3-Nitrotyrosine quantification methods: Current concepts and future challenges

Dulce Teixeira [a], Rúben Fernandes [a, b], Cristina Prudêncio [a, b], Mónica Vieira [a, b, *]

[a] *Ciências Químicas e das Biomoléculas, Centro de Investigação em Saúde e Ambiente, Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto, Portugal*
[b] *I3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal*

A B S T R A C T

Background: Measurement of 3-nitrotyrosine (3-NT) in biological samples can be used as a biomarker of nitrosative stress, since it is very stable and suitable for analysis. Increased 3-NT levels in biological samples have been associated with several physiological and pathological conditions. Different methods have been described for the detection and quantification of this molecule, such as (i) immunological methods; (ii) liquid chromatography, namely high-pressure liquid chromatography (HPLC)-based methods that use ultraviolet-visible (UV/VIS) absorption, electrochemical (ECD) and diode array (DAD) detection, liquid chromatography-mass spectrometry (LC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS); (iii) gas chromatography, such as gas chromatography-mass spectrometry (GC-MS) and gas chromatography-tandem mass spectrometry (GC-MS/MS).
Methods: A literature review on nitrosative stress, protein nitration, as well as 3-NT quantification methods was carried out.
Results: This review covers the different methods for analysis of 3-NT that have been developed during the last years as well as the latest advances in this field. Overall, all methods present positive and negative aspects, although it is clear that chromatography-based methods present good sensitivity and specificity. Regarding this, GC-based methods exhibit the highest sensibility in the quantification of 3-NT, although it requires a prior time consuming derivatization step. Conversely, HPLC does not require such derivatization step, despite being not as accurate as GC.
Conclusion: It becomes clear that all the methods described during this literature review, although accurate for 3-NT quantification, need to be improved regarding both sensitivity and specificity. Moreover, optimization of the protocols that have been described is clearly needed.

Keywords: 3-Nitrotyrosine Nitrosative stress Immunochemical method Chromatographic method Quantification methods

1. Biological markers of oxidative stress

Oxidative stress is defined as an imbalance of antioxidants and pro-oxidants in favour of the latter, potentially leading to damage [5,6]. On the other hand, Jones [6] defines oxidative stress as a disruption of redox signalling and/or control of molecular damage. The reactive oxygen species (ROS) and reactive-nitrogen species (RNS) have function in redox signalling and are produced as by-products of normal metabolic process in all aerobic organisms, at very low concentrations in cells [6,7]. Increased oxidative/nitrosative stress is characterized by inadequate cellular antioxidant defences to efficiently inactivate the overproduced ROS and RNS. A major consequence of oxidative/nitrosative stress is the damage of nucleic acid bases, proteins, lipids (including phospholipids) and carbohydrates [7,8]. This damage can compromise cell health and viability, as well as induce a variety of cellular responses like cell death by necrosis or apoptosis [8].

The molecules modified by interactions with ROS (including RNS) in the microenvironment, and those changed in response to increased redox stress are considered biomarkers of oxidative stress [2]. Fig. 1 represents a schematization of biomarkers of oxidative stress, which can be classified as molecules that are modified by interactions with ROS in the microenvironment, and molecules of the antioxidant system that change in response to increased redox stress.

A very promising approach for the assessment of oxidative stress is the detection of nitrated tyrosine (Tyr) residues in proteins [9].

2. Nitrotyrosine in physiological conditions

Tyr (4-hydroxyphenylalanine) is a non-essential amino acid and an element of the aromatic amino acids group. Most proteins found in nature contain Tyr residues in their composition, with an average abundance of about 3–4 mol % [10,11]. Tyr is moderately hydrophilic, which is explained by its hydrophobic aromatic benzene ring carrying a hydroxyl group [12,13]. As a result, Tyr is frequently surface-exposed in proteins allowing further modification, namely nitration. The nitration of Tyr residues in proteins is associated with nitrosative stress, resulting in the formation of 3-nitrotyrosine (3-NT) or other Tyr-nitrated proteins residues [10,14].

When RNS reacts with L-tyrosine and protein-associated Tyr, free 3-nitro-L-tyrosine and protein-associated 3-nitro-L-tyrosine are formed (Fig. 2) [1]. 3-NT [(2-amino-3-(4-hydroxy-3-nitrophenyl) propanoic acid)] is the result of a post-translational modification in proteins carried by RNS, such as nitric oxide (NO), derived oxidants (e.g., peroxynitrite ($ONOO^-$) and peroxynitrous acid (ONOOH)) and nitrogen dioxide radicals ($^{\bullet}NO_2$). It is formed after the substitution of a hydrogen by a nitro group (NO_2) in the *ortho* position of the phenolic ring of the Tyr residues [10,11,13,14].

The protein tyrosine nitration (PTN) is a stable post-translational modification process and does not happen randomly. The abundance of protein or Tyr residues cannot predict whether they will be the target of PTN. Furthermore, not all Tyr residues in a protein are available for nitration, which may depend on their accessibility to the solvent [10,15]. For instance, although the human serum albumin (HSA), a protein most abundantly found in plasma, contains 18 Tyr residues, an *in vitro* study showed that only two of its Tyr residues are predominantly susceptible to nitration [14,16].

The wide range of chemical and structural modifications of proteins, such as modifications affecting signal transduction pathways and cellular processes, are responsible for high levels of RNS and antioxidant enzymatic systems [17,18].

3. Metabolism of nitric oxide and its role in 3-nitrotyrosine biosynthesis

The major pathway for NO metabolism is the stepwise oxidation to nitrite and nitrate [3]. In biological fluids or buffers, NO systems are almost completely oxidized to nitrite (NO_2^-), a biologically inert metabolite of NO oxidation [19].

The oxidation of NO by molecular oxygen (O_2), physically dissolved in biological systems, originates NO_2 (nitrogen dioxide), N_2O_3 (dinitrogen trioxide) and NO_2^- (reactions 1, 2 and 3). N_2O_3 is characterized as a potent nitrosating agent, since it gives rise to the formation of the nitrosonium ion (NO^+). On the other hand, NO and NO_2^- are rapidly oxidized to nitrate (NO_3^-) in blood [3,14].

