(6)-O, respectively. When referring to these metals to the BGE in CE analysis would, thus, only be detected if the peptide was charged and not interact with the BGE/metal ions had to be found. Solutions were filtered through 0.2 μm membrane filters (Corning, New York, USA).

Peptide variants with the base sequence YAMAAMKA were synthesized by JPT Peptide Technologies (Berlin, Germany). This nonoxidized base sequence is referred to as MOMO. Single oxidized species YAM(ox)AAMKA and YAMAAM(ox)KA are called M-(3)-O (Met3 oxidized) and M-(6)-O (Met6 oxidized), respectively. When referring to M-(3)-O and M-(6)-O, the abbreviation MXO is used. The C- and N-terminus are unmodified. Isoelectric point of all peptide species: 8.5. mAbs include M-1 and MOMO were mixed each at 1 mM segments were used to avoid metal complex adhesion.

3.2 Sample/BGE preparation

For peptide analysis, metals were diluted in BGE (50 mM Na₂HPO₄, adjusted to pH 3 ± 0.05 with H₂PO₄, or HCl using a SevenExcellence pH/Ion meter S500 (Mettler Toledo) from 2 to 2000 μM, with a 1:2 dilution row (1 + 1 part). The samples were a mix of 1 mM peptide(s) and 0.5 mM His, used as a marker, in water. Regarding mAb experiments, AgF was diluted at 500 μM in mAb BGE (400 mM EACA, 2 mM TETA, 0.05% HPMC pH 5.7 ± 0.05). Preparation of mAb BGE is performed at 60 psi. Fresh capillaries were primed for separation buffer conditions by five runs with a peptide or mAb sample. After ACE separations, silver-coated electrodes were cleaned by putting them in the thiourea cleaning solution for 1 h. Gold oxide coating on electrodes was removed with 1 M NaOH overnight. Capillaries were used for max. 20 injections to avoid metal complex adhesion.

3.3 Apparatus

All experiments were carried out on a SCIEX PA800 plus System (Brea, USA) which was equipped with an UV detector, a 214 nm filter (Cat. no. 144 437; SCIEX), a temperature controlled auto sampler (±2°C), and a 30 kV power supply. Fused silica capillaries from Molex (Lisle, USA) with I.D. of 50 μm, 20 cm length from the inlet to the detection window, and a total length of 30 cm were used at 20°C. Samples were stored in the autosampler at 10°C and injected at 0.5 psi for 10 s. Polarity was positive (capillary inlet) to negative (capillary outlet) with separation voltage set at +20 kV. The currents observed under the described conditions were at around 60 μA (peptide BGE) or 25 μA (mAb BGE). Instrument control, data acquisition, and data evaluation were performed with 32 Karat 10.1 software (SCIEX). Origin software (2019 version, OriginLab Corporation, Northampton, USA) was used for data evaluation.

Before each sample injection, capillaries were flushed with 0.1 M HCl for 1 min and equilibrated for 1 min with separation buffer (peptide BGE/mAb BGE). All rinsing steps were performed at 60 psi. Fresh capillaries were primed for separation buffer conditions by five runs with a peptide or mAb sample. After ACE separations, silver-coated electrodes were cleaned by putting them in the thiourea cleaning solution for 1 h. Gold oxide coating on electrodes was removed with 1 M NaOH overnight. Capillaries were used for max. 20 injections to avoid metal complex adhesion.

4 Results and discussion

The experiments conducted were based on the hypothesis that some metal ions selectively bind Met over MetO. Adding these metals to the BGE in CE analysis would, thus, only change migration of Met containing samples whereas oxidized samples would not be affected. Literature research has brought the elements of group 10 (Ni; Pd; Pt) and 11 (Cu; Ag; Au) of the periodic table to our attention in this regard [33]. Experiments were conducted using a short peptide with the sequence YAMAAMKA. This peptide was used in the four possible Met oxidation variants (2M, M-(3)-O, M-(6)-O, MOMO, see Section 3 for details). As a marker, for detection of a possible mobility shift, a molecule that would ideally be uncharged and not interact with the BGE/metal ions had to be found. However, a BGE with pH 3 leads to a very low EOF, and uncharged molecules would not be detected in the migration timeframe of the peptides. Thus, His as a charged molecule was chosen, because it maintains its mobility at a broad range of metal concentrations (up to 4 mM tested) despite increasing ionic strength in the BGE. For practical calculations with Eq. (8), the detection peak of His was used and not the migration time of the EOF. The mobility is referred to as the relative mobility (rel. μ)

