



Absolute quantitative analysis of intact and oxidized amino acids by LC-MS without prior derivatization

Luke F. Gamon, Chaorui Guo, Jianfei He, Per Hägglund, Clare L. Hawkins, Michael J. Davies*

Dept. of Biomedical Sciences, University of Copenhagen, Copenhagen, Denmark

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ABSTRACT

The precise characterization and quantification of oxidative protein damage is a significant challenge due to the low abundance, large variety, and heterogeneity of modifications. Mass spectrometry (MS)-based techniques at the peptide level (proteomics) provide a detailed but limited picture due to incomplete sequence coverage and imperfect enzymatic digestion. This is particularly problematic with oxidatively modified and cross-linked/aggregated proteins. There is a pressing need for methods that can quantify large numbers of modified amino acids, which are often present in low abundance compared to the high background of non-damaged amino acids, in a rapid and reliable fashion. We have developed a protocol using zwitterionic ion-exchange chromatography coupled with LC-MS to simultaneously quantify both parent amino acids and their respective oxidation products. Proteins are hydrolyzed with methanesulfonic acid in the presence of tryptamine and purified by strong cation exchange solid phase extraction. The method was validated for the common amino acids (excluding Gln, Asn, Cys) and the oxidation products 3-chlorotyrosine (3-ClTyr), 3-nitrotyrosine (3-NO₂Tyr), di-tyrosine, N^ε-(1-carboxymethyl)-l-lysine, *o,o'*-di-tyrosine, 3,4,-dihydroxyphenylalanine, hydroxy-tryptophan and kynurenine. Linear standard curves were observed over ~3 orders of magnitude dynamic range (2–1000 pmol for parent amino acids, 80 fmol–20 pmol for oxidation products) with limit-of-quantification values as low as 200 fmol (*o,o'*-di-tyrosine). The validated method was used to quantify Tyr and Trp loss, and formation of 3-NO₂Tyr on the isolated protein anastellin treated with peroxynitrous acid, and for 3-ClTyr formation (over a 2 orders of magnitude range) in cell lysates and complex protein mixtures treated with hypochlorous acid.

1. Introduction

The precise characterization and quantification of species formed as a result of oxidative damage to proteins is a significant scientific challenge due to the low abundance, large variety, and heterogeneity of modifications [1]. The UniMod database currently lists over 2000 post-translational modifications (PTMs), indicating that for any given protein, the number of possible modifications may range from hundreds to thousands. However, the number of modifications, and concentration, on any single protein is typically very small [2,3]. Methods to detect, identify and quantify modifications on proteins have been recently reviewed, and this has highlighted the deficiencies in many currently

available techniques [4]. Liquid chromatography coupled with mass spectrometry (LC-MS) peptide mass-mapping (proteomics) provides rich information on the precise sites of PTMs but has limitations with regard to identification (e.g. of isobaric species) and in some cases absolute sensitivity, due to low levels of individual modifications on specific peptides. Samples therefore often require enrichment (at either the protein or peptide level) to enable detection against endogenous non-modified or highly abundant proteins (e.g. albumin for plasma samples) [5]. LC-MS approaches can also be limited by sub-optimal (< 100%) sequence coverage as a result of extensive cross-linking or other problems with digestion (particularly large proteins, those with large numbers of disulfide bonds, and those heavily decorated with

Abbreviations: ACN, acetonitrile; AGE, advanced glycation end products; BME, basement membrane extract; 3-ClTyr, 3-chlorotyrosine; CML, N^ε-(1-carboxymethyl)-l-lysine; CoV, coefficient of variation; Di-Tyr, *o,o'*-di-tyrosine; DOPA, 3,4,-dihydroxyphenylalanine; ECM, extracellular matrix; ESI, electrospray ionization; HOCl, the physiological mixture of hypochlorous acid and its conjugate anion ⁻OCl; HPLC, high-performance liquid chromatography; Kyn, kynurenine; LC-MS, liquid chromatography-mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MPO, myeloperoxidase; MS, mass spectrometry; MSA, methanesulfonic acid; NFK, N-formylkynurenine; 3-NO₂Tyr, 3-nitrotyrosine; ONOOH, the physiological mixture of peroxynitrous acid and its conjugate anion ONOO⁻; OPA, *o*-phthalaldehyde; PTM, post-translational modification; QC, quality control; SCX, strong cation exchange; SPE, solid phase extraction; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; Trp-OH, hydroxy-tryptophan; UPLC, ultra high-performance liquid chromatography

* Corresponding author.

E-mail address: davies@sund.ku.dk (M.J. Davies).

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sugars). This may lead to an incomplete picture of the pattern or extent of damage. LC-MS peptide methods are also limited by an absence of authentic standards of specific species, and therefore constrained by the assumption of equal ionization between analytes. This can result in only semi-quantitative data [4,6]. This problem can be, at least partially, circumvented by the use of isotope tagging, though this technology also has its limitations [7]. The extent of these problems is often underestimated, particularly with regard to the overall mass balance, even with relatively simple, pure, isolated proteins. Thus, in recent work studying singlet oxygen ($^1\text{O}_2$) mediated oxidation of RNase A, the extent of modification at specific amino acids (e.g. Tyr) was significantly underestimated when measured by peptide mass mapping (trypsin digestion, LC-MS) as opposed to quantification by fluorescence after acid hydrolysis and UPLC separation of the released amino acids [8]. Similarly, the mass balance for Trp residues is often very poor [9,10].

Proteins are major targets of oxidative damage, as they are the main (non-water) components of most biological systems, and highly reactive towards many oxidants including myeloperoxidase-derived hypochlorous acid (HOCl), peroxyntous acid (ONOOH), H_2O_2 and other peroxides, $^1\text{O}_2$, and peroxy radicals (ROO^\cdot), amongst others. Oxidation products formed from specific side chains have been used to detect the presence of modified proteins in biological systems. These include: 1) generic products (e.g. carbonyls, alcohols, hydroperoxides), which can be formed on multiple side-chains by a wide variety of oxidant species; 2) species formed on specific side-chains by multiple oxidants (e.g. DOPA and di-tyrosine from Tyr, *N*-formylkynurenine, NFK, and kynurenine, Kyn, from Trp); and 3) defined products formed on single side-chains by specific oxidants (e.g. chlorination of Tyr to give 3-chlorotyrosine, 3-ClTyr, by HOCl; nitration of Tyr to give 3-nitrotyrosine, 3-NO₂Tyr, by ONOOH and NO₂[·]) [4,11,12]. Some of these species (e.g. 3-ClTyr and 3-NO₂Tyr) have been used as qualitative (e.g. using immunoblotting or immunocytochemistry) and/or quantitative markers of damage, with these species providing relatively specific information about the damaging agent. The formation of modified proteins has been extensively studied in a large number of acute and chronic pathologies, including cardiovascular disease, cystic fibrosis, some cancers, asthma, neurodegenerative conditions, and rheumatoid arthritis [13–16].