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$2NO + 2NO_2 \rightarrow 2N_2O_3 \quad (2)$$

$$2N_2O_3 + 2H_2O \rightarrow 4NO_2^- + 4H^+ \quad (3)$$

In erythrocytes, NO directly and rapidly reacts with O_2 bound to haemoglobin (i.e. oxyhemoglobin ($Hb[Fe^{2+}]O_2$)), to form the chemically quite inert anion NO_3^- (reaction 4).

$$Hb\left[Fe^{2+}\right]O_2 + NO \rightarrow Hb\left[Fe^{3+}\right] + NO_3^- \quad (4)$$

Other proposed mechanism for NO_3^- formation is via oxidation of NO_2^- (derived from NO autoxidation – reaction 3) by certain oxyhemoproteins ($Hb[Fe^{2+}]O_2$), such as oxyhemoglobin or oxymyoglobin (reactions 5 and 6) [3].

$$2Hb\left[Fe^{2+}\right]O_2 + 3NO_2^- + 2H^+ \rightarrow 2Hb\left[Fe^{3+}\right] + 3NO_3^- + H_2O \quad (5)$$

$$4Hb\left[Fe^{2+}\right]O_2 + 4NO_2^- + 4H^+ \rightarrow 4Hb\left[Fe^{3+}\right] + 4NO_3^- + O_2 + 2H_2O \quad (6)$$

Concerning free radical superoxide ($O_2^{\cdot-}$), this may promptly interact with NO to produce the peroxynitrite anion ($ONOO^-$) (reaction 7) [20,21].

$$^{\cdot}NO + O_2^{\cdot-} \rightarrow ONOO^- \quad (7)$$

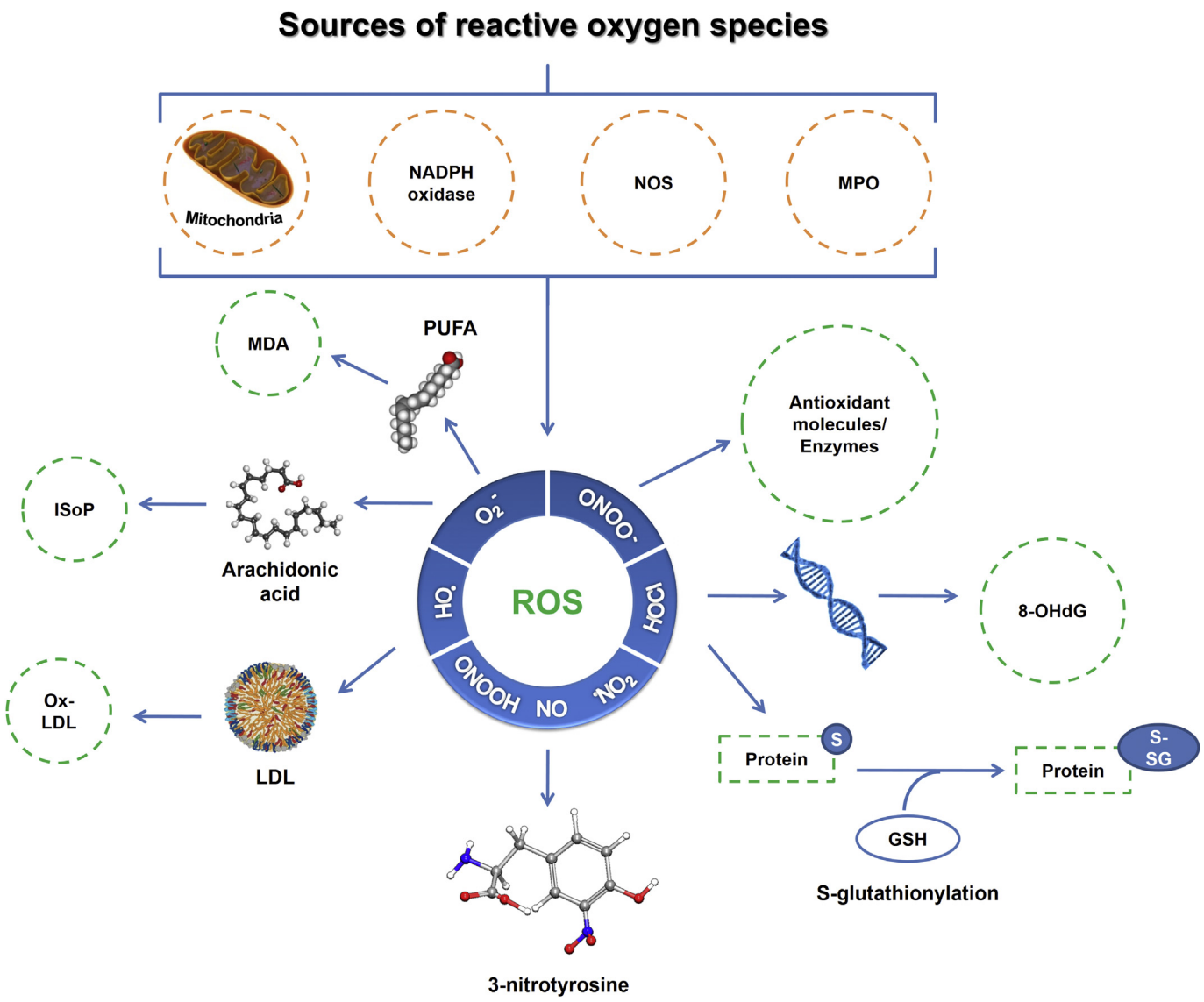

Fig. 1. Formation pathways of selected biomarkers of oxidative stress. Oxidized Low-Density Lipoprotein (Ox-LDL). Protein oxidation markers: protein nitration (3-nitrotyrosine). Oxidative DNA damage biomarkers: 8-hydroxy-2′-deoxyguanosine (8-OHdG). Antioxidant enzymes and molecules: superoxide dismutase, catalase, glutathione peroxidase, oxidized glutathione, total antioxidant capacity. GS, glutathione; reduced glutathione (GSH); oxidized glutathione (GSSG); PUFA, polyunsaturated fatty acids. Nicotinamide adenine dinucleotide phosphate (NADPH), nitric oxide synthase (NOS), mieloperoxidase (MPO). Adapted from Ho et al. [2] and Shah et al. [4].

Peroxynitrite is the extremely reactive conjugate base of peroxynitrous acid (ONOOH) (reaction 8) [3,14].

$$4ONOO^- + 4H^+ \leftrightarrow 4ONOOH \rightarrow 2NO_3^- + 2NO_2^- + O_2 + 4H^+ \quad (8)$$

In biological systems, $ONOO^-$/ONOOH system is a very strong oxidant and a potent nitrating agent, thus it has been implicated as a culprit in many diseases [22]. Peroxynitrite promotes nitration and hydroxylation in different bioorganic molecules, including proteins, lipids, thiols, sulfhydryl groups, DNA bases, and preferentially nitrates Tyr residues of protein or non-protein origins [23,24]. Peroxynitrite reacts whit CO_2 to yield a nitrosoperoxycarbonate anion ($ONOOCO_2^-$) that undergoes a fast homolysis to NO_2 and carbonate radicals ($CO_3^{\bullet-}$) (reaction 9) [18,23,25].