4.1 Mechanism of metal complexation of methionine

For an initial screening of the effects of metal ions on mobility of peptides, 2 M and MOMO were mixed each at 1 mM...
and separation in 50 mM phosphate buffer at pH 3 was carried out as a standard without observing a clear separation of the species. In a next step, the metal ions were added at concentrations of 0.25–2 mM to the BGE. Counter ions for the metals, were chosen on the basis of solubility in water. A significant alteration of mobility could be obtained for AuCl₃ and AgF, but not for NiCl₂, PdCl₂, PtCl₄, and CuCl₂ (not shown). Addition of AgF to the BGE leads to a clear shift of 2 M, M-(3)-O, and M-(6)-O, which appears to be stabilized at concentrations ≥ 250 μM (see Fig. 1A). Under these conditions, a separation of the oxidized, single oxidized and nonoxidized species can be achieved. This indicates that the binding of Ag(I) ions to 2 M, M-(3)-O, and M-(6)-O, strongly relies on the thioether groups since this is the only difference between the peptides.

Ag(I) has a strong affinity for thioether groups and is reported to form a wide range of coordination geometries, most of them ranging from linear twofold coordinate to tetrahedral complexes [34]. Sigel et al. conducted studies on dimethyl sulfide and dimethyl disulfide with several metal species including Ag(I) ions [35]. In that study, the equilibrium constants of the soft Ag(I) ion were reported to be 10⁵ times larger for thioethers than for hard ions like Ca(II). Luehrs and colleagues found, that dimethyl sulfoxide does not form any complexes with Ag(I) in aqueous solutions, as hydrogen bonding is dominant [36].

Experiments with Met have shown that silver forms [Ag-Met] complexes below pH 4.5, that solely coordinate through the thioether group [37]. At higher pH, a [Ag₂-Met]₂ complex is detected that coordinates also through the amino group, which was also confirmed in Met-containing peptides [38]. This suggests the binding of two Ag⁺ ions to 2 M and one Ag⁺ ion to M-(3)-O and M-(6)-O at pH 3 according to Eqs. (9) and (10).

\[
MXO + Ag^+ \xrightarrow{k_B \over k_D} (Ag (MXO))^+, \tag{9}
\]

\[
2M \xrightarrow{2k_B \over 2k_D} (Ag (2M))^+ \xrightarrow{2k_B \over 2k_D} (Ag_2 (2M))^{2+}. \tag{10}
\]

To prove this theory, we conducted mobility shift studies using AgF concentrations from 2 μM to 2000 μM in order to estimate the binding constants in the capillary. For single oxidized peptides, Eq. (5) can be reformulated (Eq. 11):

\[
\mu = \frac{\mu_{MXO} + K_B \left[Ag^+\right] \mu_{[Ag(MXO)]}}{1 + K_B \left[Ag^+\right]}. \tag{11}
\]

Fitting mobility plots with Eq. (11) results in accurate fits for M-(3)-O and M-(6)-O, thus, confirming the [Ag(MXO)] complex (Fig. 2). Formation constants for [Ag(MXO)] are approximately log \( K_B \approx 3.6 \) (Fig. 2, Inset table). Measurements conducted with potentiometric titration on pure Met and Met-containing dipeptides, have found a very similar value of log \( K_B \approx 3.2 \) [37, 39]. Assuming a second-order equilibrium for 2 M, we have estimated that \( K_B \) is an average of \( K_B(M-(3)-O) \) and \( K_B(M-(6)-O) \) and that the single bound species [Ag(2M)] has the same migration time as the average of M-(3)-O and M-(6)-O.

