Escola Superior de Tecnologia da Saúde do Porto

Instituto Politécnico do Porto

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Biomarkers of Nitrosative Stress. Development and validation of a new analytical method for 3-Nitrotyrosine quantification

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Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica em Saúde – Ramo de Bioquímica Aplicada, realizada sob a orientação científica da Professora Doutora Mónica Vieira, Professora Adjunta da Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto, e sob a orientação institucional da Professora Doutora Cristina Prudêncio, Professora Coordenadora com Agregação da Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto.

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I dedicate this thesis to Fernando de Oliveira

"*A ciência nunca resolve um problema*

sem criar pelo menos outros dez"

George Bernard Shaw

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Abstract

Background: The nitration of tyrosine residues in proteins is associated with nitrosative stress, resulting in the formation of 3-nitrotyrosine (3-NT). 3-NT levels in biological samples have been associated with numerous physiological and pathological conditions. For this reason, several attempts have been made in order to develop methods that accurately quantify 3-NT in biological samples. Regarding chromatographic methods, they seem to be very accurate, showing very good sensibility and specificity. However, accurate quantification of this molecule, which is present at very low concentrations both at physiological and pathological states, is always a complex task and a target of intense research.

Objectives: We aimed to develop a simple, rapid, low-cost and sensitive 3-NT quantification method for use in medical laboratories as an additional tool for diagnosis and/or treatment monitoring of a wide range of pathologies. We also aimed to evaluate the performance of the HPLC-based method developed here in a wide range of biological matrices.

Material and methods: All experiments were performed on a Hitachi LaChrom Elite® HPLC system and separation was carried out using a Lichrocart® 250-4 Lichrospher 100 RP-18 (5µm) column. The method was further validated according to ICH guidelines. The biological matrices tested were serum, whole blood, urine, B16 F-10 melanoma cell line, growth medium conditioned with the same cell line, bacterial and yeast suspensions.

Results: From all the protocols tested, the best results were obtained using 0.5% CH_3COOH:MeOH:H_2O (15:15:70) as the mobile phase, with detection at wavelengths 215, 276 and 356 nm, at 25ºC, and using a flow rate of 1 mL/min. By using this protocol, it was possible to obtain a linear calibration curve (correlation coefficient = 1), limits of detection and quantification in the order of ng/mL, and a short analysis time (<15 minutes *per* sample). Additionally, the developed protocol allowed the successful detection and quantification of 3-NT in all biological matrices tested, with detection at 356 nm.

Conclusion: The method described in this study, which was successfully developed and validated for 3-NT quantification, is simple, cheap and fast, rendering it suitable for analysis in a wide range of biological matrices.

Keywords: 3-nitrotyrosine, nitrosative stress, HPLC-DAD, quantification methods

Resumo

Introdução: A nitração de resíduos de tirosina em proteínas está associada ao *stress* nitrosativo, resultando na formação de 3-nitrotirosina (3-NT). Os níveis de 3-NT em amostras biológica têm sido associadas com numerosas condições fisiológicas e patológicas. Por esta razão, várias tentativas têm sido levadas a cabo no sentido de desenvolver métodos que quantifiquem 3-NT com precisão em amostras biológicas. Relativamente aos métodos cromatográficos, exibem, em geral, boa precisão, sensibilidade e especificidade. Contudo, a quantificação exata desta molécula, que está presente em concentrações muito baixas, tanto em condições fisiológicas como em patológicas, continua a ser uma tarefa complexa e alvo de investigação.

Objetivos: Pretendeu-se desenvolver um método de quantificação de 3-NT rápido, económico, simples e sensível, para uso em laboratórios médicos como ferramenta adicional de diagnóstico e/ou monitorização do tratamento de diversas patologias. Pretendeu-se igualmente avaliar o desempenho do método desenvolvido numa variedade de matrizes biológicas.

Material e métodos: As experiências foram realizadas no sistema HPLC Hitachi LaChrom Elite®, com separação na coluna RP-18 Lichrospher LiChroCART® 250-4 100 (5 um). O método foi validado de acordo com as diretrizes da ICH. As matrizes biológicas testadas foram soro, sangue total, urina, linha celular de melanoma B16 F-10, meio de crescimento condicionado com a mesma linha de celular, suspensões bacterianas e de levedura.

Resultado: De todos os protocolos testados, os melhores resultados foram obtidos utilizando 0.5% CH_3COOH:MeOH (15:15:70) como fase móvel, com deteção a 215, 276 e 356 nm, a 25ºC, e uma taxa defluxo de 1 mL/min. Utilizando este protocolo, foi possível obter curva de calibração linear (coeficiente de correlação = 1), limites de deteção equantificação na ordem dos ng/mL, e um tempo de análise reduido (<15 minutos por amostra). Adicionalmente, o protocolo desenvolvido permitiu a deteção e quantificação de 3.NT em todas as matrizes biológicas testadas, com deteção a 356 nm.

Conclusão: O método descrito, que foi desenvolvido e validado com sucesso para quantificação de 3-NT, é simples, barato e rápido, tornando-o apropriado para análise de uma grande diversidade de matrizes biológicas.

Palavras-chave: 3-nitrotirosina, *stress* nitrosativo, HPLC-DAD, métodos de quantificação

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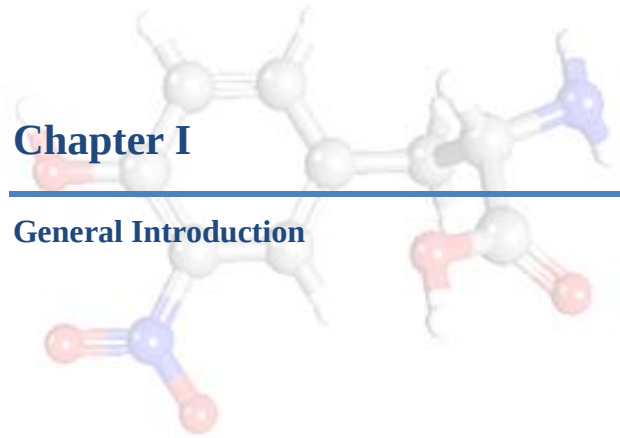
Abbreviations and acronyms

3-Br	Bromotyrosine
3-NT	3-Nitrotyrosine
ATCC	American Type Culture Collection
CAD	Coronary Artery Disease
CFU	Colony-Forming Unit
CSF	Cerebrospinal Fluid
DAD	Diode Array Detector
ECD	Electrochemical Detection
ED	Erectile Dysfunction
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization
eNOS	endothelial Nitric Oxide Synthase
GC-MS	Gas Chromatography-Mass Spectrometry
GC-MS/MS	Gas Chromatography-Tandem Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
LC	Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LoD	Limit of Detection
LoQ	Limit of Quantitation
MIP	Moleculary Imprinted Polymer
MS	Mass Spectrometry
NANC	Non-Adrenergic Non-Cholinergic
nNOS	neuronal Nitric Oxide Synthase
ODS	OctaDecylSilane
OPA	*Ortho*-phthaldialdehyde
PDE-5	Phosphodiesterase 5
PE	Penile Erection
PTN	Protein Tyrosine Nitration

RP-HPLC	Reverse Phase- High Performance Liquid Chromatography
RE	Relative error
ROS	Reactive Oxygen Species
RNS	Reactive Nitrogen Species
RSD	Relative Standard Deviation
SPE	Solid-Phase Extraction
TFA	TriFluoroAcetic Acid
Tyr	Tyrosine
UV	Ultraviolet
UV/VIS	Ultraviolet/Visible



Chapter I

General Introduction

1.1. 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 (de M Bandeira et al., 2013; Jones, 2006). On the other hand, Jones (2006) 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 (Jones, 2006; Ogino & Wang, 2007). 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 (Dalle-Donne, Rossi, Colombo, Giustarini, & Milzani, 2006; Ogino & Wang, 2007). This damage can compromise cell health and viability, as well as induce a variety of cellular responses like cell death by necrosis or apoptosis (Dalle-Donne et al., 2006).