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-} \quad (9)$$

The *in vivo* production of $ONOO^-$ leads to the nitration of Tyr residues in proteins, forming 3-NT, although it does not directly react with Tyr. Instead, it forms secondary radicals, such as $CO_3^{\bullet-}$, $^{\bullet}NO_2$ and oxo-metal complexes, which are indeed responsible for protein Tyr oxidation and nitration. The mechanism of Tyr nitration in biological systems is a two-step process (reactions 10 and 11) [23].

$$TyrH + CO_3^{\bullet-} \rightarrow Tyr^{\bullet} + HCO_3^- \quad (10)$$

$$Tyr^{\bullet} + {}^{\bullet}NO_2 \rightarrow Tyr - NO_2 \quad (11)$$

Moreover, 3-NT may be generated through multiple pathways (Fig. 3). Tyr can be nitrated by peroxidase (or heme/hemoprotein) through the *in vivo* hydrogen peroxide-dependent oxidation of nitrite to form NO_2 (reactions 12–14) [3,18,26].

$$H_2O_2 + \text{peroxidase(heme)} \rightarrow \text{oxidant(porphyrin radical)} \quad (12)$$

$$\text{Oxidant} + NO^{2-} \rightarrow {}^{\bullet}NO_2 \quad (13)$$

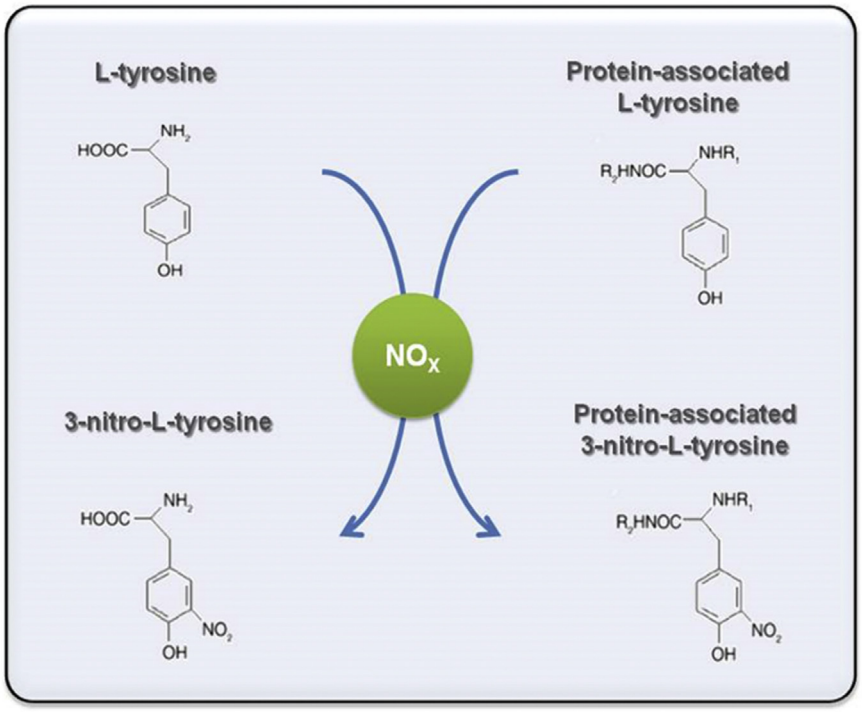

Fig. 2. Nitration of L-tyrosine to 3-nitro-L-tyrosine (Adapted from Tsikas and Caidahl [1]).

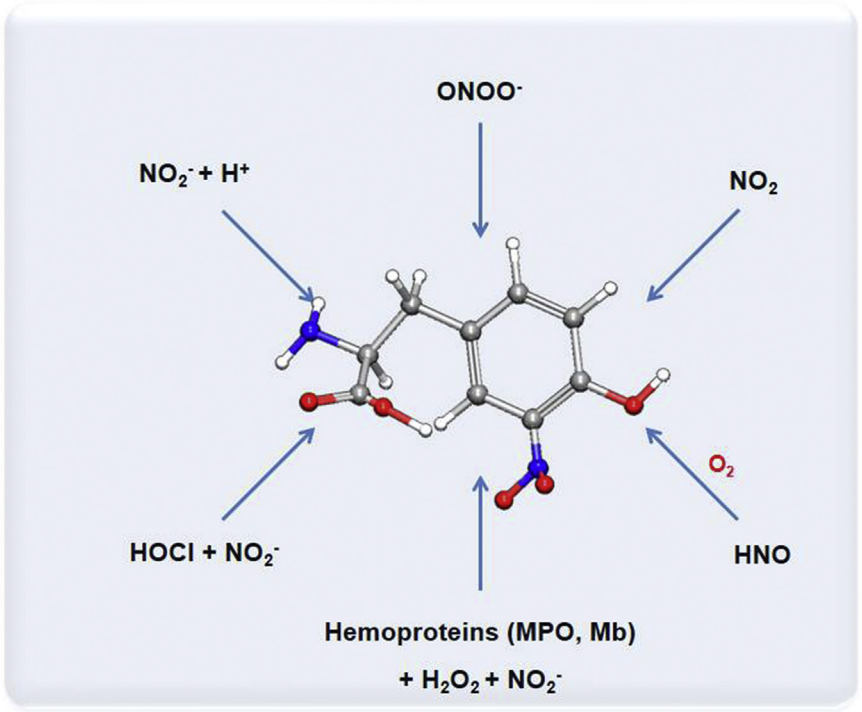

Fig. 3. Multiple pathways for the formation of 3-nitrotyrosine (Adapted from Bryan and Grisham [3]).

$$2^{\bullet}NO_2 + \mathrm{Try} \rightarrow 3 - \mathrm{NT} \qquad (14)$$

4. Association between 3-nitrotyrosine and disease

Several physiological and pathological conditions have been associated with increased nitration of proteins [15,21]. Table 1 shows the 3-NT concentration range usually found in both healthy and pathological states, as determined by different

Table 1
3-NT concentration ranges found in different biological samples from both healthy and pathological states, as determined by different methodologies.