\[
\mu_{2M} + K_B \left[Ag^+\right] \mu_{[Ag(MXO)]} + K_B \left[Ag^+\right]^2 \mu_{[Ag_2(MXO)]} = \frac{\mu_{2M} + K_B \left[Ag^+\right] \mu_{[Ag(MXO)]} + K_B \left[Ag^+\right]^2 \mu_{[Ag_2(MXO)]}}{1 + K_B \left[Ag^+\right] + K_B \left[Ag^+\right]^2}. \tag{12}
\]
ion is formed, which further hydrolyses to MetO [42]. The resulting [Au(I)–Met] complex (2) can dissociate to form free [AuCl₄⁻] or create a linear and polynuclear [Au(I)–(Met)₂], complex (3). [AuCl₂⁻] is not stable in solution at room temperature but its elimination to solid Au(0) and [AuCl₄⁻] is reported to proceed slowly [44].

According to the position and number of free Met residues, the peptides investigated in this study show different migration changes in CZE when AuCl₃ is added to the BGE. The peptide containing two Met, 2 M, shows the most significant changes. In the context of an oxidation induced by [AuCl₄⁻], it is clear that there is a signal that corresponds to the oxidized MOMO peptide that was formed out of 2 M. MALDI measurements of an equimolar mixture of AuCl₃ and 2 M peptide further confirmed that oxidation of 2 M to single oxidized and double oxidized species is induced by Au(III) [data not shown]. [Au(III)–Met] is reported to be only detectable in traces by NMR analysis after 3 min of reaction, while [Au(I)–(Met)₂]ₙ is stable for hours. This suggests that there is no direct detection of the [Au(III)–(2M)] complex taking place, but that the newly formed peak for 2 M is an [Au(I)–(2M)] species. The mobility of the newly formed peak also highly corresponds to the [Ag(2M)] complex (see Fig. 1), further sustaining this assumption. With low concentrations of AuCl₃ (2–16 μM) in the BGE, the 2 M peptide peak starts to show a small delay, without detection of the newly formed species (Fig. 1C). This indicates the complexation of 2M with Au(III), since this complex is not charged and is formed peaks first rise in intensity until approx. 500 μM AuCl₃, then intensity starts decreasing again (Fig. 1C). Adjusting the pH of the BGE. The peptide containing two Met, 2 M, shows the very similar mobility of [Au(I)(2M)] and [(Ag)₂(2M)] and the closely related chemical properties of Au(I) and Ag(I) and the very similar mobility of [Au(I)(2M)] and [(Ag)₂(2M)] observed in our data (Fig. 1), suggests that two Au(I) ions bind to one 2 M peptide under separation conditions. This difference to literature might occur due to the special conditions within separation in comparison to NMR or as a consequence
of sterical problems resulting from the size of an 8-mer peptide.

4.2 Silver for the detection of oxidative stress in proteins

In a next step, the usability of the approach in a more sophisticated system was evaluated. For this, two mAbs were tested in a modified EACA buffer system with pH 5.7. This BGE has been demonstrated to have a broad applicability for biopharmaceuticals including ACE studies [31, 32, 46]. When it comes to the adaptation of the method to proteins, some aspects need to be considered. First, with a rising pH uncharged amino groups are available in the buffer, giving a new complexation partner for Ag(I). Although the pKa of the Met amino group is approximately 9, this effect is reported to be of relevance at pH > 4.5 [37].