Numerous methods have been developed for the quantification of oxidation products derived from Cys (e.g. sulfenic, sulfinic, sulfonic acids, nitroso-Cys), Met (e.g. Met sulfoxide), Tyr (e.g. 3-ClTyr, 3-NO₂Tyr, DOPA and Di-Tyr), Trp (e.g. hydroxy-tryptophan, Trp-OH; NFK; Kyn), and a wide range of species formed by glycation/glycoxidation reactions (advanced glycation end products, AGEs, such as *N*^ε-(1-carboxymethyl)-l-lysine, CML) [17–21]. Most methods rely on reverse-phase LC separation and detection either by fluorescence (intrinsic, or after derivatization with reagents such as *o*-phthalaldehyde, OPA), UV-absorption, electrochemical detection or detection by mass spectrometry [22]. Single run HPLC/UPLC methods only allow for resolution of a limited number of analytes due to peak overlaps, and often require either derivatization or addition of ion-pairing agents to enable separation of polar amino acids [23]. These steps result in complex sample preparation protocols (and hence the potential for artefact generation) and, in the case of ion-pairing agents used in LC-MS, can result in significant signal suppression [24,25].

In this paper we present a method which enables absolute quantification of both intact amino acids and protein modification products in a single LC-MS method, without the need for derivatization. This method reduces sample handling and enables the rapid and accurate determination of the extent of loss of parent, and formation of modified species on amino acids, peptides and proteins, including cell-derived and extracellular matrix materials.

2. Materials and methods

2.1. Chemicals and reagents

Unless otherwise stated, all chemicals were purchased from commercial sources and used as received. 3-Chloro-[¹³C₆]tyrosine, [¹³C₉,¹⁵N]tyrosine, and a mix of stable isotope standards (MSK-A2-1.2) were purchased from Cambridge Isotope Laboratories (MA, USA). *N*^ε-(1-carboxymethyl)-l-lysine-d₃ was purchased from Cayman Chemicals (MI, USA). l-kynurenine-d₄ (TFA salt), 5-hydroxy-l-tryptophan and 5-hydroxy-l-tryptophan-d₄ were purchased from Toronto Research Chemicals (ON, Canada). Methanesulfonic acid (with 0.2% tryptamine) and l-DOPA-d₃ were purchased from Sigma Aldrich/Merck (Denmark). 3-Nitro-[¹³C₉,¹⁵N]tyrosine and [¹³C₁₈,¹⁵N₂]di-tyrosine were synthesized, purified by HPLC and quantified by UV absorption prior to use as an internal standard. Strata X-C cartridges (10 mg) were purchased from Phenomenex (USA). UPLC buffers were prepared using LC-MS grade acetonitrile, water, formic acid and ammonium formate purchased from VWR (Søborg, Denmark). Recombinant Anastellin was produced in *E. Coli* using a codon optimized human construct purchased from GenScript Biotech.

2.2. Synthesis of 3-nitro-[¹³C₉,¹⁵N]-tyrosine internal standard

3-Nitro-[¹³C₉,¹⁵N]-tyrosine was synthesized via reaction of [¹³C₉,¹⁵N]tyrosine with tetranitromethane. Briefly, [¹³C₉,¹⁵N]tyrosine (1 mg, 4.42 μmoles) was dissolved in 500 mM ammonium formate (4.4 mL, pH 10), treated with 1 M tetranitromethane (44.3 μL, 44.3 μmoles, 10-fold molar excess) and incubated for 1 h at 21 °C. The solution was acidified with 1% TFA, split into 5 and purified by solid phase extraction (SPE; 1 mL C18 cartridge, Supelco, USA). Cartridges were conditioned with 1 mL MeOH followed by 1 mL 0.1% TFA, the samples were then loaded, following by washing with 1 mL 0.1% TFA and eluted with 0.1% formic acid in 80% acetonitrile (ACN). 3-Nitro-[¹³C₉,¹⁵N]-tyrosine was purified by semi-preparative C18 HPLC with a gradient of water and ACN containing 0.1% formic acid. Purity was confirmed by HPLC and LC-MS analysis.

2.3. Synthesis of [¹³C₁₈,¹⁵N₂]-di-tyrosine internal standard

[¹³C₁₈,¹⁵N₂]Di-tyrosine was synthesized via reaction of [¹³C₉,¹⁵N]tyrosine with horseradish peroxidase in the presence of H₂O₂ [20]. Briefly, [¹³C₉,¹⁵N]tyrosine (1 mg, 4.42 μmoles) and horseradish peroxidase (0.03 mg) were dissolved in 100 mM ammonium formate (1.2 mL, pH 9.2) followed by addition of 30 mM H₂O₂ (10 μL, 0.3 μmoles) and incubation for 1 h at 40 °C. An additional aliquot of 30 mM H₂O₂ (10 μL, 0.1 μmoles) was then added and the solution incubated for another 1 h at 40 °C followed by acidification with 1% TFA. The sample was purified by SPE (1 mL C18 cartridge) followed by semi-preparative HPLC, as described above. Purity was confirmed by HPLC and LC-MS analysis.

2.4. Preparation of calibration standards

Individual stock solutions of Trp and the oxidation products 3-ClTyr, 3-NO₂Tyr, CML, DOPA, Di-Tyr, Trp-OH and Kyn were prepared in 0.1% formic acid in water for the native and isotopically-labelled standards and stored at −20 °C. A native standard solution was prepared by addition of Trp and oxidation product stock solutions to the amino acid standard mix solution AAS18 (Sigma Aldrich), giving a stock solution of parent amino acids (125 μM) and oxidation products (5 μM) in 0.1% formic acid. The isotopically-labelled standards were combined to prepare a stock solution of parent amino acids (125 μM)

and oxidation products (2 μM) in 0.1% formic acid.

Calibration standards were prepared by 1:1 serial dilution of solutions containing both parent amino acids (100 μM) and oxidation products (4 μM) in 0.1% formic acid. Internal standards (parent amino acids: 500 pmoles, oxidation products: 20 pmoles) were added to the dilutions and the resulting solutions were prepared for LC-MS analysis by dilution in 0.1% formic acid, purification by mixed-mode SCX SPE and resuspension in 0.1% formic acid (see also 'Sample preparation' below).

2.5. Linearity/sensitivity, reproducibility, and recovery

Calibration curves were constructed based on the ratio of peak areas of each analyte to its respective internal standard. Linearity of standard curves was assessed with a weighting factor of $1/x^2$. Four quality control (QC) samples were prepared with parent amino acids at 0.78, 3.125, 12.5 and 50 μM (subsequently referred to as: QC-1, QC-2, QC-3 and QC-4, respectively) and oxidation products at 31.25, 125, 500, 2000 nM, respectively. The corresponding amounts (in mol) injected on column were 7.8, 31.25, 125, 500 pmol (parent amino acids) and 0.3125, 1.25, 5, 20 pmol (oxidation products). QC samples were prepared for LC-MS analysis according to the sample preparation below and frozen at -20°C prior to reconstitution. Intra-day variation was assessed by re-injection of the QC-1, QC-2, QC-3, QC-4 QC standards 6 times over a 24 h period ($n = 6$). Inter-day variation was assessed by injection of the QC-1, QC-2, QC-3, QC-4 standards over four days ($n = 4$). The limit of detection (LOD) and limit of quantification (LOQ) was determined by calculation from the standard deviation of the intra-day calibrators across the linear range of the standard curve for each analyte, according to the method described by Taylor [26].