Reference	Sample (mean age ± years)	Biological sample	Method	Concentration 3-NT
Söderling et al. [54]	12 Healthy non-smoking volunteers (43 ± 9)	Plasma	GC/NCI-MS/MS	0.74 ± 0.31 nM[a]
Tsikas et al. [55]	10 Healthy volunteers (51 ± 10)	Plasma	GC tandem MS	1.149 ± 0.73 nM[a]
	6 Healthy volunteers (25 ± 3)	Plasma	GC tandem MS	2.677 ± 1.540 nM[a]
	10 Healthy volunteers (51 ± 10)	Plasma	GC-MS	4.46 ± 4.49 nM[a]
	6 Healthy volunteers (25 ± 3)	Plasma	GC-MS	5.447 ± 2.783 nM[a]
Zhang et al. [56]	20 Healthy non-smoking volunteers (41.2)	Plasma	LC-MS/MS	4.54 ± 2.75 nM[a]
	18 Healthy smokers (42.0)	Plasma	LC-MS/MS	17.42 ± 11.6 nM[a]
Radabaugh et al. [57]	40 Healthy volunteers	Plasma	LC–MS/MS	224–962 pg/mL (0.99 –4.2 nM)[b]
Misko et al. [39]	143 Healthy volunteers	Plasma	Immunoaffinity two-dimensional LC-MS/MS	536.4 pg/mL (2.49 nM)[b]
	174 Osteoarthritis patients	Plasma	Immunoaffinity two-dimensional LC-MS/MS	704.1 pg/mL (3.11 nM)[b]
Pourfarzam et al. [32]	50 Patients with no history of cardiac diseases (58.9 ± 10.3)	Plasma	HPLC-fluorescence detector 470 nm	4.4 ± 1.8 nM[a]
	50 Stable CAD patients (61.2 ± 11.23)	Plasma	HPLC-fluorescence detector 470 nm	12.8 ± 3.9 nM[a]
	50 Unstable CAD patients (59.9 ± 10.45)	Plasma	HPLC-fluorescence detector 470 nm	14.8 ± 4.8 nM[a]
Sun et al. [26]	70 Healthy Chinese volunteers	Plasma	Sandwich ELISA	7.9 ± 7 nmol/L[b]
Radabaugh et al. [57]	131 Healthy volunteers	Serum	LC–MS/MS	179–1540 pg/mL (0.79 –6.8 nM)[b]
Khan et al. [58]	25 Non-smoking healthy female volunteers (age 24–50)	Serum	Sandwich ELISA	1.1 ± 0.81 μM[b]
	24 Systemic lupus erythematosus patients (age 18–50)	Serum	Sandwich ELISA	96.52 ± 21.12 μM[b]
Tsikas et al. [59]	10 Healthy volunteers (36.5 ± 7.2)	Urine	GC–tandem MS	8.4 ± 10.4 nM
Radabaugh et al. [57]	8 Healthy volunteers	Urine	LC–MS/MS	63.5–751 pg/mL (0.28 –3.32 nM)[b]
Ryberg et al. [60]	19 Patients without history or symptoms/signs of psychiatric, neurological, malignant or systemic disorders	Cerebrospinal fluid	GC/NCI-MS/MS	0.35 ± 0.019 nM[a]
	17 Alzheimer's disease patients	Cerebrospinal fluid	GC/NCI-MS/MS	0.44 ± 0.031 nM[a]
	14 Amyotrophic lateral sclerosis patients	Cerebrospinal fluid	GC/NCI-MS/MS	0.38 ± 0.034 nM[a]
Radabaugh et al. [57]	35 Healthy volunteers	Cerebrospinal fluid	LC–MS/MS	335–5730 pg/mL (1.48 –25.33 nM)[b]
Seven et al. [61]	15 Volunteers with normal pressure hydrocephalia	Cerebrospinal fluid	ELISA based on the sandwich	573.54 ± 142.86 nM
Seven et al. [61]	20 Relapsing remitting multiple sclerosis patients before methyl prednisolone therapy	Cerebrospinal fluid	ELISA based on the sandwich	927.89 ± 244.84 nM
Lärstad et al. [62]	10 Healthy subjects (46 ± 2.6)	Exhaled breath condensate	GC/NICI/tandem MS	31 pM[a]
	8 Asthma patients (49 ± 5.1)	Exhaled breath condensate	GC/NICI/tandem MS	31pM[a]
Radabaugh et al. [57]	40 Healthy volunteers	Synovial fluid	LC–MS/MS	54.4–822 pg/mL (0.24 –3.63 nM)[b]
Ueshima et al. [63]	5 Chronic obstructive pulmonary disease patients (71.6 ± 2.4)	Sputum	HPLC-ECD	0.55 ± 0.15 pmol/mL[b]
	5 Chronic obstructive pulmonary disease patients (71.6 ± 2.4)	Saliva	HPLC-ECD	0.02 ± 0.01 pmol/mL[b]

GC/NCI-MS/MS, Gas chromatography/negative chemical ionization - mass spectrometry/mass spectrometry; GC tandem MS, Gas chromatography tandem mass spectrometry; GC-MS, Gas chromatography - mass spectrometry; LC–MS/MS, Liquid chromatography - mass spectrometry/mass spectrometry; HPLC, High Performance Liquid Chromatography; ELISA, Enzyme-Linked Immunosorbent Assay; ECD, electrochemical.
[a] Free 3-NT.
[b] Total 3-NT.

methods (discussed in detail in the following sections) and biological samples. According to Table 1, 3-NT levels in pathological conditions are slightly higher in comparison with basal levels in healthy individuals. This observation is in fact valid for several biological specimens, regardless the quantification method used.

Among the pathological conditions, there is a wide range of cardiovascular diseases, such as myocardial inflammation, heart failure and arteriosclerosis [5,27–30]. For instance, Shishehbor et al. [31] and Poufarzam et al. [32] demonstrated that 3-NT plasma levels are elevated in coronary artery disease (CAD) patients. Regarding atherosclerosis, it was found that atherosclerotic arteries have higher 3-NT levels than non-atherosclerotic blood vessels [33]. Furthermore, there is also evidence of an accumulation of 3-NT during atherogenesis [34].

