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A comparative study of proteolysis methods for the measurement of 3-nitrotyrosine residues: Enzymatic digestion versus hydrochloric acid-mediated hydrolysis☆

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Abstract

A common approach for the quantification of 3-nitrotyrosine (NY) in routine analyses relies on the cleavage of peptide bonds in order to release the free amino acids from proteins in tissues or fluids. NY is usually monitored by either GC–MS(/MS) or LC–MS/MS techniques. Various proteolysis methods have been employed to combine digestion efficiency with prevention of artifactual nitration of tyrosine. However, so far, no study was designed to compare the HCl-based hydrolysis method with enzymatic digestion in terms of reliability for the measurement of NY. The present work addresses the digestion efficiency of BSA using either 6 M HCl, pronase E or a cocktail of enzymes (pepsin, pronase E, aminopeptidase, prolidase) developed in our laboratory. The HCl-based hydrolysis leads to a digestion yield of 95%, while 25 and 75% are achieved with pronase E and the cocktail of enzymes, respectively. These methods were compared in terms of NY measurement and the results indicate that a prior reduction of the disulfide bonds ensures a reliable quantification of NY. We additionally show that the enzyme efficacy is not altered when the digestion is carried out in the presence of BSA with a high content of NY.

Keywords: 3-Nitrotyrosine; Enzymatic digestion; Acidic hydrolysis; Mass spectrometry; LC–MS/MS; BSA

1. Introduction

Since 3-nitrotyrosine (NY) was claimed to be a marker for endogenous nitrosation and nitration in proteins [1,2], much attention has been devoted to the measurement of this amino acid modification in various biological systems [3–5] in order to gain insight into the mechanisms of some pathologies and oxidative stress conditions. For instance, increased formation of NY was shown in neurodegenerative diseases such as amyotrophic lateral sclerosis [6], rheumatoid arthritis [7], Alzheimer's disease [8] but also in septic shock [9] and kidney injury induced with lipopolysaccharides (LPS) [10].

For the detection of NY residues in proteins, several anti-NY antibodies were employed for both immunohistochemistry [10] and immunoblotting [11]. The immunohistochemistry is helpful to locate the accumulation of nitrated tyrosine residues in tissues while the immunoblotting is a valuable technique to estimate the NY levels in low amounts of proteins on a relative basis. Although these methods are highly sensitive, they are limited by a possible lack of specificity of the antibodies and by potential modulation of the antibody affinity by the protein context of the epitope. Thus, they may be unsuitable for an accurate quantification of NY residues in proteins, particularly when comparing samples with moderate differences. Mass spectrometry (MS) has become an increasingly important tool in the determination of protein modifications and consequently, appears as the technique of choice to measure the levels of NY residues. Several MS approaches have been developed depending on the type of information sought. When structural data are expected in a particular protein with only a semi-quantification, the analysis by either MALDI- or (nano)electrospray-MS on a tryptic pro-

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tein digest provides reliable results. This has been successfully applied for tetranitromethane-nitrated human surfactant protein A [12], bovine serum albumin (BSA) [13–15] and angiotensin II [15]. With this approach, the accurate quantification of NY residues, when possible, implies a tedious and time consuming work which cannot be expanded for high-throughput routine analyses. Hence, the quantification of protein-bound nitrated tyrosine residues is usually conducted by analyzing free NY by either GC–MS(/MS) or LC–MS/MS after cleavage of the peptide bonds (enzymatic or acidic hydrolysis) in the protein(s) of interest. In most of the cases, an additional clean-up step is necessary prior to the analysis in order to obtain a reliable chromatographic profile and, with GC–MS(/MS) technique, a derivatization of NY must be carried out in order to convert the analyte into a volatile compound. Some steps of the sample preparation may induce an artifactual nitration of tyrosine due to the presence of significant amounts of either nitrite or nitrate in biological tissues or fluids [16–18]. Some investigators addressed the artifactual nitration of tyrosine, and efforts have been made to develop sample preparations which ensure artifact-free or artifact-controlled measurements of NY [19–21]. In essence, the acid treatment is the major source of artifactual nitration of tyrosine [1,22]. Consequently, protein hydrolysis with hydrochloric acid (6 M at 110 °C) and some derivatization conditions employed when using GC–MS and GC–MS/MS quantification may lead to an overestimation of the NY level in biological samples.

Therefore, the best approach to avoid the artifactual formation of NY during the analytical process is the combination of several enzymes for protein digestion prior to LC–MS/MS analysis. The required derivatization of NY for GC–MS(/MS) can be avoided by using the LC–MS/MS technique (unless used for a particular purpose) and the enzymatic proteolysis ensures mild conditions in terms of pH and temperature. However, the use of an enzymatic digestion does not necessarily mean that the measured level of NY exactly reflects the endogenous level as incomplete protein digestion may lead to erroneous measurements.

In the present study, we compared different proteolysis methods using either hydrochloric acid or two enzymatic digestions with BSA as model protein. The two enzymatic digestion approaches were based on the use of pronase E [23], commonly employed for the measurement of NY in proteins, and on a cocktail including pepsin, pronase E, aminopeptidase and prolidase, that was designed to ensure a more complete digestion of BSA. With this cocktail, the influence of the reduction of the disulfide bridges was also investigated. The comparison of the methods was based on the digestion yield (HPLC and amino acid analysis) and the measurement of protein-bound NY (isotope dilution LC–MS/MS). Furthermore, the influence of the nitration rate on the performance of the enzymatic digestions was evaluated.

2. Experimental

2.1. Chemicals and reagents

Hydrogen peroxide, dichloromethane, acetonitrile, 5 M NaOH, 37% HCl, ammonium acetate, ammonium formate, ammonium carbonate and tris(hydroxymethyl)aminomethane were from Merck (Darmstadt, Germany). Isoamylnitrite and triethylphosphine were purchased from Fluka (Buchs, Switzerland). Manganese dioxide was obtained from Riedel-de-Haën (Stockholm, Sweden). Diethylenetriaminepentaacetic acid, trifluoroacetic acid (TFA), BSA, pepsin, leucine aminopeptidase, prolidase and iodoethanol were provided by Sigma–Aldrich (Steinheim, Germany). Ninhydrin and pronase E were supplied by Serva (Heidelberg, Germany). Stable-isotope labelled α,β,γ,1,2,3,4,5,6-$^{13}C_9$-tyrosine (97–98 at.% ^{13}C, ($^{13}C_9$)-tyrosine) and 2,3,5,6-d_4-tyrosine (99.1 at.% ^{2}H), used for the preparation of 2,5,6-d_3-3-nitrotyrosine ((d_3)-NY) as described elsewhere [24], were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Two batches of commercial ($^{13}C_9$)-tyrosine were used to perform our experiments, and both of them contained α,β,γ,1,2,3,4,5,6-$^{13}C_9$-3-nitrotyrosine (($^{13}C_9$)-NY) as an impurity. The ($^{13}C_9$)-NY contents, measured in each batch, were 19.7 ± 0.9 and 40.7 ± 5.8 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine.