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 (Ho et al., 2013). Figure 1 represents a schematization of biomarkers of oxidative stress.

A very promising approach for the assessment of oxidative stress is the detection of nitrated tyrosine (Tyr) residues in proteins (Safinowski et al., 2009).

1.2. 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 % (Ahsan, 2013; Radi, 2013b). Tyr is moderately hydrophilic, which is explained by its hydrophobic aromatic benzene ring carrying a hydroxyl group (Bartesaghi et al., 2007; Ryberg & Caidahl, 2007). 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 (Ahsan, 2013; Tsikas, 2012).

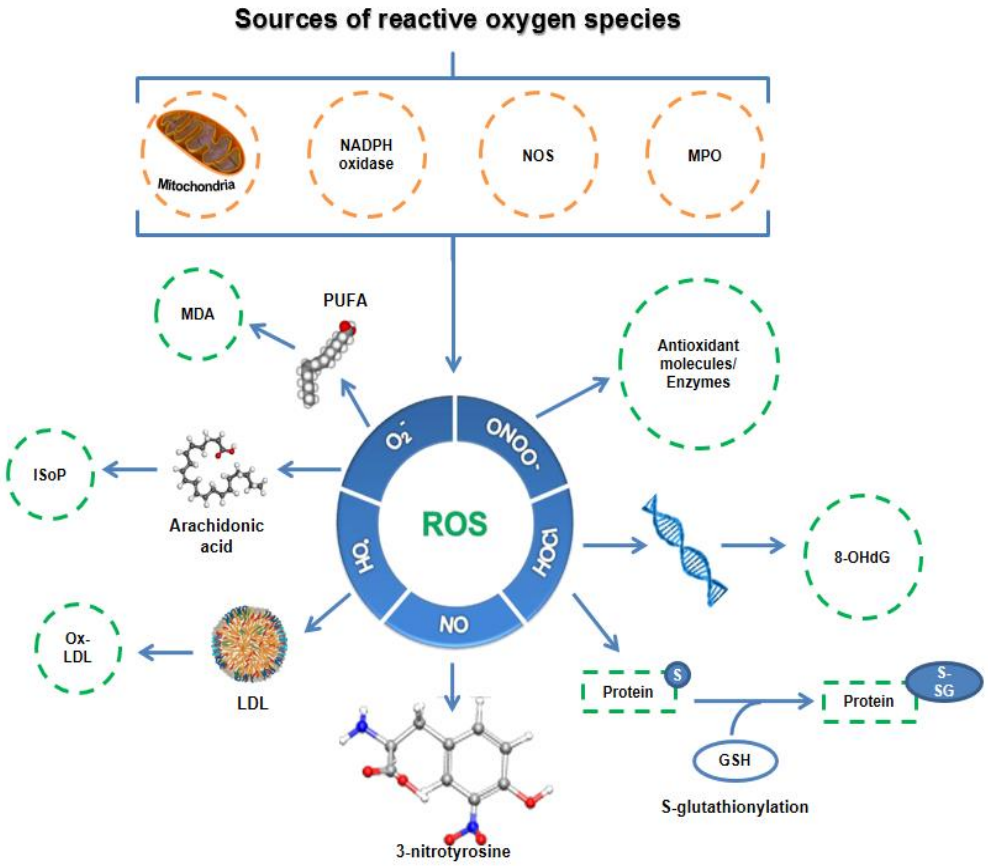


Figure 1. Formation pathways of selected biomarkers of oxidative stress.

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). Adapetd from (Ho, Karimi Galougahi, Liu, Bhindi, & Figtree, 2013) and (Shah, Mahajan, Sah, Nath, & Paudyal, 2014).

When RNS reacts with L-tyrosine and protein-associated Tyr, free 3-nitro-L-tyrosine and protein-associated 3-nitro-L-tyrosine are formed (Figure 2) (Tsikas & Caidahl, 2005). 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 (Ahsan, 2013; Radi, 2013b; Ryberg & Caidahl, 2007; Tsikas, 2012).

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 (Ahsan, 2013; Seeley, Fertig, Dufresne, Pinho, & Stevens, 2014). 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 (Jiao, Mandapati, Skipper, Tannenbaum, & Wishnok, 2001; Tsikas, 2012).

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 (Larsen, Bache, Gramsbergen, & Roepstorff, 2011; Yeo, Lee, Lee, & Kim, 2008).

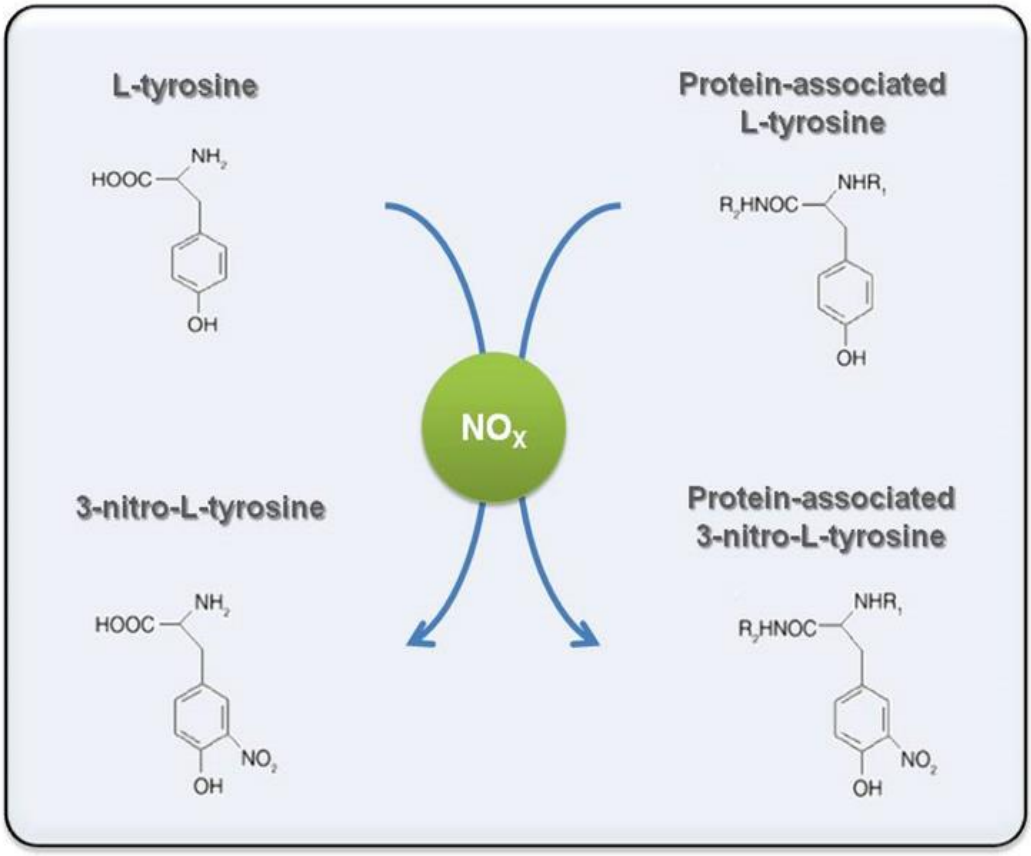


Figure 2. Nitration of L-tyrosine to 3-nitro-L-tyrosine (Adapted fromTsikas and Caidahl (2005).