Recoveries were assessed by mixed-mode strong-cation exchange (SCX) - SPE of standards dissolved in 0.1% formic acid (50 μL sample volume, parent amino acids: 100 μM , oxidation products: 4 μM) with varying concentrations of ammonium hydroxide (NH_4OH) and ACN in the elution buffer. Internal standards were added after SPE, and before drying down of the samples, in order to assess recovery of the analytes. Amino acid concentrations were then determined using the optimized LC-MS method described below.

2.6. Sample preparation

Protein samples (25 μg) were precipitated with TCA (w/v 8%) and spiked with stable isotope labelled 3-nitro- $^{13}\text{C}_9$, ^{15}N tyrosine, 3-chloro- $^{13}\text{C}_6$ tyrosine, N^ϵ -(1-carboxymethyl)-L-lysine- d_3 , L-kynurenine- d_4 , $^{13}\text{C}_{18}$, $^{15}\text{N}_2$ di-tyrosine (100 pmoles) and a mix of 17 stable isotope labelled amino acid standards (2500 pmoles, except cystine which was 1250 pmoles) before drying down using a centrifugal vacuum concentrator for 30 min at 30°C . The resulting pellet was hydrolyzed overnight in 4 M methanesulfonic acid (MSA) with 0.2% w/v tryptamine (50 μL) under vacuum at 110°C . Amino acids were partially purified by solid-phase extraction using 30 mg/1 mL mixed-mode strong cation exchange Strata X-C cartridges (Phenomenex). The columns were activated using 100% methanol (1 mL), followed by equilibration with 0.1% formic acid in water (1 mL). Samples were diluted with 0.1% formic acid in water (10 μL hydrolysate with 990 μL of 0.1% formic acid) in order to reduce competitive binding with MSA in the sample matrix. The samples were loaded onto the column and washed with 0.1% formic acid in water (1 x 0.6 mL) and 0.1% formic acid in acetonitrile (1 x 0.6 mL) followed by elution with 1% NH_4OH in 20% ACN (1 x 0.6 mL). Eluted fractions were dried at 30°C under vacuum overnight, and then dissolved in 50 μL of 0.1% formic acid.

To minimize artefactual oxidation during sample preparation, samples and aqueous buffers were made up using high purity (MilliQ) water; this contains minimal levels of trace transition metal ions that might catalyze oxidation. Protein hydrolysis was carried out in the presence of 0.2% w/v tryptamine which acts as a sacrificial target for

oxidants as a result of both its high concentration in the samples and also its ease of oxidation. The hydrolysis was also carried out under vacuum to prevent O_2 -dependent processes. After hydrolysis samples were kept at low temperatures (4°C , or frozen at -20°C to further limit oxidation reactions). The extent of recovery of individual amino acids (and hence data on loss due to all processing steps, post hydrolysis) was assessed using heavy isotope-labelled standards, with this giving good recoveries of all amino acids including readily oxidized amino acids such as Met and Trp (see Results).

2.7. Chromatography and optimized LC-MS method

Analytes were quantified by ESI LC-MS in positive ion mode using a Bruker Impact HD II mass spectrometer. Samples were separated by gradient elution using an Intakt Intrada Amino Acid 100×3.0 mm column with ACN/formic acid (Solvent A; 100/0.3) and ACN/100 mM ammonium formate (Solvent B; 20/80). Elution was initiated at 20% B for 4 min, followed by gradient elution from 20 to 100% B over 10 min, 100% B over 2 min, then returning to 20% B over 2 min and re-equilibration at 20% B for 2 min. The electrospray needle was held at 4500 V, with end plate offset of 500 V and temperature of 350°C . Nitrogen gas was used for both the nebuliser (2.0 Bar) and as the dry gas (11.0 L min^{-1}). Optimized transfer settings used a collision RF of 450/1000 V, transfer time of 15/50 μs with the basic stepping mode at 25/75% timing. Sample injections were 10 μL . MS analysis and quantification was performed at the MS [1] level.

2.8. Quantification of amino acids by HPLC with OPA derivatization

Loss of parent amino acids was quantified by HPLC analysis with pre-column derivatization using *o*-phthalaldehyde (OPA). Anastellin (AN) samples (20 μg of protein in 50 μL 100 mM sodium phosphate buffer, pH 7.4) were exposed to ONOOH (0–500 μM) for 30 min at 21°C . The protein was then precipitated by the addition of 150 μL 4% (w/v) trichloroacetic acid and incubated at 4°C for 18 h. After removal of the supernatant the protein pellet was hydrolyzed by the addition of 50 μL 4 M MSA containing 0.2% (w/v) tryptamine. Following hydrolysis for 16 h under vacuum at 110°C , the samples were neutralized with 55 μL freshly prepared 4 M NaOH. Samples were filtered using Nanosep MF 0.2 μm centrifugal filters and diluted 10-fold with 0.2 M sodium carbonate buffer, pH 10.3. Pre-column derivatization of the diluted samples (40 μL) was performed with OPA (40 μL , 1 mg mL^{-1} in 0.2 M sodium carbonate buffer, pH 10.3, with 1 $\mu\text{L mL}^{-1}$ 2-mercaptoethanol added prior to derivatization). Standard curves were generated using commercially available amino acid mixtures. The fluorescently-tagged amino acid derivatives were separated on a Shimadzu Nexera system using a Kinetex 2.6 μm EVO C18 100 \AA LC column 150×3.0 mm and eluted by gradient elution using buffer A (100 mM sodium acetate in water, pH 5.3, with 2.5% (v/v) tetrahydrofuran and 15% (v/v) methanol) and buffer B (100 mM sodium acetate (as above), with 2.5% (v/v) tetrahydrofuran and 80% (v/v) methanol) with a gradient profile as follows: 100% buffer A for 3 min, 0–10.5% buffer B linear gradient over 7.5 min, 10.5% buffer B for 5 min, 10.5–65% buffer B linear gradient over 3 min, 65–100% buffer B linear gradient over 1 min, 100% buffer B for 2 min, 100–0% buffer B linear gradient over 0.5 min, followed by equilibration at 100% buffer A for 3.5 min. A flow rate of 0.8 mL min^{-1} was used. The tagged amino acids were detected with a Shimadzu RF-20A fluorescence detector with λ_{ex} 340 nm and λ_{em} 440 nm.

2.9. Cell lysates treated with HOCl and SCN^-

J774A.1 cells (ECACC catalogue number 91051511) were maintained in DMEM supplemented with 10% (w/v) foetal bovine serum (FBS), 2 mM L-glutamine and 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin, and cultured under sterile conditions in 175 cm^2 tissue

culture flasks in humidified 5% CO₂ at 37 °C. Cells cultured to ~80% confluency in T175 flasks were lysed in Milli-Q water (10 mL), and centrifuged at 18000 g for 10 min at 4 °C to remove cell debris. Total protein content was measured by BCA assay and lysates (25 µg protein in 300 µL PBS) were then treated with HOCl alone (0, 50, 100, 200 or 400 µM) or co-treated with 200 or 400 µM HOCl with thiocyanate ions (SCN⁻; 50, 200 or 400 µM) at 37 °C for 1 h. The proteins in the treated samples were then precipitated using 8% trichloroacetic acid (TCA), hydrolyzed to free amino acids using MSA containing 0.2% tryptamine, subjected to solid phase extraction, then analyzed by LC-MS. Results represent data from three independent experiments.