In addition, diseases associated with immunological reactions appear to be connected at a very high degree with the increased formation of Tyr-nitrated proteins, such as asthma [35], systemic sclerosis [36], renal complications [4], inflammatory bowel disease [37], septic shock [38], rheumatoid arthritis and joint injury [39,40]. In relation to asthma, 3-NT expression was found to be increased in several asthmatic epithelial cells and essentially in asthmatic children [35,41]. Concerning systemic sclerosis, Shimizu et al. [36] suggested that serum 3-NT levels are significantly increased in systemic sclerosis patients compared to healthy controls. Regarding inflammatory bowel diseases, such as ulcerative colitis, the 3-NT expression is substantially increased in the inflamed colonic mucosa [37].

Furthermore, some neurological diseases and psychiatric disorders are also associated with an increased level of nitrated proteins. For example, Parakh et al. [42] reported that nitrated proteins

and high levels of 3-NT have been detected in cases of amyotrophic lateral sclerosis. Conversely, Mendonça et al. [43] demonstrated that 3-NT was expressed in both amyotrophic lateral sclerosis and control samples, with no significant difference between them. Moreover, Dietrich-Muszalska et al. [44] considered that the amount of 3-NT in plasma proteins may be important indicators of *in vivo* protein damage in schizophrenia. Besides, high levels of 3-NT have also been associated with other pathological conditions, such as Alzheimer's disease [45], Parkinson's disease [46], autism [47] and myalgic encephalomyelitis/chronic fatigue syndrome [48].

Other diseases are associated with increased protein nitration. Shu et al. [49] demonstrated that 3-NT levels in plasma samples from patients with classical Fabry disease were about six-fold higher compared with age- and gender-matched controls. The 3-NT levels are also significantly increased in diabetic patients [5,50], especially in patients with diabetic nephropathy [51] or diabetic patients with microvascular complications [52]. Regarding Chagas disease, Dhiman et al. [53] considered that 3-NT-modified proteins is an important phase in the pathophysiology of such disease, and might be useful biomarkers of disease as well. Moreover, Shah et al. [4] demonstrated that 3-NT levels were increased in plasma and serum of patients with systemic lupus erythematosus.

5. Quantification of 3-nitrotyrosine in biological samples

Since 3-NT was suggested as a biomarker of nitrosative stress, a substantial effort has been made to develop analytical methods that can be applied to biological samples [64]. Accurate quantification of substances present in biological samples at very low concentrations is, indeed, a complex task, and the particular case of 3-NT requires special concerns [1].

3-NT has been detected in several biological tissues and fluids including plasma, serum, urine, cerebrospinal fluid, synovial fluid, tissue samples and other biological samples [57]. Recently, Mergola et al. [65] developed for the first time the synthesis of a highly selective molecularly imprinted polymer (MIP) used as solid-phase extraction (SPE) sorbent for pre-concentration of 3-NT and the selective clean-up from biological sample. The results obtained suggest that this polymer can be used as an active site in a sensor so that the analyte can be directly identified in the urine of patients, where it is normally present at very low concentrations.

A common approach for 3-NT quantification is the prior cleavage of peptide bonds in order to release the free amino acids from proteins in fluids or tissues. This cleavage may be achieved by acid hydrolysis or enzymatic digestion [14,57,66]. Delatour et al. [66] compared both methods in terms of the reliability of the 3-NT measurement, providing evidence that acid hydrolysis leads to more reliable results. However, Radabaugh, et al. [57] pointed out that a prior acid hydrolysis step is more prone to artifact formation in comparison with enzymatic digestion.

Regarding quantification of 3-NT, the first approaches used different immunological methods. In fact, a large part of studies on quantification of 3-NT in biological samples has been performed using antibody-based methods, namely immunohistochemistry and western blot [13].

5.1. Immunochemical methods

5.1.1. Enzyme-linked immunosorbent assay

ELISA (enzyme-linked immunosorbent assay) is based on the basic immunology concept of the binding properties of an antibody to its specific antigen. From a general point of view, this method employs enzyme-labelled antigens and antibodies to detect a wide variety of compounds. The antigen-antibody complex is further bound by a secondary enzyme-coupled antibody, followed by the addition of a chromogenic substrate which yields a visible colour change or fluorescence, allowing the quantification of the compound [67]. One of the main advantages of ELISA technology is that it allows the simultaneous determination of standards and samples. Moreover, it does not require complex sample preparation steps [67].

Regarding 3-NT quantification, there is a wide variety of ELISA-based methods for this purpose, namely indirect, competitive, sandwich-ELISA and ELISA microarrays [68,69]. Table 2 lists some ELISA assays for the analysis of 3-NT in different biological specimens.

One of the first ELISA methods for the analysis of nitrated proteins in biological fluids was based upon a competitive model [72]. More recently, Safinowski et al. [9] performed a comparison of the performance of different commercially available immunoassays for 3-NT analysis, and concluded that all of them did not provide reliable results. They also concluded that the sandwich-based ELISA assay exhibited the worst performance, probably due to the low concentration levels of 3-NT in almost all investigated samples. ELISA requires at least two 3-NT residues for the capture and detection antibody and, in this sense, the sandwich ELISA measures only protein associated-3-NT [9,26,69]. Actually, the poor performances exhibited by different ELISA can be due to different reasons: (i) the antibodies may reveal some nonspecific binding; (ii) 3-NT may not be totally accessible to the antibody in some protein sites; and (iii) monoclonal and polyclonal antibodies used by these methods may exhibit cross-reactivity with other compounds present in biological samples [73].

Regarding microarray-ELISA assays, one of their main advantages is that they use various physically separated capture antibodies (in isolated spots), allowing an efficient way for measuring low levels of the analyte [68].

5.2. Chromatographic methods

Chromatography is a powerful analytical technique, and is widely available in different laboratory settings nowadays [74]. This technique is generally composed by two primary components: mobile phase and stationary phase [75].

Table 3 shows chromatography classifications according to the mobile and stationary phases used.

5.2.1. Liquid chromatography methods using ultraviolet, fluorescence and electrochemical detection

Liquid chromatography (LC) is one of the most extensively used methods for the determination of 3-NT (Table 4). Several different detectors have been employed, such as ultra-violet (UV), electrochemical (ECD), diode-array (DAD) and mass spectrometry (MS) [14].

HPLC (High Performance Liquid Chromatography) is a chromatographic technique that can separate a mixture of compounds, and is used in biochemistry and analytical chemistry to separate, identify, quantify and purify the active compounds of a mixture [75]. The principle behind this technique relies on the injection of the sample into a column that holds packing material (stationary phase), with further pumping of the mobile phase(s) through the column, at high pressure, and the detection through the retention times exhibited by the molecules [76].