1.3. 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 (Bryan & Grisham, 2007). In biological fluids or buffers, NO systems are almost completely oxidised to nitrite (NO_2^-), a biologically inert metabolite of NO oxidation (Shiva, 2013).

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 characterised 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 oxidised to nitrate (NO_3^-) in blood (Bryan & Grisham, 2007; Tsikas, 2012).

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

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

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

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).

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

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).

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

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

Concerning free radical superoxide (O_2^-), this may promptly interact with NO to produce the peroxynitrite anion ($ONOO^-$) (reaction 7) (Radi, 2004; Surmeli, Litterman, Miller, & Groves, 2010).

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

Peroxynitrite is the extremely reactive conjugate base of peroxynitrous acid (ONOOH) (reaction 8) (Bryan & Grisham, 2007; Tsikas, 2012).

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

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 (Goldstein & Merényi, 2008). 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 (Bircan, Balabanli, Turkozkan, & Ozan, 2011; Radi, 2013a). Peroxynitrite reacts with CO_2 to yield a nitrosoperoxycarbonate anion ($ONOOCO_2^-$) that undergoes a fast homolysis to NO_2 and carbonate radicals (CO_3^-) (reaction 9) (Kikugawa, Hiramoto, & Ohkawa, 2004; Radi, 2013a; Yeo et al., 2008).

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

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) (Radi, 2013a).

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

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

Moreover, 3-NT may be generated through multiple pathways (Figure 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) (Bryan & Grisham, 2007; Sun et al., 2007; Yeo et al., 2008).

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

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

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

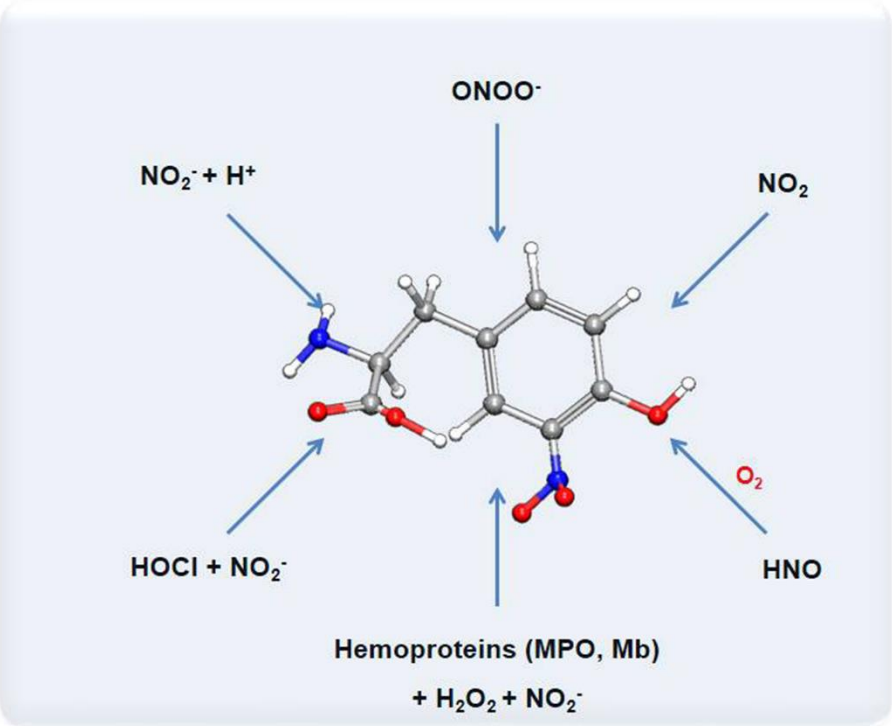


Figure 3. Multiple pathways for the formation of 3-nitrotyrosine (Adapted from (Bryan & Grisham, 2007)).

1.4. Association between 3-nitrotyrosine and disease

Several physiological and pathological conditions have been associated with increased nitration of proteins (Seeley et al., 2014; Surmeli et al., 2010). Among the pathological conditions, there is a wide range of cardiovascular diseases, such as myocardial inflammation, heart failure and arteriosclerosis (Daiber & Münzel, 2012; de M Bandeira et al., 2013; Kagota et al., 2010; Nuriel, Deeb, Hajjar, & Gross, 2008; Yakovlev & Mikkelsen, 2010). For instance, Shishehbor et al. (2003) and Poufarzam et al. (2013) 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 (Sucu et al., 2003). Furthermore, there is also evidence of an accumulation of 3-NT during atherogenesis (Upmacis, 2008).

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 (Voraphani, Gladwin, Trudeau, & Wenzel, 2013), systemic sclerosis (Shimizu et al., 2007), renal complications (Shah et al., 2014), inflammatory bowel disease (Kruidenier, Kuiper, Lamers, & Verspaget, 2003), septic shock (Teng et al., 2011), rheumatoid arthritis and joint injury (Misko et al., 2013; Winyard et al., 2011). In relation to asthma, 3-NT expression was found to be increased in several asthmatic epithelial cells and essentially in asthmatic children (Baraldi et al., 2006; Voraphani et al., 2013). Concerning systemic sclerosis, Shimizu et al. (2007) 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 (Kruidenier et al., 2003). Furthermore, some neurological diseases and psychiatric disorders are also associated with an increased level of nitrated proteins. For example, Parakh et al. (2013) reported that nitrated proteins and high levels of 3-NT have been detected in cases of amyotrophic lateral sclerosis. Conversely, Mendonça et al. (2011) 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. (2009) 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 (Mangialasche et al., 2009), Parkinson's disease (Blanchard-Fillion et al., 2006), autism (Rose et al., 2012) and myalgic encephalomyelitis/ chronic fatigue syndrome (Morris & Maes, 2014).

Other diseases are associated with increased protein nitration. Shu et al. (2014) 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 (de M Bandeira et al., 2013; Jialal, Devaraj, Adams-Huet, Chen, & Kaur, 2012), especially in patients with diabetic nephropathy (Thuraisingham, Nott, Dodd, & Yaqoob, 2000) or diabetic patients with microvascular complications (Devaraj et al., 2007). Regarding Chagas disease, Dhiman et al. (2008) 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. (2014) demonstrated that 3-NT levels were increased in plasma and serum of patients with systemic lupus erythematosus.

1.5. 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 (Guvenç, Aksoy, Kursad, Atmaca, & Yavuz, 2014). 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 (Tsikas & Caidahl, 2005).

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 (Radabaugh, Nemirovskiy, Misko, Aggarwal, & Mathews, 2008). Recently, Mergola et al. (2013) 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.

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 (Ryberg & Caidahl, 2007).

1.5.1. Immunochemical methods

1.5.1.1. 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 (Gan & Patel, 2013). 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 (Gan & Patel, 2013).

Regarding 3-NT quantification, there is a wide variety of ELISA-based methods for this purpose, namely indirect, competitive, sandwich-ELISA and ELISA microarrays (Jin &

Zangar, 2012; Weber et al., 2012). Table I lists some ELISA assays for the analysis of 3-NT in different biological specimens.