2.10. Anastellin treated with ONOOH

Anastellin (5 µM) was treated with different concentrations of ONOOH (5, 50, 500 µM) and incubated at 37 °C for 20 min in 100 mM sodium phosphate buffer, pH 7.4. Samples were prepared in triplicate. The proteins in the treated samples were then precipitated using 8% TCA, hydrolyzed to free amino acids using MSA containing 0.2% tryptamine, subjected to solid phase extraction, then analyzed by LC-MS.

2.11. Basement membrane extract (BME) treated with MPO/H₂O₂/Cl⁻ and SCN⁻

BME (20 µg) was treated with MPO (20 nM), Cl⁻ (100 mM) and H₂O₂ (27.7 µM, 1000x excess) in 0.1 M sodium phosphate, pH 7.4 (50 µL) in the presence or absence of SCN⁻ (0, 1, 10, 50 µM) and incubated at 37 °C for 30 mins. Samples were prepared in duplicate. The proteins in the treated samples were then precipitated using 8% TCA, hydrolyzed to free amino acids using MSA containing 0.2% tryptamine, subjected to solid phase extraction, then analyzed by LC-MS.

2.12. Statistics and data analysis

MS data was collected using Bruker Compass/Hystar and processed using Bruker QuantAnalysis software. Statistics (*t*-test, ANOVA, variances, standard deviation) were calculated using GraphPad Prism and Microsoft Excel.

2.13. Safety considerations

Tetranitromethane is highly toxic and can form potentially carcinogenic nitrosamines when reacted with amines (such as amino acids) at elevated temperatures. Proper precautions against inhalation of vapours and contact with skin should always be maintained.

3. Results and discussion

3.1. Column selection and chromatography for LC-MS

Previous methods for the analysis of oxidation products such as 3Cl-Tyr have typically utilised reversed-phase C18 separation [13,27,28]. We have successfully replicated these methods [28–31], but standard reversed-phase chromatography greatly limits the number of quantifiable amino acid derived analytes, as some polar compounds such as Asp, Glu, Ser elute at, or close to, the void volume. With the development of zwitterionic column chromatography, it has become possible to perform amino acid separation with MS-compatible buffers without the need for derivatization [24]. For example, the Intrada Amino Acid column (Imtakt, Japan) can be used to quantify amino acids in human serum and mouse plasma [32,33]. Preliminary experiments revealed that this type of column gave excellent peak shapes for all parent amino acids (including isobaric, baseline separation of Ile and Leu). Further optimization and method development were therefore performed using this column (see Fig. 1).

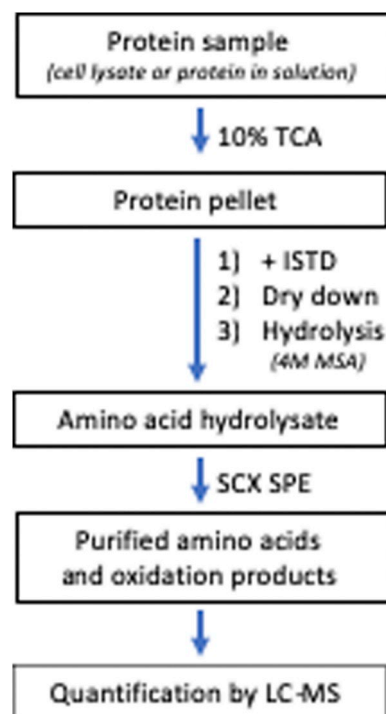


Fig. 1. Workflow for quantification of amino acids and oxidation products by LC-MS (including protein precipitation, hydrolysis and purification). For further details see the Materials and methods section. Abbreviations used: ISTD, internal standard; MSA, methane sulfonic acid; SCX SPE, strong cation exchange solid phase extraction; TCA, trichloroacetic acid.

3.2. Optimisation of MS quadrupole and transfer settings

Initial experiments revealed low molecular mass amino acids (Ala, Gly, Ser) exhibited low signal sensitivity, and therefore poor precision, under the conditions recommended by the manufacturer for low molecular mass analytes. This issue has been reported previously with Bruker QTOF mass spectrometers, and can be resolved by use of a stepping function in the quadrupole transfer settings [34]. ESI parameters were optimized by direct infusion of Ala, Gly and Ser diluted in LC-MS buffer. Optimisation of these parameters resulted in a 20–100 fold increase in signal sensitivity for these analytes (Fig. 2), though this also resulted in minor decreases (20–50%) in signal intensity for higher mass analytes such as Tyr and Met (Supplementary Table 1). This loss in signal intensity appears to be an acceptable trade-off, as it allows better LOD and LOQ concentrations for Ala, Gly, Ser.

3.3. Optimisation of solid phase extraction and sample preparation

Acid hydrolysis with MSA represents an efficient method to hydrolyse proteins to free amino acids [22]. In order to decrease the loss of Trp and derived products, which are normally lost during strong acid hydrolysis (e.g. gaseous 6 M HCl), tryptamine is often used as a sacrificial target (see also ‘Sample preparation’ section of Materials and methods) [22]. Unfortunately, tryptamine ionizes readily under electrospray conditions and therefore causes significant ion suppression during LC-MS analysis (data not shown). As such, a method was required to remove tryptamine after protein hydrolysis, without perturbing amino acid analysis. This problem is exacerbated by the high concentrations of tryptamine routinely employed (0.2% w/v) and its structural similarity to some analytes of interest (e.g. Trp and Trp-derived products). To resolve this issue, mixed-mode strong cation exchange (SCX) was employed, where tryptamine is readily retained on the resin as a result of the absence of an α -carboxylic acid group and its hydrophobicity (tryptamine is > 2 orders of magnitude more

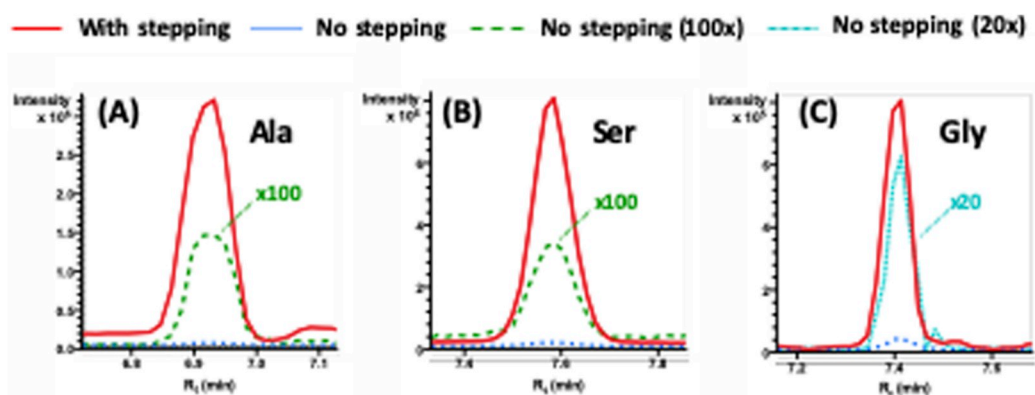


Fig. 2. Extracted ion chromatograms of low molecular mass analytes (A) Ala ($m/z = 90.0552 \pm 0.01$), (B) Ser ($m/z = 105.0370 \pm 0.01$), (C) Gly ($m/z = 76.0399 \pm 0.01$) with (red solid line) and without stepping (blue dotted line, 1x; cyan fine-dotted line, 20x; green dashed line, 100x). Scaling of 20x/100x as indicated for data without stepping. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