2.2. Nitration of non-modified BSA in the presence of peroxynitrite

The preparation of peroxynitrite was adapted from Ref. [25]. Briefly, a solution of hydrogen peroxide (in 5 M NaOH and 0.04 M diethylenetriaminepentaacetic acid) was supplemented with isoamylnitrite and incubated at room temperature (23 °C) for 3 h. Then, the aqueous phase was washed with dichloromethane and applied onto a manganese dioxide-column. The column was washed sequentially with water and 5 M NaOH to collect peroxynitrite. The concentration of peroxynitrite was calculated by UV ($\varepsilon = 1670\ M^{-1}\ cm^{-1}$ at 302 nm [26]) and was found to be 0.29 M (yield: 24%). One hundred microliters of peroxynitrite at 0.029 M (diluted in 0.1 M NaOH) was added to 12 ml of a non-modified BSA (*non-modified BSA* refers to commercial BSA which was not nitrated) solution (1 mg/ml) under vigorous stirring. After 15 min, the solution was dialyzed against water for 24 h and further aliquoted in 500 μl-fractions (500 μg nitrated BSA in each fraction) before storage at −20 °C until use.

2.3. Hydrochloric acid-mediated BSA hydrolysis

Fractions containing 500 μg of BSA (7.52 nmol) were spiked with 22 μg of a ($^{13}C_9$)-tyrosine solution (115.8 nmol) and evaporated to dryness. The dry residues were dissolved in 1 ml of HCl 6 M and the resulting solutions 24 h-incubated at 110 °C. Then, the samples were evaporated with a Speed Vac Concentrator (Savant, Farmingdale, NY, USA) and reconstituted in an appropriate solvent for purification by solid phase extraction (SPE) (*vide infra*).

2.4. BSA digestion in the presence of pronase E

BSA-containing fractions (500 μg, 7.52 nmol) were spiked with 22 μg of ($^{13}C_9$)-tyrosine solution (115.8 nmol) and evaporated to dryness. The dry residues were reconstituted in 1 ml

of 0.1 M sodium acetate, and 15 μl of a pronase E solution (1 mg/ml) in 2 M Tris buffer (pH 8.2) was added. Samples were incubated either for 24 or 48 h at 37 °C (pronase incubations at 50 °C were not considered as protein precipitation may occur with biological material at such a temperature). For 48 h-incubations, another 15 μl of the pronase E solution was added after 24 h of incubation. The enzymatic digestion was stopped by adding 10 μl of TFA to the samples prior to evaporation to dryness with a Speed Vac concentrator. The dry residues were dissolved in 1 ml of 10 mM ammonium acetate and purified by SPE as described below.

2.5. Multi-enzymes digestion of BSA

Fractions containing 500 μg of BSA (7.52 nmol) were supplemented with 22 μg of ($^{13}C_9$)-tyrosine solution (115.8 nmol) prior to evaporation to dryness. The dry residues were dissolved in 1 ml of 0.02 M HCl, and 2 × 18 μl of a pepsin solution (1 mg/ml in 0.02 M HCl) were added for twice 1 h-incubation at 37 °C. Then, the samples were buffered with 250 μl of 2 M Tris (pH 8.2), 2 × 15 μl of a pronase E solution (1 mg/ml in Tris buffer 2 M) were added and twice incubated at 37 °C for 1 h. The samples were further supplemented with 20 μl of an aminopeptidase solution (0.295 mg/ml or 7.08 U/ml in water) and 14 μl of a prolidase solution (0.76 mg/ml or 67 U/ml in 2 M Tris buffer) simultaneously. The incubation was continued for 24 h at 37 °C. Finally, 2 × 15 μl-portions of the pronase E solution were added into the samples for a 1 h-incubation at 37 °C. The enzymatic digestion was stopped with 10 μl of TFA, and the samples were evaporated to dryness prior to purification by SPE as described below.

2.6. Reduction of the disulfide bridges

This step was adapted from methods reported elsewhere [27,28]. Typically, aliquots of 500 μg BSA were reconstituted in 1 ml of 1 M ammonium carbonate (pH 10.5). The reduction reagent was prepared by mixing 5 μl of triethylphosphine and 20 μl of iodoethanol in 975 μl of acetonitrile. One hundred microliters of reduction reagent was applied into the ammonium carbonate solution of BSA and the mixture was incubated 1 h at 37 °C. Samples were evaporated to dryness with a Speed Vac concentrator, and redissolved in 200 μl of water for a second evaporation to dryness before an enzymatic digestion.

2.7. Solid phase extraction

Dried samples were reconstituted in 1 ml of 10 mM ammonium acetate (pH 6.7) and the resulting solutions were spiked with 3 ng of (d_3)-NY and 1.5 ng of ($^{13}C_9$)-NY. Samples were loaded onto preconditioned octadecylsilyl 500 mg Bond Elut cartridges (Varian, Middelburg, The Netherlands). The cartridges were washed with 1 ml of 2 mM ammonium acetate, tyrosine was eluted with 1 ml of methanol–2 mM ammonium acetate (5:95, v/v) and NY with 1.5 ml of methanol–2 mM ammonium acetate (20:80, v/v). The fractions containing NY were loaded onto preconditioned aminopropyl 500 mg Isolute cartridges (Separtis, Grellingen, Switzerland) and 2 ml of 2 mM ammonium formate (pH 3.5) was used to wash the stationary phase. The elution of NY was carried out with 1.5 ml of water–acetic acid (25:75, v/v). Fractions were evaporated to dryness and the reconstitutions were made with 60 μl of water for NY while 600 μl was used for tyrosine. Samples were kept at 4 °C for further LC–MS/MS analysis. Details about the performance of the method can be found elsewhere [21,29,30].