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

Compound	Biological specimen	ELISA Type	Reference
3-NT	Bronchoalveolar lavage	Sandwich ELISA	(Fitzpatrick, Brown, Holguin, & Teague, 2009)
3-NT-containing proteins	Plasma	Competitive ELISA	(Dietrich-Muszalska et al., 2012)
3-NT-modifed proteins	Plasma	Competitive ELISA	(J. Khan et al., 1998)
3-NT	Plasma and serum	Competitive luminescence assay (CLIA)	(Safinowski et al., 2009)
3-NT	Plasma	Solid phase ELISA	(Safinowski et al., 2009)
3-NT	Plasma and serum	Sandwich ELISA	(Sun et al., 2007)
3-NT	Plasma and sputum	ELISA microarray	(Jin & Zangar, 2012)
3-NT	Plasma	Sandwich ELISA	(Jialal et al., 2012)
3-NT	Serum	Sandwich ELISA	(Shimizu et al., 2007)
Protein bound 3-NT	Serum	Sandwich ELISA	(F. Khan, Siddiqui, & Ali, 2006)
Protein bound 3-NT	Plasma and serum	Indirect ELISA	(Weber et al., 2012)

One of the first ELISA methods for the analysis of nitrated proteins in biological fluids was based upon a competitive model (J. Khan et al., 1998). More recently, Safinowski et al.

(2009) 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 (Safinowski et al., 2009; Sun et al., 2007; Weber et al., 2012). 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 (Sodum, Akerkar, & Fiala, 2000).

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 (Jin & Zangar, 2012).

1.5.2. Chromatographic methods

Chromatography is a powerful analytical technique, and is widely available in different laboratory settings nowadays. Chromatography is a technique for separation of complex mixtures and was described at the beginning of the twentieth century by Russian–Italian botanist M. S. Tswett (Wixom & Gehrke, 2011). This technique is generally composed by two primary components: mobile phase and stationary phase (Naushad & Khan, 2014). Table II shows chromatography classifications according to the mobile and stationary phases used.

Table II. 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)

1.5.2.1. 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 III). Several different detectors have been employed, such as ultra-violet (UV), electrochemical (ECD), diode-array (DAD) and mass spectrometry (MS) (Tsikas, 2012).

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 (Naushad & Khan, 2014). 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. The retention time is based on differences in the migration rate through the column arising from different partitions of the sample in the stationary phase, the molecules being analysed, and the solvent(s) used. Commonly used solvents contain miscible combinations of water and organic liquids, the most common being methanol and acetonitrile. The gradient elution is responsible for separating the analyte mixture as a function of the affinity of the analyte for the mobile phase. The choice of solvents, additives and gradient can be influenced by the nature of both the stationary phase and the analyte (Meyer, 2013).

HPLC is the standard technique for analysing amino acids, being the technique used by most laboratories (Ryberg & Caidahl, 2007). 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 (Kaur & Halliwell, 1994). 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 detection limit of 0.2 µM (Kaur & Halliwell, 1994). Variants of this method have been used in several studies to determine 3-NT. Hitomi et al. (2007) 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 (Yang, Zhang, & Pöschl, 2010) . 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 (Herce-Pagliai, Kotecha, & Shuker, 1998; Yang et al., 2010). 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 (Ryberg & Caidahl, 2007).

HPLC-Diode Array Detector. Comparatively to UV detection, DAD allows the simultaneous detection at different wavelengths (Dong, 2006). 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 (Mergola et al., 2013). Recently, Selzle et al. (2013) 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 (Selzle et al., 2013).

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 (Herce-Pagliai et al., 1998). For instance, Pourfarzam et al. (2013) 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 (Pourfarzam et al., 2013). 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) (W.-Z. Zhang, Lang, & Kaye, 2007). The limitation of this method is that several fluorescent compounds are produced during the derivatization process (Herce-Pagliai et al., 1998).

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 (Nuriel et al., 2008).

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 (Nuriel et al., 2008).

1.5.2.2. 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 (Ryberg & Caidahl, 2007; Tsikas & Duncan, 2014). 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 (Tsikas & Caidahl, 2005). 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 artefacts formation (Ryberg & Caidahl, 2007; Tsikas & Duncan, 2014).

Nevertheless, liquid chromatography-mass spectrometry (LC-MS) does not offer the necessary selectivity for 3-NT measurement in biological sample, especially in human plasma (Ryberg & Caidahl, 2007; Tsikas & Duncan, 2014). 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 (Ryberg & Caidahl, 2007).

Chen & Chiu (2008) developed a highly specific and accurate LC/MS/MS assay that allows simultaneous analysis of protein-bound 3-NT and 3-bromotyrosine (3-BT) in human urine. This proteins function as non-invasive biomarkers for *in vivo* PTN and bromination.

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

Table III. 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
3-NT, Tyr (Guvenç et al., 2014)	Brain tissue	Inertsil® ODS C_{18} 4.6 x 250; 5	50 mM sodium acetate (pH 4.2), 10% methanol (v/v)	0.8	DAD 278 nm
3-NT (Sodum et al., 2000)	Proteins from blood plasma	Two ultrasphere ODS 4.6 x 150; 3 4.6 x 46; 5	50 mM sodium acetate (pH 4.7), 5% methanol (v/v)	0.7	UV 280 nm
3-NT, *p*-nitro-L-phenylalanine, and L-Tyr (Tsikas, Mitschke, Suchy, Gutzki, & Stichtenoth, 2005)	Urine	Nucleosil 100-5 C_{18} 4.0 x 250; 5	50 mM $(NH_4)_2SO_4$ in water-methanol (95:5, v/v) (pH 5.5)	1.0	UV 276 nm
3-NT, Tyr (W.-Z. Zhang et al., 2007)	Plasma	Nova-Pak C_{18} 3.9 × 150; 4	**Mobile phase A:** ACN and 0.02M 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 0.02M phosphate buffer (pH 6.5; 10:90 v/v), 500 µL/L TFA (pH 3.5)	1.0 (34°C)	UV 540 nm
3-NT (Ishida, Hasegawa, Mukai, Watanabe, & Nishino, 2002)	Plasma	SD ODS 3.0 x 150; 5	100 mM phosphate buffer solution, 5% methanol (v/v)	0.5 (25°C)	ECD
3-NT (Hitomi et al., 2007)	Rat plasma	SC-50ODS 3.0 x 150; 5	200 mM phosphate buffer containing 5mg/mL EDTA, 2% ACN	0.5	ECD

Table III. (continued)

Compound	Sample	Column[a]	Eluents	Flow rate (mL/min)	Detection method
3-NT (Bircan et al., 2011)	Animal spleen tissues	Microtech Scientific C_{18} 1.0 x 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
Free 3-NT Assay (Pourfarzam et al., 2013)	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
3-NT residues in protein (Yang et al., 2010)	Bovine serum albumin and ovalbumin	Grace Vydac 2.1 x 250; 5	0.1% (v/v) TFA in water and ACN	0.3	DAD 280 and 357 nm
Tyr and 3-NT metabolites (Blanchard-Fillion et al., 2006)	Undifferentiated human teratocarcinoma NT2 and rat pheochromocytoma PC12 cell lines	Octadodecyl silica gel reverse-phase column 4.6 x 250; 5	0.1% TFA in ultra pure water (solvent A) and 100% ACN (solvent B)	1.0	DAD 215, 275, 365 nm
Tyr and 3-NT (Kikugawa et al., 2004)	Gaseous nitrogen oxide species	Inertsil ODS-2 4.6 x 250; 5	0.5% (v/v) acetic acid:methanol (29:1, v/v)	1.0	UV-VIS detector 280 nm
3-NT (Ueshima et al., 2007)	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
Free 3-NT (F. Khan et al., 2006)	Serum	C_{18} reversed phase column 4.6 x 250; 5	500 mM potassium phosphate buffer (pH 3.5), 10% methanol (v/v)	0.8	UV 274 nm

[a] Column dimensions in the following order: internal diameter (in mm) x length (in mm); particle size (in µm).