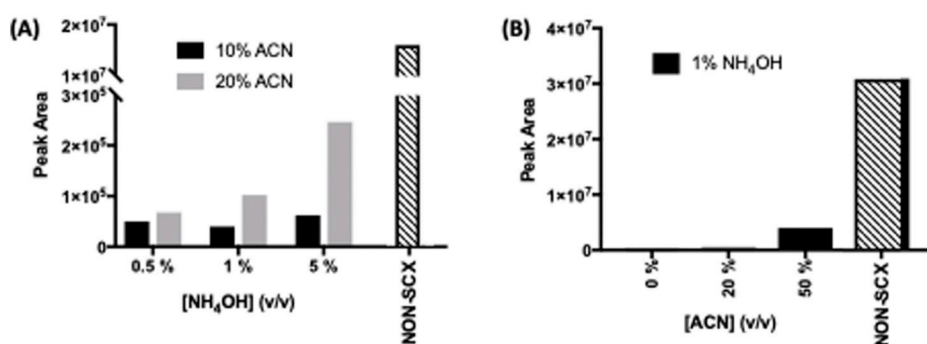


Fig. 3. Elution of tryptamine with varying concentrations of NH_4OH and ACN after binding to mixed-mode SCX material. Peak areas of eluted tryptamine during optimisation of (A) NH_4OH concentration (0.5–5% w/v in water, 10 or 20% ACN; black bars, 10% ACN; grey bars, 20% ACN) and (B) ACN concentration (0–50% v/v in water, 1% NH_4OH). Samples injected directly (bars with diagonal lines, NON-SCX; without SCX SPE purification) contain the maximum concentration of tryptamine expected on column.

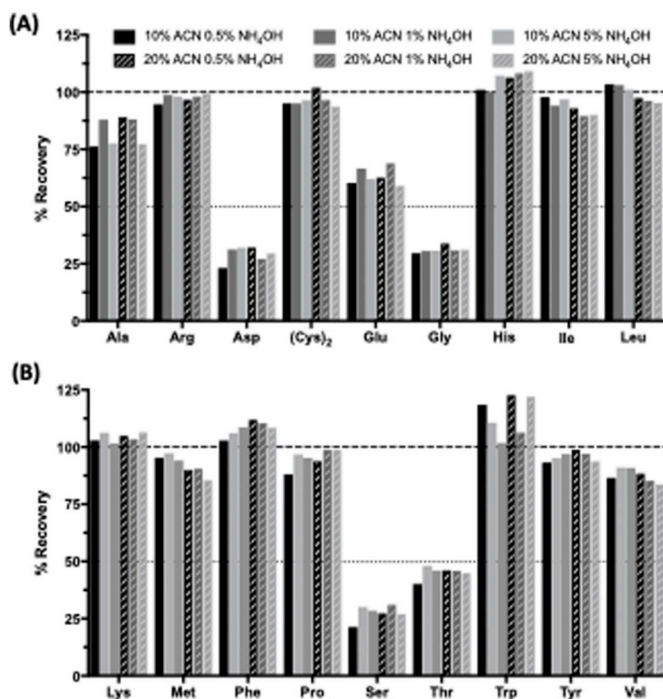


Fig. 4. Recoveries after mixed-mode SCX solid phase extraction of amino acids diluted in methanesulfonic acid containing 0.2% w/v tryptamine. Amino acids are presented in alphabetical order: (A) Ala to Leu, (B) Lys to Val. Amino acids were eluted with 0.5–5% NH_4OH and 10 or 20% ACN in water as indicated.

hydrophobic than the most non-polar amino acid, Trp, with estimated $\log P = 1.5$ vs -1.1 ; ChemAxon).

Methanol was used initially for washing of the SCX SPE resin and

Table 1
Linearity and limits of detection for individual amino acids and oxidation products.

Analyte	Calibration Range	Coefficient (r^2)	LOD (pmol)	LOQ (pmol)
3-ClTyr	0.08–20 pmol	0.9970	0.156	0.521
3-NO ₂ Tyr	0.08–20 pmol	0.9997	0.423	1.41
Ala	2–1000 pmol	0.9914	5.90	19.7
Arg	2–1000 pmol	0.9920	0.924	3.08
Asp	30–1000 pmol	0.9753	32.8	109
CML	0.08–20 pmol	0.9851	0.174	0.580
(Cys) ₂	10–500 pmol	0.9872	0.102	0.340
DOPA	0.08–20 pmol	0.9974	28.2	94.1
Di-Tyr	0.08–20 pmol	0.9995	0.0619	0.206
Glu	2–1000 pmol	0.9958	9.39	31.3
Gly	30–1000 pmol	0.9540	13.1	43.7
His	2–1000 pmol	0.9975	4.79	16.0
Trp-OH	0.08–20 pmol	0.9955	2.27	7.55
Ile	2–1000 pmol	0.9921	5.37	17.9
Kyn	0.08–20 pmol	0.9995	0.117	0.391
Leu	2–1000 pmol	0.9964	3.16	10.5
Lys	2–1000 pmol	0.9995	7.99	26.6
Met	2–1000 pmol	0.9769	1.41	4.69
Phe	2–1000 pmol	0.9985	2.08	6.92
Pro	2–1000 pmol	0.9791	1.43	4.77
Ser	3–1000 pmol	0.9836	22.6	75.5
Thr	4–1000 pmol	0.9956	8.04	26.8
Trp	2–1000 pmol	0.9767	0.781	2.60
Tyr	2–1000 pmol	0.9884	1.29	4.31
Val	2–1000 pmol	0.9920	9.89	33.0

elution of the amino acids, as recommended by the manufacturer (100% MeOH during washing, 5% NH_4OH in MeOH for elution). However, preliminary analysis of samples of HOCl-treated proteins prepared using this method, with subsequent untargeted analysis using a combination of XCMS Online and DCA-Hal (a tool designed for detecting Cl or Br containing compounds) [35], revealed a number of ions

Table 2
Precision and accuracy for individual amino acids and oxidation products.