2.8. Analysis of BSA and BSA hydrolysates by HPLC-UV

The HPLC system consisted of a Hewlett-Packard Series 1050 device (Agilent, Geneva, Switzerland) equipped with a SymmetryShield RP8 column (2.1 mm i.d. × 150 mm, 3.5 μm particle size) from Waters (Milford, MA, USA). With TFA 0.1 vol.% as solvent A and acetonitrile as solvent B, the gradient was: from 0 to 8 min: 100% of solvent A; from 8 to 30 min: a linear gradient to solvent A–solvent B (50:50, v/v). The flow rate was set at 300 μl/min and the wavelength detection selected at 277 nm.

2.9. Calculation of the proteolysis yield

The composition of amino acids in non-modified BSA was performed with a High Performance Analyzer System 6300 from Beckman Coulter (Fullerton, CA, USA) as an adaptation from Refs. [31,32]. Amino acids were separated on a cation exchange column and the detection was carried out by post-column derivatization with ninhydrin. Derivatized amino acids were detected by photometry using wavelengths at 570 and 440 nm. The amino acids were quantified by external calibration and the resulting amounts were summed to estimate the digested portion of non-modified BSA. The proteolysis yield (expressed in %) was obtained with the ratio: measured digested portion of non-modified BSA/amount of non-modified BSA subjected to digestion. With 6 M HCl, asparagine, glutamine, methionine, tryptophan and cysteine were not considered for the calculation as these residues are not stable under such conditions.

2.10. Measurement of NY by LC–MS/MS

The analysis of NY residues in proteins was reported in detail elsewhere [21,29]. Briefly, the LC–MS/MS system consisted of a HP series 1100 (Hewlett-Packard, Palo-Alto, CA, USA) coupled to a Finnigan MAT TSQ 7000 tandem mass spectrometer (San Jose, CA, USA) equipped with the API 2 interface. The column was a microbore XTerra MC C_{18} (1.0 mm i.d. × 150 mm, 3.5 μm particle size) from Waters (Milford, MA, USA) and the positive electrospray was chosen as ionization mode. The acquisition was performed in the selected reaction monitoring mode and the transitions were: NY: *m/z* 227 → 181 (collision energy (CE) set at 20 eV) as quantifier and *m/z* 259 ([M + methanol + H]$^+$) → 181 (CE set at 24 eV) as qualifier; (d_3)-NY: *m/z* 230 → 184 as quantifier and *m/z* 262 → 184 as qualifier; ($^{13}C_9$)-NY: *m/z* 236 → 189 as quantifier and *m/z* 268 → 189 as qualifier; tyrosine: *m/z*

182 → 165 (CE set at 22 eV) as quantifier and *m/z* 182 → 136 (CE set at 28 eV) as qualifier; ($^{13}C_9$)-tyrosine: *m/z* 191 → 174 as quantifier and *m/z* 191 → 144 as qualifier. Dwell time per transition: 400 ms.

2.11. Control of the artifactual nitration of tyrosine

To evaluate the potential artifactual nitration of tyrosine during the proteolysis, we developed a strategy based on the monitoring of ^{13}C-labeled NY, generated by the nitration of ^{13}C-labeled tyrosine supplemented in the protein sample prior to the hydrolysis step. This was described in detail elsewhere [21], and only a short overview of the method is given hereafter. The amount of added ($^{13}C_9$)-tyrosine is chosen in order to obtain concentrations of both tyrosine (from the sample) and ($^{13}C_9$)-tyrosine at the same order of magnitude in the final extract. During the proteolysis, any contamination which may nitrate tyrosine residues reacts similarly with both protein-originating tyrosine and spiked ($^{13}C_9$)-tyrosine, leading to the formation of NY and ($^{13}C_9$)-NY, respectively. The hydrolysate is further supplemented with (d_3)-NY (internal standard used for an accurate quantification of NY and ($^{13}C_9$)-NY) prior to sample work-up and LC–MS/MS analysis. The measured number of ($^{13}C_9$)-NY per ($^{13}C_9$)-tyrosine in the sample is compared to a reference value (a non-hydrolyzed standard containing a mixture of ($^{13}C_9$)-tyrosine, ($^{13}C_9$)-NY and (d_3)-NY) in order to evaluate a possible artifactual conversion of tyrosine into NY). Typical chromatograms of artifact-free and hydrolysis-nitrated biological samples are depicted in Fig. 1, where only the traces of NY, ($^{13}C_9$)-NY and (d_3)-NY are shown.

3. Results

3.1. Artifactual nitration of tyrosine

Our approach, encompassing the monitoring of the potential artifactual nitration of tyrosine, was validated by comparing the level of ($^{13}C_9$)-NY in sodium nitrite-contaminated non-modified BSA hydrolyzed with either 6 M HCl or pronase E (Fig. 2). Results, obtained after SPE and LC–MS/MS analysis, show that non-modified BSA which was not treated with sodium nitrite did not induce any artifactual nitration of tyrosine residues whatever the hydrolysis method used; the ($^{13}C_9$)-NY level ranged from 18.2 to 25.8 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine, while the reference value for the molar ratio obtained from a mixture of standards which was not submitted to proteolytic conditions was measured at 19.7 ± 0.9 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine. In contrast, the level of ($^{13}C_9$)-NY increased in a time-dependent manner when non-modified BSA was supplemented with sodium nitrite at an amount of 6.7 μg (97 nmol) per mg of BSA (15 nmol) and hydrolyzed with 6 M HCl. After a 3 h-incubation, values of 28.6 ± 1.2 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine was obtained, while the level reached 95.7 ± 4.6 and 124.6 ± 6.1 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine when the incubation was conducted for 6 or 24 h, respectively. In contrast, when non-modified BSA contaminated with sodium nitrite was digested by pronase E, the molar ratio of ($^{13}C_9$)-NY to ($^{13}C_9$)-tyrosine remained almost constant during the 24 h incubation period (the slight increase at 24 h should be rationalized in terms of experimental variations rather than artifact induction).