Next, the affinity of silver toward free thiol groups is known to be higher than for thioethers, which means that Ag(I) may bind to Cys residues first. However, this effect should be constant for stressed and unstrained proteins, thus, resulting in similar mobility shifts for stressed and unstrained proteins. Additionally, most Cys residues are covalently bound in disulfide bonds, which were shown to have a lower affinity to Ag(I) than thioethers [35]. For our experiment, two standard mAbs, mAb1 and mAb2, each with a pl of 8.5 and with 14 Met residues were used as model biopharmaceuticals. These samples were stressed with 1% H₂O₂ for 1 or 6 h. For both mAbs, only minor CZE-profile changes are observed when they are exposed to H₂O₂, indicating increased oxidation (Fig. 4A and B). When these samples were injected in the presence of 500 μM Ag⁺ in the BGE, a mobility shift of unstressed/nonoxidized and immediately (1 h) stressed samples occurred, while highly stressed samples (6 h) were not affected (Fig. 4C and D). This indicated binding of Ag(I) to free thioether groups and, thus, demonstrated, that stressed mAbs, containing MetO, were not affected by the presence of Ag(I) in the BGE while unstressed samples were changing their mobility (Fig. 4). Alhazmi and colleagues conducted several ACE studies on the effect of metal ions, including Ag(I), on proteins [47–49]. For these studies, a Tris(hydroxymethyl)-aminomethan (tris) BGE at pH 7.4 was used. Results were ambiguous for Ag: some proteins as human serum albumin was reported to have an increased mobility, while others are not affected or show mobility decrease. This can be explained by the complex formation of Ag at higher pH. In this case, the participation of amino groups in complex formation leads to polynuclear [(Ag)₃(Met)₃] complexes [39]. Additionally, Kₐ for amino and thioether groups are reported to be in the same range, leading to a competitive situation. Since there are usually more accessible amino groups than thioether groups in a protein, this increased number of interactions may affect protein structure and, thus, mobility in very different ways. For these reasons, it is expected that the method presented here will show best results in terms of specificity at low pH (ideally < 4.5).

5 Concluding remarks

Methionine oxidation is linked to decreased stability of proteins and is, thus, of great interest as a critical quality attribute in pharmaceutical industry. For development of an easy and cost-efficient method for determination of the oxidation state in proteins, we apply ACE in combination with metal agents in the BGE. This approach has been tested with six different metals: Ni, Pd, Pt, Cu, Ag, and Au. The best results were obtained by using Au and Ag. Our data have shown that Au(III) is reduced to Au(I) and Met is oxidized to MetO in the capillary. Au(I) can then complex Met containing peptides and change their mobility in ACE. AuCl₃ can, thus, be used together with thioethers containing samples for studying behavior of Au(I) in CZE at concentration around 250–500 μM. Additionally, AuCl₃ can be of interest as an alternative oxidant for stressing biological samples.

The specific interaction of Ag(I) with thioethers could be successfully exploited in ACE to separate species with different oxidation status of their Met residues. Studies with peptides have shown a clear separation of nonoxidized, single-oxidized, and double-oxidized species, which could not be separated by classical CZE. Moreover, even different types of single-oxidized species were distinguished. Our data suggest the binding of one Ag(I) ion to one Met residue, which increases the overall charge of the analyte. The binding constant for 8-mer peptides containing one Met residue was determined to be log Kₐ ≈ 3.6 and are well comparable to literature values obtained by amperometric titration.
Figure 4. Effects of the AgF addition on the mobility of stressed mAb samples: mAb1 (A) and mAb2 (B) were stressed with 1% H₂O₂ for 1 h (red lines) and 6 h (green lines) and compared with unstressed samples (black lines) in a metal-free BGE. The same BGE was modified with 500 μM AgF and the experiment was repeated for mAb1 (C) and mAb2 (D). BGE: 400 mM EACA, 2 mM TETA, 0.05% HPMC, pH 5.7; separation voltage: 20 kV; capillary: fused silica. All lines were shifted according to the marker (His).

Experiments with mAbs have shown that the binding of silver ions also affects mobility of large proteins. However, mobility changes obtained for mAbs are relatively low compared to those of peptides, because of the low abundance of Met in the protein. Hence, for proteins the method has the ability to be used as an indicator for protein oxidation stress. If a more detailed resolution is necessary, the method needs to be used on peptide level.

The presented new ACE method for the detection of Met oxidation is economical and specific. Additionally, it is easily accessible as using standard equipment and techniques and has the advantage of rapid analysis times. It is expected to become a valuable tool for MetO detection and characterization.

Data availability statement
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

6 References
[1] Cleland, J. L., Powell, M. F., Shire, S. J., Crit. Rev. Ther. Drug Carrier Syst. 1993, 10, 307–377.
[2] Vogt, W., Free Radic. Biol. Med. 1995, 18, 93–105.
[3] Bernstein, H. D., Poritz, M. A., Strub, K., Hoben, P. J., Brenner, S., Walter, F., Nature 1989, 340, 482–486.
[4] Gellman, S. H., Biochemistry 1991, 30, 6633–6636.

The authors have declared no conflict of interest.