Analyte	QC 1			QC 2		
	Nominal Conc.	Mean Conc.	CoV (%)	Nominal Conc.	Mean Conc.	CoV (%)
3-ClTyr	0.3125 pmol	0.382 ± 0.0332	8.7	1.25 pmol	1.39 ± 0.0666	4.8
3-NO ₂ Tyr	0.3125 pmol	0.403 ± 0.125	31.0	1.25 pmol	1.30 ± 0.155	11.9
Ala	7.8 pmol	21.0 ± 1.16	5.5	31.25 pmol	45.3 ± 2.27	5.0
Arg	7.8 pmol	11.6 ± 0.335	2.9	31.25 pmol	38.7 ± 0.516	1.3
Asp	7.8 pmol	16.7 ± 4.10	24.5	31.25 pmol	36.7 ± 9.65	26.3
CML	0.3125 pmol	0.356 ± 0.0509	14.3	1.25 pmol	1.38 ± 0.0450	3.3
(Cys) ₂	3.9 pmol	4.48 ± 0.687	7.7	15.6 pmol	16.6 ± 0.594	1.8
DOPA	0.3125 pmol	7.893 ± 6.458	81.8	1.25 pmol	21.3 ± 13.6	64.0
Di-Tyr	0.3125 pmol	0.304 ± 0.0710	23.4	1.25 pmol	1.15 ± 0.063	5.5
Glu	7.8 pmol	11.3 ± 2.88	25.5	31.25 pmol	34.4 ± 1.96	5.7
Gly	7.8 pmol	42.6 ± 5.13	12.0	31.25 pmol	65.6 ± 8.16	12.5
His	7.8 pmol	12.4 ± 0.511	4.1	31.25 pmol	35.2 ± 0.92	2.6
Trp-OH	0.3125 pmol	0.908 ± 0.556	61.2	1.25 pmol	2.01 ± 0.635	31.6
Ile	7.8 pmol	41.4 ± 1.57	3.8	31.25 pmol	58.1 ± 2.35	4.0
Kyn	0.3125 pmol	0.491 ± 0.0815	16.6	1.25 pmol	1.28 ± 0.250	19.6
Leu	7.8 pmol	30.1 ± 1.20	4.0	31.25 pmol	49.8 ± 1.15	2.3
Lys	7.8 pmol	9.46 ± 1.20	12.7	31.25 pmol	33.8 ± 2.33	6.9
Met	7.8 pmol	8.93 ± 1.30	14.6	31.25 pmol	27.7 ± 1.75	6.3
Phe	7.8 pmol	13.6 ± 0.517	3.8	31.25 pmol	41.3 ± 1.23	3.0
Pro	7.8 pmol	13.0 ± 0.300	2.3	31.25 pmol	39.9 ± 0.41	1.0
Ser	7.8 pmol	33.4 ± 5.26	15.7	31.25 pmol	67.3 ± 7.98	11.9
Thr	7.8 pmol	13.9 ± 1.46	10.5	31.25 pmol	45.7 ± 3.78	8.3
Trp	7.8 pmol	29.8 ± 0.292	1.0	31.25 pmol	54.3 ± 0.491	0.9
Tyr	7.8 pmol	11.3 ± 0.342	3.0	31.25 pmol	38.3 ± 0.834	2.2
Val	7.8 pmol	81.0 ± 4.24	5.2	31.25 pmol	69.9 ± 2.11	3.0

Analyte	QC 3			QC 4		
	Nominal Conc.	Mean Conc.	CoV (%)	Nominal Conc.	Mean Conc.	CoV (%)
3-ClTyr	5 pmol	5.24 ± 0.194	3.7	20 pmol	19.4 ± 0.430	2.2
3-NO ₂ Tyr	5 pmol	4.92 ± 0.256	5.2	20 pmol	19.1 ± 0.452	2.4
Ala	125 pmol	135 ± 5.11	3.8	500 pmol	432 ± 8.70	2.0
Arg	125 pmol	133 ± 1.57	1.2	500 pmol	489 ± 4.74	1.0
Asp	125 pmol	135 ± 22.9	17.0	500 pmol	483 ± 12.5	2.6
CML	5 pmol	5.17 ± 0.148	2.9	20 pmol	18.8 ± 0.234	1.2
(Cys) ₂	62.5 pmol	63.2 ± 1.98	1.6	250 pmol	244 ± 9.88	2.0
DOPA	5 pmol	10.0 ± 5.92	59.4	20 pmol	10.1 ± 3.59	35.5
Di-Tyr	5 pmol	4.29 ± 0.108	2.5	20 pmol	16.4 ± 0.626	3.8
Glu	125 pmol	131 ± 8.17	6.2	500 pmol	500 ± 11.9	2.4
Gly	125 pmol	168 ± 9.85	5.9	500 pmol	479 ± 37.1	7.7
His	125 pmol	132 ± 4.85	3.7	500 pmol	501 ± 3.86	0.8
Trp-OH	5 pmol	8.64 ± 3.19	36.9	20 pmol	26.9 ± 6.35	23.6
Ile	125 pmol	158 ± 2.64	1.7	500 pmol	503 ± 5.41	1.1
Kyn	5 pmol	3.34 ± 0.439	13.1	20 pmol	14.5 ± 2.13	14.7
Leu	125 pmol	142 ± 2.07	1.5	500 pmol	439 ± 4.85	1.1
Lys	125 pmol	128 ± 6.40	5.0	500 pmol	495 ± 6.05	1.2
Met	125 pmol	119 ± 3.36	2.8	500 pmol	586 ± 16.7	2.9
Phe	125 pmol	150 ± 4.14	2.8	500 pmol	570 ± 11.7	2.1
Pro	125 pmol	127 ± 1.27	1.0	500 pmol	436 ± 1.76	0.4
Ser	125 pmol	176 ± 14.5	8.2	500 pmol	524 ± 19.6	3.7
Thr	125 pmol	145 ± 7.59	5.2	500 pmol	529 ± 15.7	3.0
Trp	125 pmol	143 ± 0.965	0.7	500 pmol	446 ± 3.23	0.7
Tyr	125 pmol	136 ± 1.63	1.2	500 pmol	487 ± 4.95	1.0
Val	125 pmol	183 ± 3.07	1.7	500 pmol	522 ± 1.98	0.4

consistent with methylated products. These were tentatively identified as the methyl esters 3-ClTyr-OMe and 3,5-Cl₂-Tyr-OMe, with these detected for both parent amino acids and internal standards. The use of ACN instead of MeOH in the SPE protocol eliminated these artefacts.

Elution conditions were optimized by varying the concentration of NH₄OH (to disrupt ionic interaction) and ACN (to disrupt hydrophobic interaction) in eluting amino acids from Strata X-C cartridges. Only limited quantities of tryptamine were found to be eluted off the resin with ACN concentrations < 20% and NH₄OH concentrations < 5% (Fig. 3A). Significant elution was observed with high concentrations of ACN (> 50%) and 1% NH₄OH (Fig. 3B). Elution at 0.5, 1 or 5% NH₄OH and 10 or 20% ACN gave excellent recovery of all the common amino

acids, with many close to 100% (Fig. 4A and B). The polar amino acids Asp, Gly, Ser and Thr exhibited some breakthrough during binding/washing, leading to recoveries of 25–50%. The optimal elution conditions, with good amino acid recovery and negligible tryptamine elution, were determined as 1% NH₄OH with 20% ACN.