ACN, acetonitrile; EDTA, ethylenediamine tetraacetic acid; TFA, trifluoroacetic acid.

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

Compound	Sample	Column[a]	Eluents	Flow rate	Detection method
3-NT (Seeley et al., 2014)	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/mim	LC-MS/MS
3-chlorotyrosine, 3-BT, and 3-NT (Gaut, Byun, Tran, & Heinecke, 2002)	Plasma	C_{18} reversed phase column Zorbax 1.0 x 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-NT and 3-BT (Chen & Chiu, 2008)	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
3-NT (Thornalley et al., 2003)	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
Free amino acid and protein 3-NT (Radabaugh et al., 2008)	Biological fluids	Immunoaffinity column: Targa 4.6 x 30 C_{18} reversed-phase column: Betasil 2.1 x 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

Table IV. (continued)

Compound	Sample	Column[a]	Eluents	Flow rate	Detection method
3-NT, 3-BT, Dibromotyrosine (Kato et al., 2009)	Urine	ODS-SR 2 × 150; 5	0.05% formic acid/CH_3CN (95:5)	Not stated	LC/MS/MS
Free 3-NT (Hui et al., 2012)	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
3-NT (Nemirovskiy et al., 2009)	Plasma	Immunoaffinity column: Targa 4.6 x 30 C_{18} reversed-phase column: 2.1 x 100	Immunoaffinity column: 1% formic acid solution C_{18} reversed-phase column: 100% 10 mM NH_4OAc in H_2O and 80% ACN	1 mL/min	2D LC–MS/MS

[a] Column dimensions in the following order: internal diameter (in mm) x length (in mm); particle size (in µm)

ACN, acetonitrile; TFA, trifluoroacetic acid.

1.5.2.3. Gas chromatography-mass spectrometry methods

GC-MS methodology is used for the analysis of volatile and thermally stable small molecules (Ryberg & Caidahl, 2007). 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 (Tsikas & Duncan, 2014). Derivatization, however, often accounts for 3-NT artefact formation, which is may be responsible for high variation of basal plasmatic 3-NT levels (Tsikas & Caidahl, 2005). 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 (Tsikas & Caidahl, 2005; Tsikas et al., 2005). Table V **s**ummarises 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 (Tsikas & Duncan, 2014).

Gaut et al. (2002) 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 (Tsikas, 2012).

In the study performed by Söderling et al. (2003), 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 (Söderling et al., 2003).

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 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 (Tsikas & Duncan, 2014). Overall, the main

disadvantage of using chromatographic methods is that they involve time-consuming sample preparation procedures (Weber et al., 2012).

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

Compound	Sample	Column[a]	Eluents	Detection method
3-NT (Tsikas et al., 2005)	Urine	Optima 5-MS 30 x 0.25; 0.25	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa collision pressure)	GC–MS/MS
Protein-associated 3-NT and 3-nitrotyrosinoalbumin (Tsikas et al., 2003)	Plasma	Optima 5-MS 30 x 0.25; 0.25	Helium (55 kPa); Methane (530 Pa) and Argon (0.27 Pa)	GC–MS/MS
3-NT (Ryberg et al., 2004)	CSF	DB-1 MS column 15 × 0.32 ; 0.25	Helium (3psi); Methane (750-800 Pa)	GC–MS/MS
3-NT (Söderling et al., 2003)	Plasma	DB5-MS column 30 x 0.25; 0.25	Helium (41 kPa); Methane (0.27-1.1 kPa)	GC–MS/MS
Free 3-NT (Lärstad, Söderling, Caidahl, & Olin, 2005)	Exhaled breath condensate (EBC)	DB5-MS column 30 x 0.25; 0.25	Helium (38 kPa); Methane (0.97-0.99 kPa)	GC/NICI/tandem MS

[a] Column dimensions in the following order: internal diameter (in m) x length (in mm); particle size (in µm)

1.6. Objectives of this thesis

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 nitrosative stress state, has been associated with a wide range of diseases. Previous published studies have shown that other biomarkers of oxidative stress may be a useful tool for therapeutic monitoring (Joly & Grunfeld, 2014; Peixoto, 2012). In this sense, the main goal of this project was to develop a sensitive, simple, low-cost 3-NT quantification method for use in medical laboratories as a tool for diagnosis and/ or treatment monitoring of a wide range of pathologies.

This goal was achieved through the following tasks:

(i) Development and validation of the method for 3-nitrotyrosine quantification (chapter II);
(ii) Evaluation of the applicability of the aforementioned chromatographic method in different samples (chapter III).

Lastly, chapter IV shows the overall conclusions, limitations as well as future perspectives of this study.

Chapter II

Development and validation of the method for 3-nitrotyrosine quantification

2.1. Introduction

2.1.1. General concept of a High-performance liquid chromatography system

An HPLC system is composed by several specialized sectors interconnected to each other by a thin tube system that allows the movement of the mobile phase between them (Figure 4). The major components of a classic HPLC system are described in detail below (Dong, 2006; Rouessac & Rouessac, 2013).

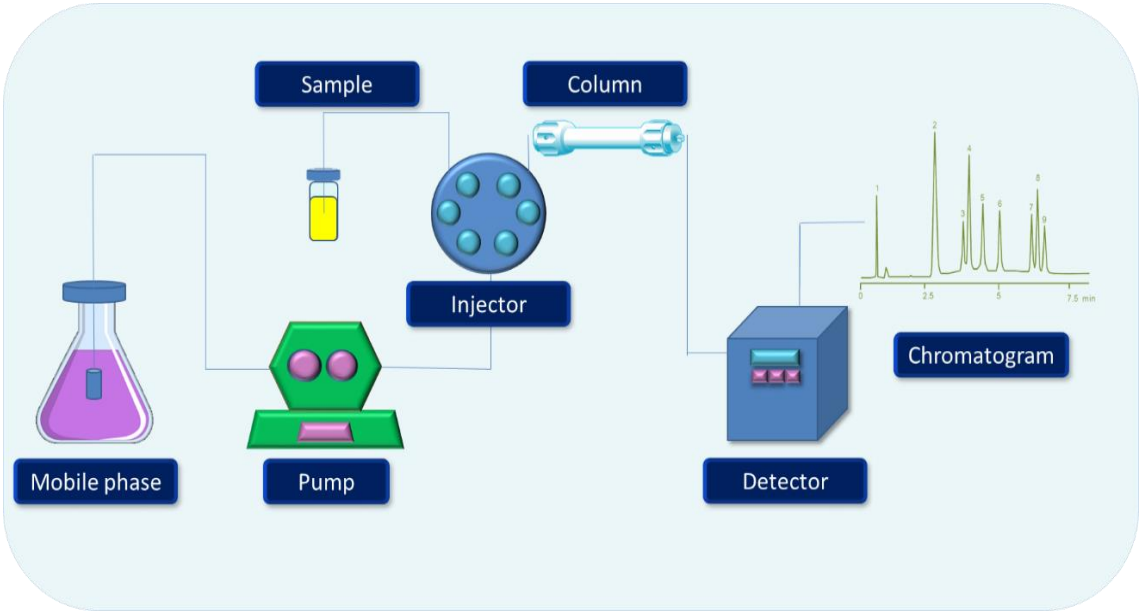


Figure 4 Schematic representation of a modular HPLC instrument.

The chromatography allows the separation of a complex mixture into its individual compounds. The separation is performed between a mobile phase and a stationary phase. The HPLC is composed by mobile-phase reservoirs, pumps, mixer to mix the solvents, a valve into which the sample is injected, a guard column, a column containing the stationary phase, a detector, and a recorder.