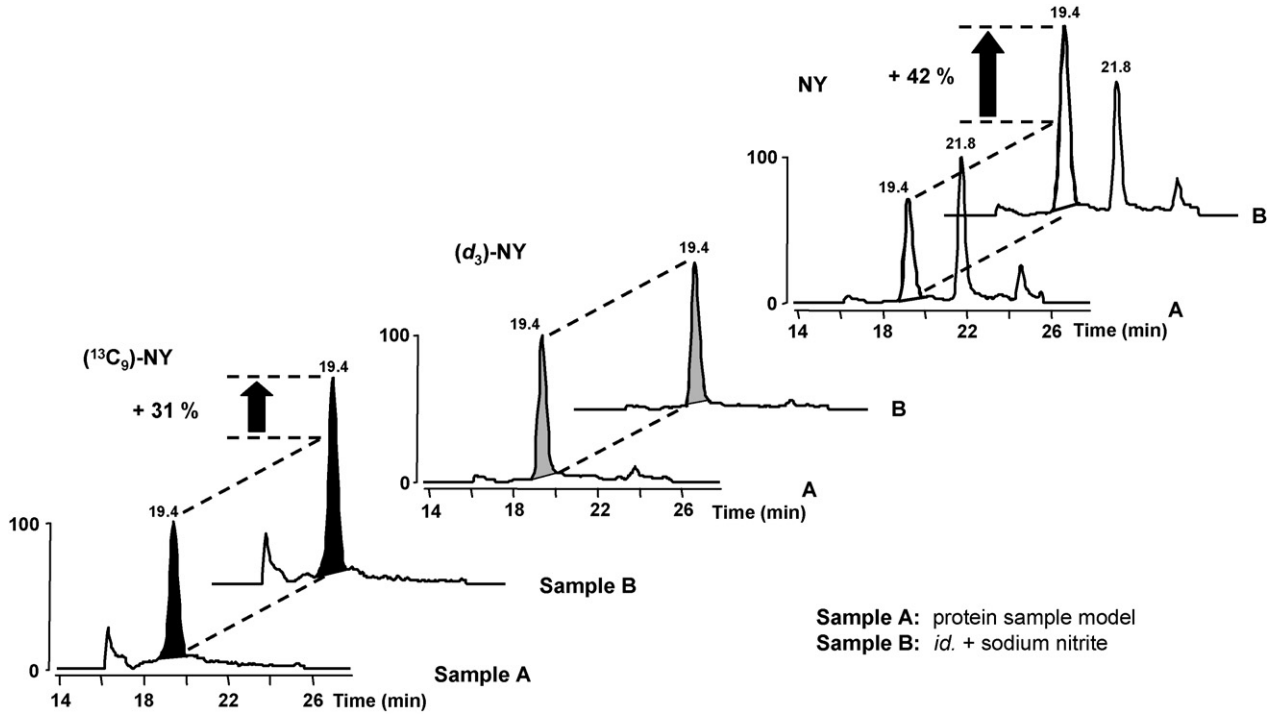

Fig. 1. Chromatographic profiles of NY (transition *m/z* 227 → 181), (d_3)-NY (transition *m/z* 230 → 184) and ($^{13}C_9$)-NY (transition *m/z* 236 → 189) in our model sample (sample A) and the same one supplemented with sodium nitrite (sample B), after acidic hydrolysis with 6 M HCl. The peak at 21.8 min corresponds to an unknown contamination, while tyrosine elutes at 7.6 min.

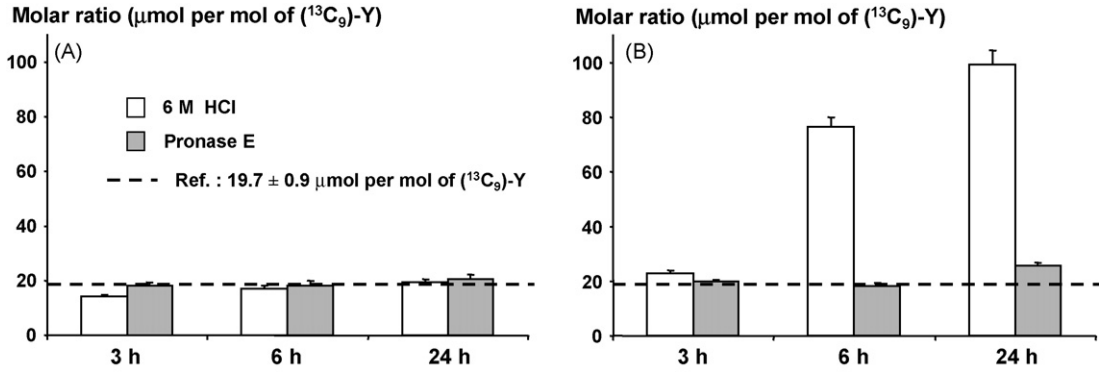

Fig. 2. Time course of the molar ratio of ($^{13}C_9$)-NY to ($^{13}C_9$)-tyrosine in hydrolysates of non-modifed BSA without external addition of sodium nitrite (A) and in hydrolysates of BSA supplemented with sodium nitrite at 6.7 μg per mg of BSA (B). Both incubations were submitted to either 6 M HCl-based hydrolysis or digestion with pronase E (Ref. stands for Reference: a mixture of standards which was not submitted to any proteolytic conditions prior to analysis).

Table 1
Levels of NY and ($^{13}C_9$)-NY[a] in BSA samples hydrolyzed with hydrochloric acid or digested with enzymes

	Non-modified BSA		Nitrated BSA[b]	
	NY[c]	($^{13}C_9$)-NY[d,e]	NY[c]	($^{13}C_9$)-NY[d,e]
6 M HCl ($n = 3$)	38.3 ± 10.7	41.6 ± 1.5	5256 ± 522	41.0 ± 7.5
Pronase E	18.9 ± 5.6	36.1 ± 3.3	2190 ± 759	40.3 ± 10.6
Multi-enzymes digestion	21.9 ± 2.3	43.7 ± 6.7	4062 ± 506	36.4 ± 3.1
Reduction + multi-enzymes digestion	37.6 ± 6.2	37.2 ± 5.9	5544 ± 582	45.3 ± 1.9

[a] Mean ± S.D., $n = 4$ unless specified.
[b] For details, refer to Section 2.2.
[c] μmol per mol of tyrosine.
[d] μmol per mol of ($^{13}C_9$)-tyrosine.
[e] The reference was set as 40.7 ± 5.8 μmol ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine.

The measurement of ($^{13}C_9$)-NY was also carried out in each sample of BSA nitrated with peroxynitrite (Table 1), and the molar ratio ranged from 36.4 to 45.3 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine with a reference value at 40.7 ± 5.8 μmol of ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine. This suggests that no product linked to the addition of peroxynitrite mediated the nitration of tyrosine residues in nitrated BSA in a detectable manner using either a 24 h-incubation time under acidic conditions or one with enzymes at neutral pH (48 h-incubation).