3.4. Linearity and sensitivity

Linearity ranges were assessed by injection of 12 standards, corresponding to 11 serial 1:1 dilutions of the highest concentration standard. The determined calibration ranges including coefficients of determination (R²), limits of detection (LOD) and limits of quantification

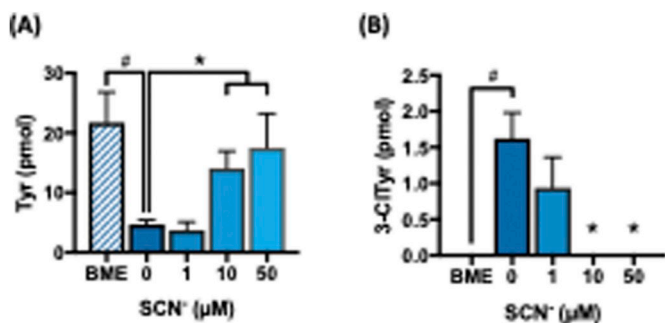


Fig. 5. Quantification of total (A) Tyr and (B) 3-ClTyr on extracellular matrix proteins from basement membrane extracts (BME) treated with an MPO/H₂O₂/Cl⁻ system in the absence or presence of increasing concentrations of SCN⁻ (0–50 μM). BME (20 μg) was treated with MPO (20 nM), Cl⁻ (100 mM) and H₂O₂ (27.7 μM, 1000-fold excess) in 0.1 M sodium phosphate at pH 7.4 (50 μL) and incubated at 37 °C for 30 min. # Indicates statistical significance of the oxidized versus control samples in the absence of SCN⁻ (0 μM SCN⁻) as determined by Student's t-test. * Indicates statistical significance against the oxidized sample in the absence of SCN⁻ (0 μM SCN⁻) as determined by one-way ANOVA with post-hoc analysis using Dunnett's multiple comparison test.

(LOQ), are presented in Table 1. R² values were > 0.99 for most analytes, ranging from 0.9540 (Gly) to 0.9995 (Di-Tyr, Kyn, Lys). The sensitivities of the lowest molecular mass analytes such (Gly, Ser) were modest in comparison to other analytes (dynamic range roughly one order-of-magnitude less), even after method optimisation (see above). LOQs ranged from 200 fmol (Di-Tyr) to 110 pmol (Asp) on column.

3.5. Intra- and inter-day reproducibility

Intra-day and inter-day precision and accuracy values for QC standards at four different concentrations are presented in Table 2. The QC-1 concentration represents the lowest concentration QC standard and reveals excellent reproducibility of high abundance analytes (i.e. parent amino acids), with a coefficient of variation (CoV) of < 20% for the majority of the parent amino acids. Amongst the oxidation products, only 3-ClTyr, CML and Kyn showed CoVs below 20% at the QC-1 concentration. However, while this may indicate these amino acids are above the LOQ at the QC-1 concentration, using Taylor's method as a more stringent determination of LOQ, most amino acids are still below

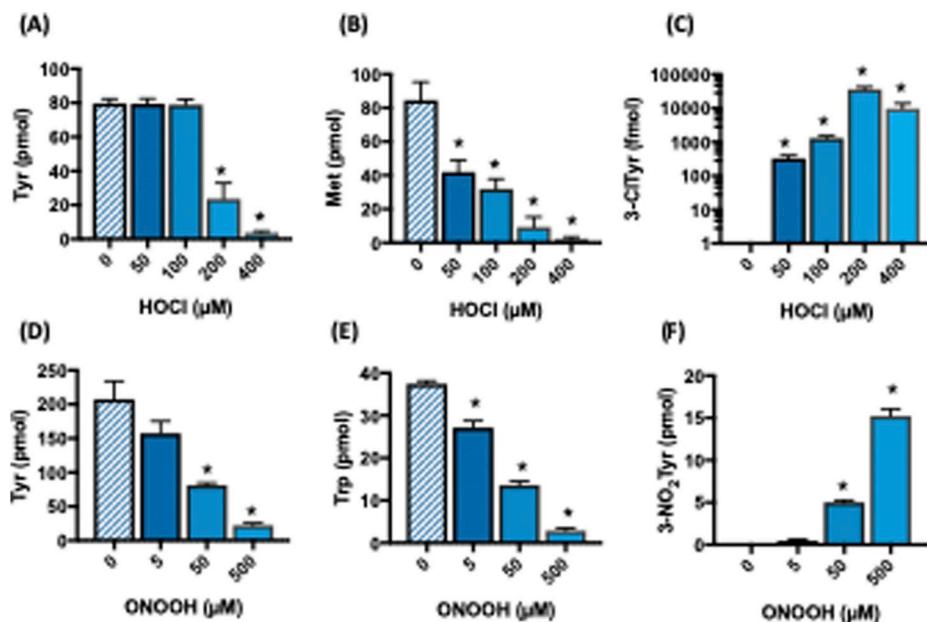


Fig. 6. Quantification of total (A) Tyr, (B) Met and (C) 3-ClTyr in HOCl-treated cell lysates. Quantification of (D) Tyr, (E) Trp and (F) 3-NO₂Tyr in ONOOH-treated anastellin. Cell lysates from J774A.1 cells were treated with HOCl (0–400 μM) at 37 °C for 1 h. Anastellin (5 μM) was treated with ONOOH (0–500 μM) at 37 °C for 30 min. Linear scales with the exception of a log₁₀ scale in panel (C). Data are presented as absolute concentrations normalised to Phe. * Indicates statistical significance against the untreated control sample as determined by one-way ANOVA with post-hoc analysis using Dunnett's multiple comparison test.

the LOQ indicated (Table 1). With the exception of Asp, all parent amino acids showed excellent reproducibility at the QC-2, QC-3 and QC-4 QC concentrations (CoV 0.4–12.5%). 3-ClTyr, 3-NO₂Tyr, CML, Di-Tyr and Kyn all showed excellent reproducibility at the QC-2, QC-3 and QC-4 QC concentrations. Whilst DOPA and Trp-OH gave linear standard curves with high r² values, they exhibited relatively poor precision across the complete range of QC concentrations (CoV of 23.6–81.8%). Unfortunately, the isobaric species *ortho* and *meta*-Tyr, which are products of Phe oxidation, could not be resolved under the optimized conditions. This is therefore a limitation of this assay.

3.6. Application of optimized method to purified proteins, extracellular matrix preparations and cell lysates

A number of chronic inflammatory diseases have been associated with elevated levels of oxidized amino acids, including 3-ClTyr and 3-NO₂Tyr generated from endogenous protein Tyr residues [17,36,37]. Using the method described above, we were able to simultaneously measure both parent and oxidized amino acids in a number of different oxidation systems and samples (purified proteins, basement membrane extracellular matrix extract and cell lysates). In contrast to HPLC/UPLC methods, where relatively high amounts of protein are required (high μg – low mg quantities), adequate signal-to-noise ratios were obtained for detection and quantification, with < 25 μg of starting protein.

The extracellular matrix is a known target of oxidative damage (reviewed [38,39]) and particularly at sites of inflammation due to the generation of oxidants extracellularly (e.g. HOCl by MPO, and ONOOH from the reaction of NO[•], from nitric oxide synthase enzymes, and O₂^{-•}, from NADPH oxidases and other sources), the high abundance of ECM, the low levels of antioxidant defences and repair systems, and a slow rate of turnover. The ECM is therefore likely to accumulate modifications, and data has been presented to support this hypothesis [40–43].