HPLC is the standard technique for analysing amino acids, being the technique used by most laboratories [13]. One of the first chromatographic methods for quantifying 3-NT uses isocratic reversed phase HPLC and UV absorbance detection at 274 nm, as first reported by Kaur and Halliwell [77]. This HPLC-based method uses column with C-18, eluent (500 mM KH_2PO_4—F_3PO_4 with 10% methanol (v/v)), an acidic mobile phase (pH 3.01), and with a

Table 2
ELISA assays for the analysis of 3-NT in different biological specimens.

Compound	Biological specimen	ELISA type	Sensitivity/Detection range/Standard curve/LOD/LOQ for 3-NT
3-NT [70]	Bronchoalveolar lavage	Sandwich ELISA	2.0 nmol/L (Sensitivity)
3-NT [50]	Plasma	Sandwich ELISA	N.S.
3-NT [9]	Plasma	Solid phase ELISA	3.12–200 nM (Detection range)
3-NT-containing proteins [71]	Plasma	Competitive ELISA	10 to 500 nM (Standard curve)
3-NT-modifed proteins [72]	Plasma	Competitive ELISA	10 μM–10 mM (Standard curve)
3-NT [26]	Plasma and serum	Sandwich ELISA	1.8 nmol/L (Sensitivity)
Protein bound 3-NT [69]	Plasma and serum	Indirect ELISA	1.82 ± 0.56 pmol/mg protein (LOD) 4.29 ± 1.56 pmol/mg protein (LOQ)
3-NT [9]	Plasma and serum	Competitive luminescence assay (CLIA)	1.68 nM–101 μM (Detection range)
3-NT [68]	Plasma and sputum	ELISA microarray	N.S.
3-NT [36]	Serum	Sandwich ELISA	N.S.
Protein bound 3-NT [58]	Serum	Sandwich ELISA	2 nM (LOD)

LOD, limit of detection; LOQ, limit of quantification; N.S.,not stated.

Table 3
Chromatographic techniques used based on samples characteristics.

Chromatographic technique	Mobile phase	Stationary phase	Sample
Gas chromatography	Gas	Solid/liquid	Gaseous sample (ordinary temperatures) Samples that vaporise (when heated)
Liquid chromatography	Liquid	Solid/liquid	Liquid samples Solid samples (solvent-soluble)

detection limit of 0.2 μM [77]. Variants of this method have been used in several studies to determine 3-NT. Hitomi et al. [78] performed an optimization of 3-NT separation process, showing that it depends on the mobile phase used. Moreover, the retention time depends on two factors, (i) the pH of the mobile phase, and (ii) the concentration of acetonitrile. The retention time was prolonged by a powerfully acidic mobile phase.

HPLC-UV detector. The HPLC-UV allows the detection of 3-NT as free amino acid, and associated with peptides and proteins as well [79]. Tyr and 3-NT have a maximum absorbance at 280 nm in solution at pH 3.5. Additionally, 3-NT has a second absorbance at 357 nm. However, this second wavelength is more selective and, therefore, more suitable for detection purposes. In basic solutions (pH 9.5), 3-NT has a maximum absorbance at 430 nm [79,80]. The main drawback of this method is the lack of selectivity and sensitivity. HPLC coupled to ECD has the potential to be more selective than HPLC-UV or HPLC-fluorescent methods [13].

HPLC-Diode Array Detector. Comparatively to UV detection, DAD allows the simultaneous detection at different wavelengths [81]. Although HPLC-DAD is the most commonly used method for *in vitro* 3-NT analysis, it is not enough sensitive for *in vivo* quantitative analysis [65]. Recently, Selzle et al. [82] developed a simple and efficient HPLC-DAD method for the determination of the nitration degree of small amounts of the birch pollen allergen Bet v 1. This method can be photometrically calibrated by the amino acids Tyr and 3-NT without the need for nitrated protein standards. The study also reported that this new method can be used in the investigation of the reaction kinetics and mechanism of protein nitration [82].

HPLC-fluorescence detection. Since 3-NT is not a fluorescent compound, it can only be detected using a fluorescence detector after structural modifications, such as the reaction of the amino group of 3-NT with a suitable derivatizing reagent [80]. For instance, Pourfarzam et al. [32] used 4-fluoro-7-nitrobenzo-2-oxa-l,3-diazole (NBD-F) as derivatization reagent. This reagent has been reported in several studies and the results obtained were comparable among them [32]. The fluorescent dyes used in the derivatization step of amino acids significantly improve the sensitivity and specificity of detection. NBD-F has a 10-fold higher sensitivity than *ortho*-phthaldialdehyde (OPA) [56]. The limitation of this method is that several fluorescent compounds are produced during the derivatization process [80].

HPLC-Electrochemical detector. HPLC using ECD is relatively low-cost and has sufficient sensitivity for the measurement of 3-NT at moderately low basal levels found in most biological samples from healthy individuals. These ECD-based methods have the potential to be more selective than HPLC-UV or HPLC-fluorescence methods, with a sensitivity about 100-fold greater [29].

The reproducibility of chromatogram signal intensity and retention time of 3-NT are determined by the quality of the C18 column used, as well as the appropriate maintenance of the electrochemical cell. Such reproducibility is ensured by (i) routine washing of the column with methanol, (ii) ensuring that potentials are not applied when the mobile phase is not flowing, and (iii) periodic reconditioning of the electrode when a performance loss is detected [29].

5.2.2. Liquid chromatography-mass spectrometry methods

One of the ways to overcome the limitations of HPLC is to couple it with an MS. This combination is advantageous for robust and unambiguous identification of compounds, especially when in the MS/MS mode [13,83]. Analytical methods based on MS-methodology are normally accepted as gold standards for the analysis of endogenous substances in biological fluids, owing to their high accuracy [1]. The advantage of this technique is that it does not require sample derivatization to increase its volatility, unlike gas chromatography-mass spectrometry (GC-MS), since it increases the likelihood of artifacts formation [13,83].

Nevertheless, liquid chromatography-mass spectrometry (LC-MS) does not offer the necessary selectivity for 3-NT measurement in biological sample, especially in human plasma [13,83]. One way to improve the selectivity and sensitivity for measurement is to use HPLC Hypercarb columns in triple-stage quadrupole LC–ESI (Electrospray ionization) -MS/MS. The Hypercarb columns are composed of porous spherical carbon particles and the separation is based on the stronger retention time for polar compounds [13].