3.2. Completeness of the digestion

Initially, the completeness of the digestion of non-modified BSA was controlled by HPLC-UV and the profiles of the extracts obtained with either 6 M HCl or pronase E were compared at 277 nm (Fig. 3). Our results show that no BSA was detectable when the hydrochloric acid conditions were used for the hydrolysis, indicating that the albumin was cleaved completely. In contrast, non-modified BSA was still detectable (at a low level) in the extract obtained by the digestion with pronase E. From the chromatographic profile, the occurrence of several peaks in the retention time range 15–24 min strongly suggested that numerous peptides remain undigested by pronase E. This was consistent with the results of the amino acids analyser which show that only 24.9 ± 4.2% (mean ± S.D., $n = 4$) of the non-modified BSA was cleaved as free amino acids using pronase E for 48 h, whereas the best digestion was achieved with 6 M HCl (digestion yield of 94.3 ± 6.0%, $n = 4$). Interestingly, in the time range of 0–24 h, the use of 2 wt.% pronase E appeared to be efficient enough to cleave non-modified BSA partially (digestion yield of 20.8 ± 3.4%, $n = 4$) while a second addition of pronase E (2 wt.%) at 24 h for a total incubation duration of 48 h did

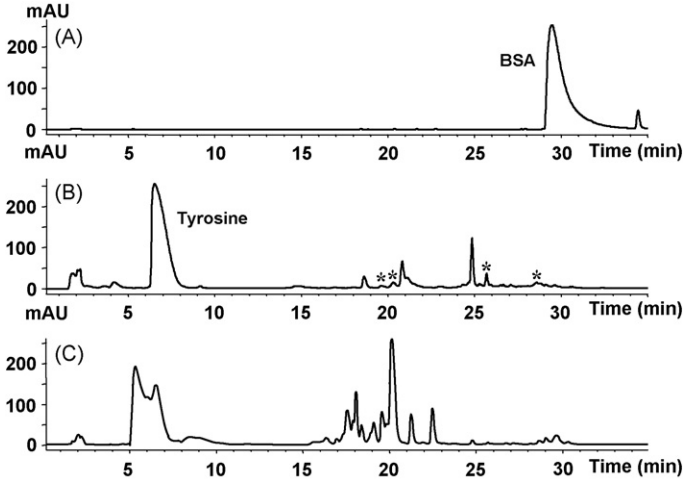

Fig. 3. HPLC-UV chromatographic profiles (detection wavelength at 277 nm) of BSA (A), BSA hydrolysates obtained with 6 M HCl (B, peaks marked with asterisks are also present in the blank) or 48 h-incubation in the presence of pronase E (C).

not improve significantly the efficiency of the digestion which increased to 24.9 ± 4.2% only. This demonstrated that, after a 24 h-incubation, the incomplete digestion of the peptides was mainly due to a lack of reactive sites for pronase E and not due to the autolysis of the enzyme. Therefore, we developed a strategy based on a multi-enzymes digestion to increase the proteolysis efficacy. The digestion encompassed a first incubation in the presence of pepsin to cleave BSA at the C-terminus of the residues tyrosine, phenylalanine, tryptophan and leucine [33]. A subsequent treatment with pronase E was aimed at generating unspecific cleavages prior to incubation with aminopeptidase and prolidase simultaneously to form short peptides [34] and cleave proline-containing loops [35]. Finally, another treatment with pronase E was applied to further digest the remaining peptides and ensure a cleavage as complete as possible. With this approach, the non-modified BSA was digested with a yield of 72.3 ± 4.6% ($n = 4$) when no preliminary reductive cleavage of cystine disulfide bonds was performed, and this value increased to 73.9 ± 2.1% ($n = 4$) when the cysteine residues were alkylated in the presence of triethylphosphine and iodoethanol.

The amino acid profile of a blank sample (no BSA) digested according to the multi-enzyme method led to a 8%-weight-load in the amino acids profile of digested non-modified BSA; this was taken into account for the calculation of the digestion yield.

3.3. Measurement of the NY level in commercial BSA

Our results indicate that the digestion of non-modified BSA with several enzymes, even if good, did not allow a complete release of the amino acids when compared to the hydrochloric acid-mediated hydrolysis (*vide supra*). Therefore, in order to study the influence of this trend on the measurement of the nitrated tyrosine residues, the LC–MS/MS technique was used to quantify NY in commercial BSA (either non-modified or nitrated) digested by either chemical or enzymatic methods. In each sample, the level of ($^{13}C_9$)-NY was measured to check for any potential artifactual nitration of tyrosine which may have led to an overestimation of NY; this is of particular interest for 6 M HCl since acidic conditions promote the nitration of the aromatic ring of tyrosine residues. No significant increase in the level of ($^{13}C_9$)-NY was obtained either in hydrochloric acid- or enzyme-based proteolysis method when compared to the level in the reference (Table 1). Since the HCl-based hydrolysis of non-modified BSA was shown to be complete and artifact-free, and due to the fact that NY is stable in 6 M HCl over 24 h [21], we assumed that the level of 38.3 μmol NY per mol of tyrosine was the real value in our batch of non-modified BSA. Our measurements show that the NY-to-tyrosine molar ratio obtained with a pronase E-based digestion is approximately half the value found with 6 M HCl. When the multi-enzymes digestion was applied, a slight increase (by 14%) in the level of NY was observed when compared to a digestion using pronase E but still, the value was significantly lower than the reference obtained with 6 M HCl. In a sharp contrast, the level of NY was similar to the reference (37.6 μmol NY versus 38.3 μmol NY per mol of tyrosine) when the multi-enzymes digestion was preceded by a reduction of the disulfide bridges.

This comparison was extended to a peroxynitrite-nitrated BSA in order to investigate a potential loss of enzymatic efficiency when the extent of nitration of BSA was increased. The measurements of ($^{13}C_9$)-NY indicated that no artifactual nitration of tyrosine potentially generated by any compound linked with the addition of peroxynitrite occurred during the proteolysis; in the samples, 36–45 μmol ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine was found while the reference was at 40.7 ± 5.8 μmol ($^{13}C_9$)-NY per mol of ($^{13}C_9$)-tyrosine. Again, the NY-to-tyrosine molar ratios in nitrated BSA obtained with either pronase E or the multi-enzymes digestion (without preceding S–S bond cleavage of cystine residues) were lower than the value resulting from the hydrolysis with 6 M HCl (58 and 23% decreases, respectively). In the case of a multi-enzymes digestion, the level of NY was not significantly different from that obtained with 6 M HCl when a preceding reduction of the disulfide bridges was applied.