Pump. The role of the pump is to force the mobile phase through the column which packing is fairly compact. The pump allows to maintain a constant flow regardless of the pressure. The pressure depends on (i) the flow rate, (ii) the viscosity of the mobile phase and (iii) the particle size of the stationary phase. Generally, the pump contains two pistons, and is capable of delivering an eluent of fixed (isocratic) or variable composition to create an elution gradient (Baker, Dunn, Lajtha, & Holt, 2007; Rouessac & Rouessac, 2013).

Injector. The injector allows to introduce a precise volume of sample onto the head of the column in a rapid manner. The importance of these injectors relies on the minimization of flow disturbances due to the dynamic regime of the mobile phase, which permits a stable flow from the column to the detector (Rouessac & Rouessac, 2013).

Column. The column is regarded as the major component of an HPLC system. The column is a straight stainless steel calibrated tube and coated with an inert material, such as spherical silica gel beads, polymers and alumina (Kazakevich & LoBrutto, 2007). This column should be changed from time to time and it is recommended, prior to analysis, to pass the samples through a filter of pore size less than 0.5μm in order to enlarge column's time of life (Rouessac & Rouessac, 2013).

Stationary phase. The stationary phase is the second medium in which the compounds initially dissolved in the mobile phase will interact. The spherical silica gel is the basic material mostly used in HPLC columns packaging (Rouessac & Rouessac, 2013). The silica matrices are robust and very polar and form a three-dimensional network. This matrix can be chemically modified by a derivatisation process through hydrophobic ligands, providing the original surface with a higher level of hydrophobicity. For instance, the ligand dimethyl octadecylsilane (ODS) is often used in such derivatisation process, being mostly applied in protocols for the reverse phase-HPLC (RP-HPLC) analysis of peptides and proteins (Fanali, Haddad, Poole, Schoenmakers, & Lloyd, 2013; Rouessac & Rouessac, 2013).

Mobile phase. The interaction between the mobile and the stationary phases affects the retention time of the analytes. If the stationary phase is polar, the mobile phase should be less polar. On the other hand, if the stationary phase is non-polar, the mobile phase should be polar. Regarding the latter case, the mobile phase is often made up of water with a modifying organic solvent (e.g., methanol or acetonitrile) (Buszewski & Noga, 2012; Rouessac & Rouessac, 2013).

Detector. Detectors permit continuous registration of specific physical and chemical proprieties of the column effluent (Kazakevich & LoBrutto, 2007). HPLC-based methods employ a wide range of detection strategies based on the analytes proprieties, such as spectrophotometry, fluorescence detection and refractive index (Baker et al., 2007; Rouessac & Rouessac, 2013).

2.1.2. Theory of reversed phase chromatography

In simplistic terms, chromatography involves passing a mixture dissolved in the mobile phase trough a stationary phase, which separates the analyte to be measured from other molecules present in the mixture. Such separation process is based on the differential

partitioning effect between the mobile and stationary phases (Mullangi, Sharma, & Srinivas, 2012).

Reversed-phase chromatography involves the separation of molecules on the basis of their hydrophobicity. This separation depends on the hydrophobic binding interaction between the solute molecules from the mobile phase and the immobilized hydrophobic ligands from the stationary phase (Fanali et al., 2013) (Figure 5).

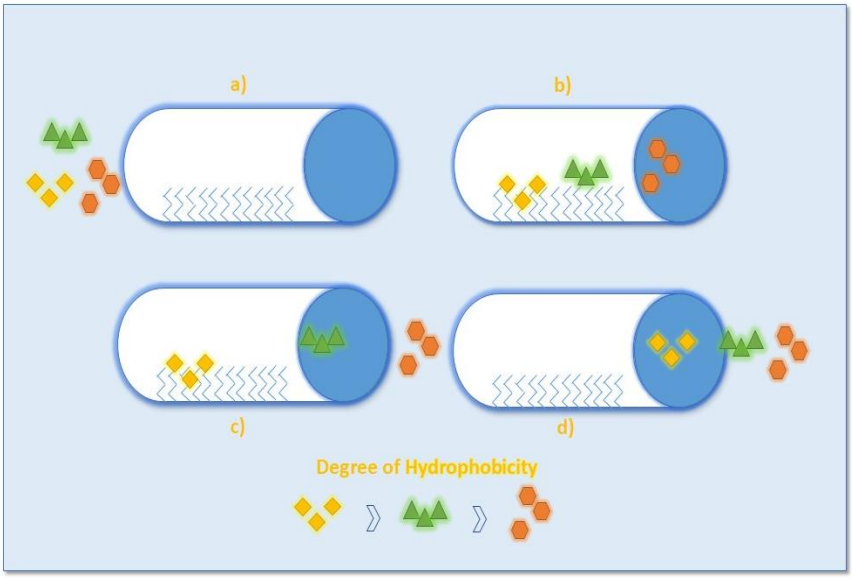


Figure 5. Principle of separation of reversed-phase chromatography.

2.1.2.1. Critical parameters in reversed phase chromatography

Column length. The essential parameters of any chromatographic column are its (i) permeability, (ii) efficiency, and (iii) retention ability (Guiochon, 2007) .

The resolution of small organic molecules in reversed phase separations is more sensitive to column length than in high molecular weight molecules. The resolution of small peptides may sometimes be improved by increasing column length. In the case of small changes in the organic modifier concentration of the mobile phase, the resolution is increased by using a longer column (Biotech, 2002).

The column length may not be a critical parameter in the separation resolution when gradient elution is used. Using elution gradient is particularly advantageous in biological samples that contain a mixture of molecules with different adsorption affinities (Gad, 2007).

Flow rate. One important factor in the resolution of small molecules in reversed phase separations is the flow rate. Nevertheless, larger biomolecules appear to be insensitive to flow rate. In analytical protocols, the flow rate is especially important during the loading of

a large number of samples. Flow rate may also influence the dynamic binding capacity of the gel, which reflects the kinetics of the solute binding process during the loading stage (Biotech, 2002).

Temperature. The temperature is a critical factor in reversed phase chromatography, particularly when analysing small molecules. It is known that column temperature affects both time retention and selectivity. In this case, increasing the temperature is theoretically effective (Guillarme, Heinisch, & Rocca, 2004). Increased temperatures are known to decrease mobile phase viscosity used in reversed phase chromatography (Guillarme et al., 2004). In turn, viscosity influences the method resolution, as a decrease in this parameter leads to an increased resolution (Clark, 2004).

Mobile phase. Mobile phase used in reversed phase chromatography is commonly made up of strong acids with large concentrations of organic solvents. However, and when physiological conditions needs to be mimicked, a buffer (e.g. phosphate buffer) is often used as mobile phase (Taylor, 2014).

2.1.3. HPLC method development

Analytical method development and validation is a four-step process. Briefly, HPLC method development includes (i) selection of the method and system, (ii) selection of initial conditions, (iii) method optimization, and (iv) validation.

2.1.3.1. Selection of the method and initial system

The first step is to check whether similar protocols have previously been published, allowing a more cohesive and less time consuming start point (Murugan et al., 2013). In this initial step, the type of HPLC is selected, and the physicochemical properties of the molecule as well as the sample preparation process are evaluated (Fergusom & Huet, 2011; Gupta, Jain, Gill, & Gupta, 2012; Murugan et al., 2013).