Chen & Chiu [84] developed a highly specific and accurate LC/MS/MS assay that allows simultaneous analysis of protein-bound 3-

Table 4
Different HPLC-based methods used for the determination of 3-NT in biological samples.

Compound	Sample	Column[a]	Eluents	Flow rate (mL/min)	Detection method	LOD LOQ for 3-NT
3-NT [24]	Animal spleen tissues	Microtech Scientific C_{18} 1.0 × 50; 5	50 mM H_3PO_4, 50 mM citric acid, 40 mg/L EDTA, 100 mg/L octane sulfonic acid, 5% methanol (v/v) (pH 3.1 with KOH)	0.05	ECD	N.S. N.S.
3-NT residues in protein [79]	Bovine serum albumin and ovalbumin	Grace Vydac 2.1 × 250; 5	0.1% (v/v) TFA in water and ACN	0.3	DAD 280 and 357 nm	4.3 pmol N.S.
3-NT, Tyr [64]	Brain tissue	Inertsil® ODS C_{18} 4.6 × 250; 5	50 mM sodium acetate (pH 4.2), 10% methanol (v/v)	0.8	DAD 278 nm	6.7 nM N.S.
Tyr and 3-NT [25]	Gaseous nitrogen oxide species	Inertsil ODS-2 4.6 × 250; 5	0.5% (v/v) acetic acid:methanol (29:1, v/v)	1.0	UV–VIS detector 280 nm	N.S. N.S.
3-NT, Tyr [56]	Plasma	Nova-Pak C_{18} 3.9 × 150; 4	**Mobile phase A:** ACN and 0.02 M phosphate buffer (pH 6.5; 90:10 v/v), 375 μL/L TFA, 5 mL/L 2-propanol (pH = 4.5) **Mobile phase B:** ACN and 0.02 M phosphate buffer (pH 6.5; 10:90 v/v), 500 μL/L TFA (pH 3.5)	1.0 (34 °C)	UV 540 nm	0.5 nM N.S.
3-NT [85]	Plasma	SD ODS 3.0 × 150; 5	100 mM phosphate buffer solution, 5% methanol (v/v)	0.5 (25 °C)	ECD	<10 fmol N.S.
Free 3-NT Assay [32]	Plasma	Nova-Pak C_{18} 3.9 × 150; 4	Sodium phosphate buffer (0.1 M, pH 7.2), methanol (52.5:47.5 v/v)	1.0	UV 470 and 540 nm	0.2 nM 0.6 nM
3-NT [73]	Proteins from blood plasma	Two ultrasphere ODS 4.6 × 150; 3 4.6 × 46; 5	50 mM sodium acetate (pH 4.7), 5% methanol (v/v)	0.7	UV 280 nm	1 pmol ≈400 pmol
3-NT [78]	Rat plasma	SC-50ODS 3.0 × 150; 5	200 mM phosphate buffer containing 5 mg/mL EDTA, 2% ACN	0.5	ECD	N.S. N.S.
Free 3-NT [58]	Serum	C_{18} reversed phase column 4.6 × 250; 5	500 mM potassium phosphate buffer (pH 3.5), 10% methanol (v/v)	0.8	UV 274 nm	0.20 nM N.S.
3-NT [63]	Sputum and saliva	C_{18} reversed phase column 3.0 × 150; 5	100 mM sodium phosphate buffer (pH 5.0), 5% methanol (v/v)	0.5	ECD	10 fmol N.S.
Tyr and 3-NT metabolites [46]	Undifferentiated human teratocarcinoma NT2 and rat pheochromocytoma PC12 cell lines	Octadodecyl silica gel reverse-phase column 4.6 × 250; 5	0.1% TFA in ultra pure water (solvent A) and 100% ACN (solvent B)	1.0	DAD 215, 275, 365 nm	N.S. N.S.
3-NT, *p*-nitro-L-phenylalanine, and L-Tyr [59]	Urine	Nucleosil 100-5 C_{18} 4.0 × 250; 5	50 mM $(NH_4)_2SO_4$ in water-methanol (95:5, v/v) (pH 5.5)	1.0	UV 276 nm	6 pmol N.A.

ACN, acetonitrile; EDTA, ethylenediamine tetraacetic acid; TFA, trifluoroacetic acid; LOD, limit of detection; LOQ, limit of quantification; N.S.,not stated.
[a] Column dimensions in the following order: internal diameter (in mm) x length (in mm); particle size (in μm).

NT and 3-bromotyrosine (3-BT) in human urine. This proteins function as non-invasive biomarkers for *in vivo* PTN and bromination.

Table 5 describes several liquid chromatography-based methods/protocols for determination of 3-NT in biological samples that have been published over the last year.

5.2.3. Gas chromatography-mass spectrometry methods

GC-MS methodology is used for the analysis of volatile and thermally stable small molecules [13]. When molecules to be analysed, such as amino acids, do not meet these features, a further derivatization step is required. In this sense, analysis of 3-NT by this methodology requires prior chemical derivatization and/or modification of the functional groups (i.e., p-OH, a-NH_2, and COOH) to increase volatility and thermal stability (i.e., reduction of the aromatic NO_2 group). In addition, derivatization improves GC and MS behaviour of 3-NT [83]. Derivatization, however, often accounts for 3-NT artifact formation, which is may be responsible for high variation of basal plasmatic 3-NT levels [1]. Nevertheless, GC-tandem MS is described as a methodology that allows the accurate quantification of free 3-NT in human plasma at basal levels. GC-tandem MS is more accurate than GC-MS [1,59]. Table 6 summarises different gas chromatography-based methods for determination of 3-NT in biological samples.

The GC-MS and GC-MS/MS methods for the analysis of 3-NT involve the preparation of perfluorinated derivatives. Perfluorinated compounds are strong electron-capturing species, thus offering assays of particularly high sensitivity [83].

Gaut et al. [87] reported that GC–MS was 100-fold more sensitive than LC–tandem MS for the analysis of 3-NT. The major drawback of this method is that 3-NT is not suitably separated from Tyr, nitrite and nitrate prior to GC–MS analysis or derivatization. Such separation could potentially be achieved, for instance, by HPLC [14].

In the study performed by Söderling et al. [54], the derivatization method used was based on the reduction of the nitro group of 3-NT by dithionite, heptafluorobutyric acylation and subsequent methyl derivatization. Their results demonstrated excellent GC and MS properties, namely low background and a favourable fragmentation pattern [54].