The chromatographic profile of a blank sample, *i.e.* in the absence of externally added BSA, treated with the multi-enzymes method did not exhibit any peak above the background in the traces corresponding to the transitions of NY. With our analytical conditions the limit of detection was 0.72 μmol NY per mol tyrosine.

4. Discussion

4.1. Performance of the proteolysis methods

Our results clearly demonstrate that enzymatic digestion of proteins are attractive alternatives to hydrochloric acid-based hydrolysis to prevent the artifactual generation of NY from the reaction of tyrosine with nitrite or nitrate present in biological fluids and tissues. However, a potential disadvantage of an enzymatic proteolysis is the autolysis of the enzyme(s) which leads to an incomplete digestion of the substrate and may contribute to the level of amino acids of interest in the medium.

Furthermore, the activity of some enzymes may be too specific to completely cleave proteins/peptides, leading to a trend in the measurement. In the case of BSA, we have shown that the digestion using pronase E (commonly employed for the analysis of NY) is not complete and lead to an underestimation of the level of NY residues. Interestingly, we also show that the partial digestion of BSA is mainly due to a lack of activity of pronase E towards the peptides in the digestion medium and not only to its autolysis. The combination of several digestion steps in the presence of pepsin, pronase E, aminopeptidase and prolidase significantly improves the digestion yield. When the cystine disulfide bonds in BSA are first reduced, the digestion efficacy is only slightly improved; however, this allows to obtain NY levels similar to those detected with 6 M HCl proteolysis.

It should be pointed out that these results have been obtained on a single protein (BSA), and additional investigations should be carried out to confirm these results with other proteins or in complex protein mixture. One should keep in mind that the low level of NY determined after pronase E digestion may be rationalized in terms of a lack of reactivity of the enzyme on particular sites of BSA-originating peptides. It is likely that these

peptides contain tyrosine and NY residues in such a ratio that the measurement of free NY and tyrosine in the extract lead to an under-estimation of the endogenous level of NY (NY-rich peptides are poorly digested while tyrosine residues are released in a quantitative manner). For any structural reason, this may not happen in another protein and the measured value may be similar to the target value (NY and tyrosine residues are digested in such a way that the ratio NY/tyrosine reflects the endogenous one) or a possible case would be a digestion which releases NY with a good yield while numerous tyrosine residues remain undigested. In the later case, the measured value exceeds the target value.

4.2. Measurements in biological samples

Several authors have reported basal levels of protein-bound NY in various tissues. In the light of the present results it appears interesting to compare the values in the literature and, on the basis of the proteolysis methods reported here, interpret them in terms of relevance. NY residues were quantified in proteins of rat liver, heart and plasma as well as human plasma (Table 2). In rat liver, the levels range from 0.18 to 479 μmol of NY per mol of tyrosine depending of the assay employed. The highest values were obtained by Leeuwenburgh et al. who cleaved liver proteins with 6 M HCl in the presence of phenol and benzoic acid (1% each), measuring NY as the *N*-propyl-heptafluorobutyryl derivative by isotope dilution negative-ion chemical ionization GC–MS [36]. Under these hydrolysis conditions, the proteins were completely converted into a mixture of free amino acids; however there is some concern about a possible nitration of tyrosine residues during the sample preparation. In terms of biological significance, these measurements suggest that the proteins contained up to 4 NY per 10,000 tyrosine residues approximately, which raises the question of cell viability at such a high content of tyrosine modifications. Phenol and benzoic acid were added during hydrolysis to avoid artifactual nitration, but it was demonstrated that these compounds only partly prevent the sample preparation-mediated nitration of tyrosine [21]. In addition, the derivatization of NY was conducted in the presence of *n*-propanol–HCl (3:1, v/v) without any tyrosine preservative, suggesting a possible formation of artifact at this stage. These investigators have also measured NY residues in skeletal muscles of rat and their results did not show any significant difference between young (9 months) and old animals (24 months) [36], whereas Western blot and HPLC (gas phase hydrolysis) analyses performed on the SERCA2a isoform of sarcoplasmic reticulum Ca-ATPase of rat skeletal muscle revealed a three to four-fold increase in NY levels in 28-month old relative to 5-month old animals [37]. The two sets of data suggest that the endogenous level of NY measured in rat muscle by GC–MS as the *N*-propyl-heptafluorobutyryl derivative following a HCl-based proteolysis is hidden by a high background induced by the artifactual nitration of tyrosine residues. This suggests that the age-related increase in NY in rat liver (if any), attempted to be observed by Leeuwenburgh et al. [36], is masked by a high background of artifactual nitration in the detected signal.

Interestingly, the levels of NY in rat liver obtained by enzymatic digestion were found to be 42- and 2200-fold lower using proteinase K [38] and pronase E [11], respectively. On the basis of our results obtained with BSA, a certain underestimation of the NY level using pronase E seems possible but a comprehensive study should be carried out to confirm this assumption. Nevertheless, the discrepancies between the measurements obtained with proteinase K and pronase E demonstrate that proteolytic digestion with enzymes requires a meticulous optimization to ensure a reliable quantification.

So far, rat heart proteins have never been digested with enzymes prior to NY analysis, and only hydrochloric acid was used for that purpose. The results published in the literature support the fact that the hydrolysis of proteins from biological samples with 6 M HCl should be carried out with extreme care to avoid an overestimation of the protein-bound NY level. When HCl was used in the vapor phase, the NY levels were found to be less than 15 μmol NY per mol tyrosine as measured

Table 2
Measurements of protein-bound NY in rat and human tissues and fluids

Tissue/fluid	Value	Proteolysis method	Reference
Rat liver[a]	9.5 ± 1.1	Proteinase K (1 U/10 mg protein, 18 h at 55 °C)	Skinner et al. [38]
	311–479	6 M HCl + 1% phenol + 1% benzoic acid (24 h at 110 °C)	Leeuwenburgh et al. [36]
	0.18 ± 0.09	Pronase E (2 mg/10 mg protein, 16 h at 50 °C)	Girault et al. [11]
Rat heart[a]	154 ± 31	6 M HCl + 1% phenol (24 h at 110 °C)	Crowley et al. [39]
	112–141	6 M HCl + 1% phenol + 1% benzoic acid (24 h at 110 °C)	Leeuwenburgh et al. [36]
	<15	Vapor-phase HCl (110 °C)[b]	Yi et al. [19]
Rat plasma[a]	0.37 ± 0.32	Pronase E (2 mg/10 mg protein, 16 h at 50 °C)	Shigenaga et al. [22]
	23 ± 12	Pronase (1 mg/10 mg protein, 16 h at 50 °C)	Sodum et al. [40]
	9.7 ± 5.1	6 M HCl (24 h at 110 °C)	Delatour et al. [21]
	4.6 ± 0.8	6 M HCl (24 h at 110 °C)	Delatour et al. [29]
Human plasma[c]	11.9 ± 1.8	4 M NaOH (16 h at 120 °C)	Frost et al. [20]
	0.6 ± 0.4	Pronase from *S. griseus* (2 mg/10 mg protein, 18 h at 50 °C)	Söderling et al. [44]

[a] μmol per mol Y.
[b] Time not specified.
[c] pmol per mg protein.

by LC–MS/MS [19], whereas it was estimated to be in the range 110–160 μmol NY per mol tyrosine using liquid phase conditions using GC–MS [36,39].