Physicochemical proprieties of the molecule to study play an important role in method development (Carini, Kaiser, Ortega, & Bassani, 2013; Gupta et al., 2012). The properties to take into account usually include (i) solubility (which influences the solvent selection), (ii) polarity (to choose the solvent and composition of the mobile phase), (iii) pKa and (iv) pH (Gupta et al., 2012; Murugan et al., 2013). The sample should ideally be dissolved in the mobile phase. When not possible, additives may be used, such as formic acid, acetic acid and salts (Murugan et al., 2013).

2.1.3.2. HPLC conditions

Selection of the optimal conditions are pivotal for a successful method development process. However, a wide range of parameters needs to be carefully examined in advance.

Buffer selection. The selection of buffer is based on the desired pH. The recommended pH range for reversed phase on silica-based packing is between 2 and 8. Buffers have a pKa close to the recommended pH range and are usually used to adjust the pH of the mobile phase (Fergusom & Huet, 2011). The pH of the mobile phase is an important parameter to bear in mind since it determines the chromatographic retention of various analytes with acid-base properties (Roses, 2004).

Buffer concentration. For small molecules, the buffer concentration range is usually 10-50nM, with an organic solvent content <50%. The most common buffers used in reversed phase protocols are sodium and potassium salts (Gupta et al., 2012).

Isocratic or gradient separations. Regarding the mobile phase composition, two main strategies are commonly used: isocratic or gradient elution. When applying an isocratic elution, the mobile phase composition and the velocity of the compounds moving through the column remains constant throughout the separation process (Gupta et al., 2012). On the other hand, a gradient elution implies that the mobile phase composition is changed during the analysis, which can be achieved through different approaches (linear, segment, convex and concave gradient). The selection of the gradient mode to be used should allow a reduced time analysis as well as a high resolution (V. S. Joshi, Kumar, & Rathore, 2015).

2.1.3.3. Method optimization

After a general selection of the initial conditions, it is essential to optimize them so that satisfactory sensitivity and efficient chromatographic separation can be achieved. The optimization process comprises different stages, and the time consumed on this step depends on the number of variables to be adjusted. Experimental variables often include pressure, temperature, column length, mobile phase and flow rate (Wang, Carr, & Stoll, 2010).

2.1.3.4. Method Validation

The validation step is extremely important since it assures that the analytical method optimized is suitable for the intended purpose. Validation data should provide detailed description of the method, including demonstration of compliance with acceptance criteria

approved by a recognised entity following current good laboratory practice (FDA, 2015). This step is of particular importance for methods to be used in medical laboratories (Kumar, Sreenivasa Reddy, Managuli, & Pai K, 2015).

Q2 (R1) guideline from International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) is considered the primary reference for recommendations and definitions on validation of analytical methods (FDA, 2015; ICH, 2005; Sonawane, Poul, Usnale, Waghmare, & Surwase, 2014). Directive 96/23/EC concerns to the performance of analytical methods and to the interpretation of results (European Commission, 2002).

2.1.3.5. Validation of parameters

The parameters defined by ICH for validation of analytical methods are briefly described in Table VI.

Table VI. Parameters defined by ICH for validation of analytical methods

Parameters	Description
Specificity	ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc." This definition implies the identification of an analyte and a purity test.
Linearity	The linearity in an analytical procedure that allows to obtain test results directly proportional to the concentration (amount) of analyte in the sample; The linearity should be determined at least with a series of five patterns for which the expected concentrations should cover 80%-120% of the desired concentration. The result should be proportional to the concentrations of the analytes and it should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares.
Accuracy	The accuracy of an analytical method is defined by the closeness of test results obtained by that method to the true value or to an accepted reference value. Accuracy may be determined through different ways: (i) by analysing a sample of known concentration (reference material), and comparing the measured value to the true value; (ii) by comparing test results from the new method with those of a second well-characterized procedure, the accuracy of which is stated and/or defined; (iii) based on the recovery of known amounts of analyte (minimum to prepare in triplicate at three levels) and (iv) based on the recovery of spiked analyte with standard additions.

Table VI. (continued)

Parameters	**Description**	
Precision	Precision is an analytical procedure that enables to evaluate the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions.	
	Repeatability	Repeatability express the precision operating over a short time interval under the same condition (intra-assay precision); It should be assessed using a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each), or from a minimum of six determinations at 100% of the test concentration.
	Intermediate precision	The intermediate precision consists in the comparison of the results of a method run within a single laboratory over a number of days. This precision may reflect discrepancies in results obtained from different operators, different instruments standards and reagents from different suppliers or others.
	Reproducibility	Reproducibility is assessed between laboratories by means of an inter-laboratorial trial.
Range	The range of an analytical produce provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.	
Limit of detection	The limit of detection (LoD) is the point at which the lowest concentration of analyte in a sample can be detected but not essentially quantitated as an exact value.	
	Visual evaluation	Visual evaluation is determined by the (i) analysis of samples with known concentration of analyte and by (ii) establishing the minimum level at which the analyte can be reliably detected.
	Signal-to-Noise	The signal-to-noise ratio determination is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is normally considered satisfactory for estimating the detection limit.

Table VI. (continued)

Parameters	**Description**	
	Standard deviation of the response and slope	The LoD may be expressed as: $\boldsymbol{LoD = \frac{3.3\sigma}{S}}$ σ = the standard deviation of the response S = the slope of the calibration curve The σ may be estimated by the following ways: Based on the Standard Deviation of the Blank - analysing an appropriate number of blank samples and calculating the standard deviation Based on the Calibration Curve - using samples containing an analyte in the range of LoD. The standard deviation may be calculated by the residual standard deviation of a regression line or by the standard deviation of y-intercepts of regression lines.
Limit of quantification	The limit of quantification (LoQ) is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy.	
	Visual evaluation	Visual evaluation determined by the (i) analysis of samples with known concentrations of analyte and by (ii) setting the minimum level at which the analyte can be quantified with acceptable accuracy and precision
	Signal-to-Noise	The signal-to-noise ratio determination is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected.
	Standard deviation of the response and slope	The LoQ may be expressed as: $\boldsymbol{LoQ = \frac{10\sigma}{S}}$ σ = the standard deviation of the response S = the slope of the calibration curve The σ may be estimated by following ways: (i) Based on the Standard Deviation of the Blank - analysing an appropriate number of blank samples and calculating the standard deviation (ii) Based on the Calibration Curve - using samples containing an analyte in the range of LoQ. The standard deviation may be calculated by the residual standard deviation of a regression line or by the standard deviation of y-intercepts of regression lines.

Table VI. (continued)

Parameters	Description
Robustness	Robustness should be determined during the development phase, and provides an indication of the procedure's analysis reliability in relation to deliberate variations in method. Parameters that affect robustness often include (i) pH, (ii) mobile phase composition; (iii) flow rate, (iv) column temperature, and (v) column type.

Reference: (FDA, 2015; Huber, 2010; ICH, 2005)

2.1.4. Aim

The aim of this chapter was to develop a suitable method for use in most clinical laboratories. Therefore, and taking into account all the positive and negative features of the methods described throughout Chapter I, we defined as major pre-requisites i) availability of the equipment in a wide range of medical laboratories, ii) reduced pre-analysis steps, and iii) cost-effective analysis. After an exhaustive analysis, we found that an HPLC-based method would perfectly meet the previous pre-requisites.

In chapter II is described the first three steps of the method development stage: i) selection of the method and system, (ii) selection of initial conditions, (iii) method optimization and method validation. Regarding optimization, different mobile phases were tested in order to find one exhibiting well-defined peaks, a good LoD and LoQ, a good linearity and reduced time analysis.