In fact, the major strategies used to avoid artifacts formation are (i) isolation of 3-NT by SPE and HPLC; (ii) reduction of 3-NT to

Table 5
Different liquid chromatography-based methods used for the determination of 3-NT in biological samples.

Compound	Sample	Column[a]	Eluents	Flow rate	Detection method	LOD LOQ for 3-NT
Free amino acid and protein 3-NT [57]	Biological fluids	Immunoaffinity column: Targa 4.6 × 30 C_{18} reversed-phase column: Betasil 2.1 × 100; 5	Immunoaffinity column: 1% formic acid C_{18} reversed-phase column: 100% 10 mM ammonium acetate acetonitrile	Immunoaffinity column: 1 mL/min C_{18} reversed-phase column: 0.3 mL/min	Immunoaffinity LC–MS/MS	5 pg/mL (22.1 pM) 5 pg/mL (Lower LOQ)
3-NT [15]	Microglial Cell Lysate	EASY-Spray™ 250 × 0.075; 2	0.1% formic acid in water (mobile phase A) and 0.1% formic acid in ACN (mobile phase solvent B)	350 nL/min	LC-MS/MS	N.S. N.S.
Free 3-NT [86]	Plasma	Altima HP 100 × 2.1 mm; 3 Kinetex 100 × 2.1; 2.6	0.01% acetic acid in ultrapure water (mobile phase A) + 0.01% acetic acid in ACN (mobile phase B)	0.2 mL/min	LC-MS/MS	0.034 nM 0.112 nM
3-chlorotyrosine, 3-BT, and 3-NT [87]	Plasma	C_{18} reversed phase column Zorbax 1.0 × 150; 5	Methanol/water/acetic acid (4/95/1,v/v/v, pH 3 – solvent A) + methanol/water/acetic acid (95/4/1, v/v/v, pH = 3.2 -solvent B)	0.05 mL/min	LC–MS/MS	3.2 fmol N.S.
3-NT [88]	Plasma and Urine	HypercarbTM[c] 2.1 × 50; 5	0.1% TFA with a linear gradient of 10–50% acetonitrile	0.2 mL/min	LC-MS/MS	0.022 pmol N.S.
3-NT and 3-BT [84]	Urine	Reversed phase C_{18} column 2.0 × 150; 5	0.01% formic acid (pH 3.2) to 25% methanol in 0.01% formic acid	0.2 mL/min	LC–ESI/MS/MS	44 fmol N.S.
3-NT, 3-BT, Dibromotyrosine [89]	Urine	ODS-SR 2 × 150; 5	0.05% formic acid/CH_3CN (95:5)	Not stated	LC/MS/MS	1 nM N.S.

ACN, acetonitrile; TFA, trifluoroacetic acid; LOD, limit of detection; LOQ, limit of quantification; N.S., not stated.
[a] Column dimensions in the following order: internal diameter (in mm) x length (in mm); particle size (in μm).

Table 6
Different gas chromatography-based methods used for the determination of 3-NT in biological samples.

Compound	Sample	Column[a]	Eluents	Detection method	LOD LOQ for 3-NT
3-NT [60]	CSF	DB-1 MS column 15 × 0.32; 0.25	Helium (3psi); Methane (750–800 Pa)	GC–MS/MS	N.S. N.S.
Free 3-NT [62]	Exhaled breath condensate (EBC)	DB5-MS column 30 × 0.25; 0.25	Helium (38 kPa); Methane (0.97–0.99 kPa)	GC/NICI/ tandem MS	0.56 pM 1.70 pM
Protein-associated 3-NT and 3-nitrotyrosinoalbumin [55]	Plasma	Optima 5-MS 30 × 0.25; 0.25	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa)	GC–MS/MS	N.S. N.S.
3-NT [54]	Plasma	DB5-MS column 30 × 0.25; 0.25	Helium (41 kPa); Methane (0.27–1.1 kPa)	GC–MS/MS	0.03 nM 0.3 nM
3-NT [59]	Urine	Optima 5-MS 30 × 0.25; 0.25	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa collision pressure)	GC–MS/MS	4 amol 125 pM

[a] Column dimensions in the following order: internal diameter (in m) x length (in mm); particle size (in μm), LOD, limit of detection; LOQ, limit of quantification; N.S., not stated.

3-amimotyrosine before sample derivatization, thus artifactual nitration of Tyr does not influence the measurement; (iii) use of high concentrations of reactive aromatic compounds (i.e., phenol) to capture nitrosating species; and (iv) quantification of artifactual 3-NT formation, by incorporating stable-isotope labelled Tyr (e.g., 13 C6 -Tyr) into each sample [83]. Overall, the main disadvantage of using chromatographic methods is that they involve time-consuming sample preparation procedures [69].

6. Conclusions

As previously stated, nitration of proteins is a common process that occurs under physiological conditions. On the other hand, a significant increase in the extent of this process, induced by an increased nitrative stress state, has been associated with a wide range of diseases.

3-NT has been pointed out as a potential biological tool for the therapeutic monitoring of this process. However, PTN is typically a low-yield process, thus it requires sensitive analytical methods for its quantification. Regarding this, the quantification of 3-NT poses some

challenges, namely: (i) endogenous levels are extremely low, and near the limits of detection/quantitation for most of the currently available analytical methods; (ii) 3-NT must be detected and quantified in the presence of a vast excess of Tyr, thus requiring high selective method (avoiding interferences from other closely related molecules; and (iii) changes in 3-NT levels may be very slight.

In summary, a wide range of methods for 3-NT detection and quantification has been developed during the last years, all of them presenting positive and negative aspects. Regarding ELISA-based methods, and despite being the least time-consuming and straightforward methods, it has become clear that they do not provide the most accurate results. On the other hand, and in relation to chromatographic methods, they seem to be very accurate, showing very good sensibility and specificity. GC-based methods exhibit the highest sensibility in the quantification of 3-NT. However, and owing to 3-NT chemical properties, a derivatization step prior analysis is required, which ends up being time-consuming for the analyst. Moreover, derivatization reactions often induce artifacts formation, which may further influence the final analysis. Conversely, HPLC does not require such derivatization step, despite being not as accurate as GC. Regarding this matter, and in order to improve both sensitivity and specificity of HPLC-based methods, further optimization of the protocols that have been described is clearly needed.

Conflict of interest

The author declares no conflict of interest.

Acknowledgements

This work was supported by an Integration into Scientific Research Grant (B Int-ICD/IPP-BST/CISA/01/2014) funded by Centro de Investigação em Saúde e Ambiente (CISA).

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