For the detection of NY in rat plasma proteins, two methods using enzymatic digestion with pronase yielded inconsistent data; 23 ± 12 μmol NY per mol tyrosine was obtained by liquid chromatography-electrochemical detection [40], while 62-fold lower levels were found with an approach based on the conversion of NY into 3-aminotyrosine and analysis by the same technique [22]. The approach developed by Shigenaga and co-workers encompasses several steps (acetylation, extraction, *O*-deacetylation and reduction) which can induce some losses of material and lead to a slight underestimation of NY, particularly because no internal standard was used. Each step implies an efficient chemical reaction but some analyte loss during the sample preparation can never be excluded. In addition, the stability of 3-aminotyrosine in aqueous solution is limited [41], and this method may also be suspected to under-estimate the levels of NY. This may also explain, at least in part, the low level obtained by Girault et al. [11] in rat liver, using the method developed by Shigenaga et al. [22]. Rat plasma proteins were also measured with 6 M HCl combined with LC–MS/MS, and protein-bound NY was found in the range of 4–10 μmol NY per mol tyrosine [21,29]. Interestingly, with a 95% confidence interval, the value of 9.7 ± 5.1 μmol NY per mol tyrosine ($n = 6$) [21] is not statistically different from 23 ± 12 μmol NY per mol tyrosine ($n = 3$) [40], and the higher value obtained with a pronase-based proteolysis may be rationalized in terms of a lower digestion of some tyrosine-rich peptides. It should be mentioned that the two sets of values obtained with rat plasma by Delatour et al. [21,29] can not be considered as different; indeed, when a *t*-test (5% level) is carried out with 9.7 ± 5.1 μmol NY per mol tyrosine ($n = 6$) [21] and 4.6 ± 0.8 μmol NY per mol tyrosine ($n = 2$) [29], the values do not appear statistically different.

With human plasma, an alkaline hydrolysis was proposed to cleave proteins and the artifactual nitration of tyrosine was controlled based on the measurement of trideuterated NY after supplementation of the samples with tetradeuterated tyrosine and analysis by GC–MS [20]. Unlike some particular amino acids (cysteine, serine, arginine and threonine), both tyrosine and NY are stable in 4 M NaOH at 120 °C (16 h) and thus, it could be assumed that the value at 11.9 ± 1.8 pmol NY per mg protein (which corresponds to 35.5 ± 5.5 μmol NY per mol tyrosine) is a good estimation of the endogenous level. However, when Frost et al. [20] applied their method for the measurement of free NY in human plasma, they found a concentration at 64 nM while several recent studies applying LC–MS/MS and GC–MS/MS techniques suggest that it should rather range of 0.7 to 2.8 nM [19,42–44]. Therefore, even if Frost et al. [20] claim the use of an artifact-free method, the reliability of their measurements in human plasma is questionable, and the level at 12 pmol NY per mg protein appears to be overestimated. This concern was discussed in detail elsewhere [4]. In contrast, when pronase was employed for the analysis of NY residues in human plasma, the basal level was found to be 0.6 pmol per mg of protein [44]. Interestingly, the quantification of the NY residues in human serum albumin (HSA) isolated by affinity column chromatography provided basal levels within a range of 0.4–1.55 μmol NY per mol tyrosine [43]. With a HSA molecular mass at 66 446 [45] and taking into account the nineteen NY residues in the protein (Swissprot database, www.expasy.org/uniprot/P02768), the NY level in HSA may be expressed as 0.11–0.44 nmol NY per mg HSA, which is more than 100-times lower than the value reported by Frost et al. [20]. Since the results of the present study suggest that pronase E slightly underestimates (about two-fold) the number of NY residues in proteins, a realistic estimation of the NY level in human plasma proteins and/or serum albumin would be 0.2–1.0 pmol per mg of protein. However, it is likely that the efficacy of pronase E is protein structure-dependent and the observation found for BSA in the current study should be confirmed in other protein systems.

5. Conclusion

A prerequisite to the employment of biomarkers as potential indicators of certain pathologies or degenerative conditions is the availability of validated analytical methods that are accurate and reproducible. This is a major concern for the quantification of NY residues in proteins since the measurement of this nitration product of tyrosine may be subjected to artifacts and/or incomplete digestion of proteins. In the present paper, we show that the digestion of BSA with pronase E, an enzyme commonly used for the analysis of protein-bound NY, does not lead to a total release of the amino acids while a complete digestion was achieved with a chemical hydrolysis in 6 M HCl at 110 °C. Therefore, an enzymatic digestion was developed to ensure an efficient cleavage of the peptide bonds of BSA prior to LC–MS/MS analysis. Our process encompasses a first digestion in the presence of pepsin prior to a second incubation with pronase E. The sample is further treated with a mixture aminopeptidase–prolidase, and a last incubation in the presence of pronase E. In addition, our results indicate that a preliminary reduction of the disulfide bridges significantly improves the measurement of NY residues in BSA.

These results may explain, at least in part, some conflicting data in the literature, and point out the major role of the protein digestion efficacy in the reliability of the measurement. The values reported in the literature suggest that the employment of enzymatic digestion should be carefully optimized to generate reliable values.