Treatment of basement membrane extracts (BME), a specialized extracellular matrix that underlies epithelial and endothelial cells (e.g. in the skin and blood vessels, respectively), with a MPO (20 nM)/H₂O₂ (27.7 μM)/Cl⁻ (100 mM) system to form HOCl, gave rise to a significant loss of parent Tyr (78% loss, Fig. 5A) and concomitant formation of 3-ClTyr (1.62 pmol, Fig. 5B). Addition of an alternative competing substrate (SCN⁻) for Compound I of MPO resulted in a dose-dependent increase in the concentration of parent Tyr detected, consistent with less modification of Tyr (corresponding to 35% and 19% loss of Tyr at 10 μM and 50 μM SCN⁻, respectively; Fig. 5A). A

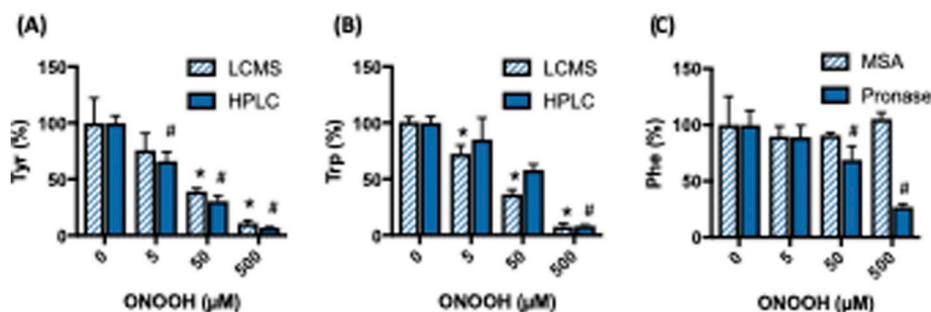


Fig. 7. Quantification of total (A) Tyr, (B) Trp in ONOOH-treated anastellin as determined by LCMS or HPLC (with OPA derivatization). Anastellin (5 μM) was treated with ONOOH (0–500 μM) at 37 $^{\circ}\text{C}$ for 30 min. Yield of Phe (C) after hydrolysis with MSA or pronase digestion. Data are presented as relative to the control condition (untreated). */# Indicates statistical significance against the untreated control sample (LCMS or HPLC, respectively, Panel A/B; MSA or pronase, respectively, Panel C) as determined by one-way ANOVA with post-hoc analysis using Dunnett's multiple comparison test.

concomitant decrease in the yield of 3-ClTyr was also observed (0.94 pmol at 1 μM SCN^- , below LOD at 10 and 50 μM SCN^- ; Fig. 5B). These data are consistent with previous reports on the protective effects of SCN^- against MPO induced, HOCl-mediated, protein damage in human plasma [28].

To determine whether the method is also applicable to more complex systems with a greater number of components and potentially interfering species, experiments were also carried out with lysates from murine J774A.1 macrophage-like cells. The lysates (25 μg protein) were treated with increasing doses of reagent HOCl (0–400 μM , see Materials and methods and Fig. 6). Analysis of the extracted cellular proteins showed a loss of parent Tyr (70 and 95% loss of Tyr with 200 and 400 μM HOCl, respectively; Fig. 6A) and detection of 3-ClTyr (320 fmol–36 pmol, Fig. 6C) with the concentrations detected increasing in a dose-dependent manner with higher HOCl concentrations. Slightly lower levels of 3-ClTyr were detected at 400 μM than 200 μM , probably as a result of increased conversion of 3-ClTyr to the di-chlorinated species 3,5-dichlorotyrosine (3,5-Cl₂Tyr), a known reaction with high HOCl excesses [44]. The formation of this di-chlorinated product is supported by a dose-dependent increase in MS peaks from ions with m/z 250.0038 assigned to this species (data not shown). The observed increase in 3-ClTyr could be quantified over 3 orders of magnitude, illustrating the large dynamic range of the developed assay. A concomitant loss of protein-bound Met, a kinetically-important target of HOCl [44], was also observed (50% loss of Met at 100 μM HOCl, 89% at 200 μM , Fig. 6B) together with the corresponding product Met sulfoxide (confirmed by comparison with an authentic standard). The latter product was not quantified. A dose dependent loss of Arg, His and Lys were also observed (Supplementary Fig. 1) as expected given the known reactivity of these side chains with HOCl [44], but no significant loss was detected for the other amino acids quantified in these experiments (data not shown).

The potential detection of other oxidation products was also explored. Thus, treatment of the isolated ECM protein component anastellin, which is a fragment of the first Type III domain of the key matrix protein fibronectin [45], with the nitrating agent ONOOH resulted in a dose-dependent loss of Tyr (24, 61 and 89% loss, at 5, 50 and 500 μM ONOOH, respectively; Figs. 6D and 7A) and Trp (27, 64 and 92% at 5, 50 and 500 μM ONOOH respectively; Figs. 6E and 7B), and a corresponding increase in the levels of the nitrated product 3-NO₂Tyr (0.48, 5 and 15.2 pmol at 5, 50, and 500 μM ONOOH respectively; Fig. 6F).

An alternative digestion method using the enzyme pronase, was examined with ONOOH-treated anastellin, but this resulted in relatively poor digestion at high oxidant doses (e.g. 500 μM), as indicated by reduced concentrations of amino acids such as Phe that do not react rapidly with ONOOH (Fig. 7). This poor digestibility of the modified protein may arise from poor accessibility of the proteolytic enzymes to putative cleavage sites in the modified protein (e.g. for steric or electronic reasons), but may also arise from the formation, with this protein, of insoluble aggregates at high oxidant excesses (He et al., unpublished data). Amino acid loss in ONOOH-treated anastellin was also determined by HPLC using OPA derivatization, a technique commonly used for amino acid analysis [22], and compared with the MS data

(Fig. 7A–C) The loss of parent amino acids (e.g. Tyr, Trp; Fig. 7A and B) with increasing ONOOH concentrations was consistent between the two techniques, though the HPLC method is limited to the quantification of parent amino acids, and cannot be used to determine the presence or concentration of low-abundance, or unknown, reaction products in the same analysis runs. The detection of these products using HPLC requires the use of alternative conditions and/or detectors (e.g. Ref. [4,40,42]), and therefore increases overall handling and analysis times.

4. Conclusions

Herein we demonstrate a versatile LC-MS assay for the simultaneous quantification of amino acids and a number of downstream oxidation products. LC-MS parameters were optimized to enable detection of low (Ala, Gly and Ser) and higher molecular mass analytes in single runs. The method was validated and tested for intra-/inter-day precision and accuracy, with this showing a dynamic range of 2–3 orders of magnitude for most analytes. Application of the method in three different biologically-relevant experimental systems (isolated proteins, mixtures of ECM proteins and cell lysates) with three different oxidant systems (MPO/H₂O₂/Cl⁻, reagent HOCl and reagent ONOOH) illustrates the versatility of the assay, with this allowing quantification of 3-ClTyr and 3-NO₂Tyr, as well as concomitant loss of the parent amino acids Tyr, Trp and Met in a single chromatographic analysis.

Declaration of competing interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.redox.2020.101586>.

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