References

[1] H. Ohshima, M. Friesen, I. Brouet, H. Bartsch, Food Chem. Toxicol. 28 (1990) 647.
[2] B. Halliwell, FEBS Lett. 411 (1997) 157.
[3] M.W. Duncan, Amino Acids 25 (2003) 351.
[4] D. Tsikas, K. Caidahl, J. Chromatogr. B 814 (2005) 1.
[5] D. Tsikas, A. Mitschke, M.-T. Suchy, F.-M. Gutzki, D.O. Stichtenoth, J. Chromatogr. B 827 (2005) 146.
[6] M.F. Beal, R.J. Ferrante, S.E. Browne, R.T. Matthews, N.W. Kowall, R.H. Brown, Ann. Neurol. 42 (1997) 646.
[7] H. Kaur, B. Halliwell, FEBS Lett. 350 (1994) 9.
[8] K. Hensley, M.L. Maidt, Z. Yu, H. Sang, W.R. Markesbery, R.A. Floyd, J. Neurosci. 18 (1998) 8126.

[9] Ø.A. Strand, A. Leone, K.-E. Gierchsky, K.A. Kirkeboen, Crit. Care Med. 28 (2000) 2779.
[10] K. Bian, K. Davis, J. Kuret, L. Binder, F. Murad, Am. J. Physiol. 277 (1999) F33.
[11] I. Girault, A.E. Karu, M. Schaper, M.H. Barcellos-Hoff, T. Hagen, D.S. Vogel, B.N. Ames, S. Christen, M.K. Shigenaga, Free Rad. Biol. Med. 31 (2001) 1375.
[12] K.D. Greis, S. Zhu, S. Matalon, Arch. Biochem. Biophys. 335 (1996) 396.
[13] A. Sarver, N.K. Scheffler, M.D. Shetlar, B.W. Gibson, J. Am. Soc. Mass Spectrom. 12 (2001) 439.
[14] K. Jiao, S. Mandapati, P.L. Skipper, S.R. Tannenbaum, J.S. Wishnok, Anal. Biochem. 293 (2001) 43.
[15] A.-S. Petersson, H. Steen, D.E. Kalume, K. Caidahl, P. Roepstorff, J. Mass Spectrom. 36 (2001) 616.
[16] D.S. Winlaw, G.A. Smythe, A.M. Keogh, C.G. Schyvens, P.M. Spratt, D.S. Macdonald, Lancet 345 (1995) 390.
[17] D. Tsikas, F.-M. Gutzki, S. Rossa, H. Bauer, C. Neumann, K. Dockendorff, J. Sandmann, J.C. Frölich, Anal. Biochem. 244 (1997) 208.
[18] A.H. Cross, P.T. Manning, R.M. Keeling, R.E. Schmidt, T.P. Misko, J. Neuroimmun. 88 (1998) 45.
[19] D. Yi, B.A. Ingelse, M.W. Duncan, G.A. Smythe, J. Am. Soc. Mass Spectrom. 11 (2000) 578.
[20] M.T. Frost, B. Halliwell, K.P. Moore, Biochem. J. 345 (2000) 453.
[21] T. Delatour, J. Richoz, J. Vuichoud, J.H. Stadler, Chem. Res. Toxicol. 15 (2002) 1209.
[22] M.K. Shigenaga, H.H. Lee, B.C. Blount, S. Christen, E.T. Shigeno, H. Yip, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 3211.
[23] M. Trop, Y. Birk, Biochem. J. 116 (1970) 19.
[24] T. Delatour, P.A. Guy, R.H. Stadler, R.J. Turesky, Anal. Biochem. 302 (2002) 10.
[25] R.M. Uppu, W.A. Pryor, Methods Enzymol. 269 (1996) 322.
[26] J.S. Beckman, J. Chen, H. Ischiropoulos, J.P. Crow, Methods Enzymol. 233 (1994) 229.
[27] U.T. Ruegg, J. Rudinger, Methods Enzymol. 47 (1977) 111.
[28] J.E. Hale, J.P. Butler, V. Gelfanova, J.-S. You, M.D. Knierman, Anal. Biochem. 333 (2004) 174.
[29] T. Delatour, J. Richoz, P. Vouros, R.J. Turesky, J. Chromatogr. B 779 (2002) 189.
[30] T. Delatour, Anal. Bioanal. Chem. 380 (2004) 515.
[31] AACC Method 07-01, Method Am. Assoc. Cereals Chem. 07-01 (1986) 1.
[32] C. Martín-Hernández, S. Bénet, L. Marvin-Guy, J. Agric. Food Chem. 53 (2005) 8607.
[33] J.S. Fruton, in: A. Meister (Ed.), Advances in Enzymology and Related Areas of Molecular Biology, vol. 44, Wiley and Sons, New York, NY, 1976, p. 1.
[34] H. Kim, W. Lipscomb, in: A. Meister (Ed.), Advances in Enzymology and Related Areas of Molecular Biology, vol. 68, Wiley and Sons, New York, NY, 1994, p. 153.
[35] B.T. Kurien, N.C. Patel, A.C. Porter, S. Kurono, H. Matsumoto, H. Wang, R.H. Scofield, Anal. Biochem. 331 (2004) 224.
[36] C. Leeuwenburgh, P. Hansen, A. Shaish, J.O. Holloszy, J.W. Heinecke, Am. J. Physiol. 274 (1998) R453.
[37] R.I. Viner, D.A. Ferrington, A.F.R. Hühmer, D.J. Bigelow, C. Schöneich, FEBS Lett. 379 (1996) 286.
[38] K.A. Skinner, J.P. Crow, H.B. Skinner, R.T. Chandler, J.A. Thompson, D.A. Parks, Arch. Biochem. Biophys. 342 (1997) 282.
[39] J.R. Crowley, K. Yarasheski, C. Leeuwenburgh, J. Turk, J.W. Heinecke, Anal. Biochem. 259 (1998) 127.
[40] R.S. Sodum, S.A. Akerkar, E.S. Fiala, Anal. Biochem. 280 (2000) 278.
[41] M.K. Shigenaga, Methods Enzymol. 301 (1999) 27.
[42] E. Schwedhelm, D. Tsikas, F.-M. Gutzki, J.C. Frölich, Anal. Biochem. 276 (1999) 195.
[43] D. Tsikas, E. Schwedhelm, F.K. Stutzer, F.-M. Gutzki, I. Rode, C. Mehls, J.C. Frölich, J. Chromatogr. B 784 (2003) 77.
[44] A.-S. Söderling, H. Ryberg, A. Gabrielsson, M. Lärstad, K. Torén, S. Niari, K.A. Caidahl, J. Mass Spectrom. 38 (2003) 1187.
[45] P.J. Thornalley, M. Argirova, N. Ahmed, V.M. Mann, O. Argirov, A. Dawnay, Kidney Int. 58 (2000) 2228.