2.2. Material and Methods

2.2.1. Instrument and software

All experiments were performed on a Hitachi LaChrom Elite® HPLC system (Hitachi High - Technologies Corporation, Tokyo, Japan) composed by HTA L-2130 LaChrom Elite quaternary pumps (Hitachi High-Technologies Corporation), L-2200 LaChrom Elite autosampler (Hitachi High-Technologies Corporation), L-2300 LaChrom Elite column heater (Hitachi High-Technologies Corporation), L-2455 LaChrom Elite photo DAD (Hitachi High-Technologies Corporation). EZChrom Elite Compact Software Version 3.3.2. (Agilent Technologies, Inc., Santa Clara, CA, United States) was used for data collection and treatment.

2.2.2. Reagents and consumables

3-Nitro-L-tyrosine was purchased from Santa Cruz Biotechnology, Inc. (Bergheimer, Heidelberg, Germany). L-Tyrosine was purchased from AppliChem - BioChemia GmbH (Ottoweg, Darmastadt, Germany). Glacial acetic acid (100%) was purchased from Merck S.A. (Algés, Portugal). Methanol (HPLC GOLD Ultra Gradient) was purchased from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). Ultrapure water was obtained from the Water Purification System TKA Barnstead™ GenPure™ capsule 0.2µm (Thermo Fisher Scientific, Wilmington, DE, EUA). Trifluoroacetic acid was purchased from Biochem Chemopharma (Ligne, Cosne sur Loire, France). LiChrospher® 100 RP-18 (5 µm) LiChroCART® 250-4 was purchased from Merck S.A. (Algés, Portugal). Membrane filters 0.45µm, 47mm, were purchased from Advantec®, Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). Puradisc^{TM}, 0.2 µm, 25mm sterile and endotoxin free filters were purchased from Whatman^{TM} (GE Healthcare UK Limited, Buckinghamshire, UK). 2 mL syringes were purchased from Terumo® Medical Corporation (Leuven, Belgium).

2.2.3. Analytical procedure

2.2.3.1. Mobile phase – 500 mM KH_2PO_4 (pH 3.5)

KH_2PO_4 solutions were prepared in three different concentrations: 500 mM plus 10% MeOH, 50 mM, and 25 mM. All solutions were adjusted to pH 3.5 using a 1M H_3PO_4 solution.

2.2.3.2. Mobile phase – 0.5% CH_3COOH:MeOH:H_2O

0.5% CH_3COOH:MeOH:H_2O solutions were prepared according the following proportions: 29:1:70 (Kikugawa et al., 2004), 30:0:70, and 15:15:70.

All mobile phases, including those prepared in 2.2.3.1, were filtered through a 0.45µm membrane.

2.2.3.3. Calibration standards

0.5 mg/mL 3-NT and Tyr stock solutions were made using the aforementioned mobile phases as solvents. All stock solutions were filtered through a filter membrane device. The first assays were performed using standard solutions containing either 3-NT or Tyr in the following concentrations (100000; 50000; 25000; 10000; 5000 ng/mL). In the subsequent assays, standard solutions containing both 3-NT and Tyr were prepared in the following concentrations (50000; 25000; 10000; 5000; 2500; 1250; 625 and 312.5 ng/mLStandard

solutions were prepared by diluting the respective stock solution into the desired mobile phase. When required, heat was used in order to better dissolve the reagents. These standard solutions were used for calibration purposes.

2.2.3.4. Chromatographic conditions

Table VII shows the chromatographic conditions used in all assays described in this study.

Table VII. Chromatographic conditions

Flow rate*	Volume of injection	Detection
1mL/min	25 µL	190-400 nm

* When 500 mM KH_2PO_4, 10% MeOH was used, assays were carried out at flow rate of 0.8 mL/min

2.2.4. Method optimization

2.2.4.1. Optimization of Tyr standard preparation

0.4 mg/mL Tyr stock solutions were prepared using the following solvents:

(a) PBS pH 7.35;
(b) ultrapure water;
(c) acidified ultrapure water;
(d) mobile phase (0.5% CH_3COOH:MeOH:H_2O 15:15:70, v/v)

2.2.4.2. Optimization of the temperature

In order to find out the best operating temperature, a wide range of temperatures (15, 20, 35, 45, 55 and 65ºC) were assayed using two standard solutions (50000ng/mL and 25000 ng/mL) and two samples (serum).

2.2.5. Method validation

All the methods tested were validated according to ICH guidelines for validation of analytical procedures (ICH, 2005).

2.2.5.1. Specificity

The specificity of the methods was determined by comparing chromatograms obtained from 3-NT and Tyr spiked-samples.

2.2.5.2. Linearity

Six to eight standard solution concentrations comprising the range between 312.5 - 50000 ng/mL were assayed. Calibration curves were constructed by plotting average peak area versus concentrations. The linearity was evaluated using regression analysis.

2.2.5.3. Accuracy

Accuracy was determined by measuring recovery in 3-NT-spiked serum samples (3 different concentration levels: 25000, 1000, and 50 ng/mL) and in standard solutions (50000, 25000, 10000, 5000, 2500, 1250, 625, 312.5 ng/mL).

2.2.5.4. Precision

Precision was determined by means of the repeatability (intraday precision). The repeatability was evaluated by analysing standard solutions (8 concentration levels: 50000, 25000, 10000, 5000, 2500, 1250, 625, 312.5 ng/mL) and 3-NT-spiked serum samples (3 different concentration levels: 25000, 1000, 50 ng/mL).

2.2.5.5. LoD and LoQ

LoD and LoQ were determined using the following equations:

$$\boldsymbol{LoD = \frac{3.3\sigma}{S}}$$

$$\boldsymbol{LoQ = \frac{10\sigma}{S}}$$

Where δ is the standard deviation of the blank and S is the slope retrieved from the calibration curve data.

2.2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.02 (La Jolla, CA, USA). Calibration curves equations were obtained using linear regression analysis.

2.3. Results and discussion

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 most 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 to 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.

The development and validation of an HPLC-based method are, as for any other analytical method, an important requirement for quality assurance purposes. Thus, our HPLC assays were performed and the results interpreted according to ICH guidelines (ICH, 2005) and 96/23/EC directive (European Commission, 2002).

Firstly, the critical parameters (wavelength of detection, composition of mobile phase, optimum pH, temperature and concentrations of standard solutions) were studied in detail in order to develop an effective method for quantification of 3-NT. All chromatographic assays were performed using a C_{18} column, which is regarded as the most appropriate type for RP-HPLC analysis of peptides and proteins (Aguilar, 2004).

Concerning the wavelength of detection, DAD was used in order to obtain 3-NT and Tyr absorbance spectra. 3-NT and Tyr-containing solutions were run and detected within the ultraviolet range (190-400 nm). Maximum absorbance values were found at 215, 276 and 356 nm for 3-NT (Figure 6), and 196, 223 and 274 nm for Tyr (Figure 7). Accordingly, we defined 215, 276 and 356 nm as the optimal wavelengths of detection for our subsequent assays.

With regards to the second critical parameter, mobile phases were prepared with different methanol concentrations. The composition of the mobile phases to be assayed were selected based on the (i) information shown in Table VIII, and (ii) pH range (Ahuja & Dong, 2005; D. D. Joshi, 2012). In general, a pH range between 2-3 is a good starting point in a method development process. This pH range suppresses the ionization of most acidic compounds and the ionization of any silanol groups present on the column. On the other hand, basic compounds may be ionized under these conditions. Nevertheless, operating at high pH values to suppress such ionization process may be detrimental for most columns (Ahuja & Dong, 2005).

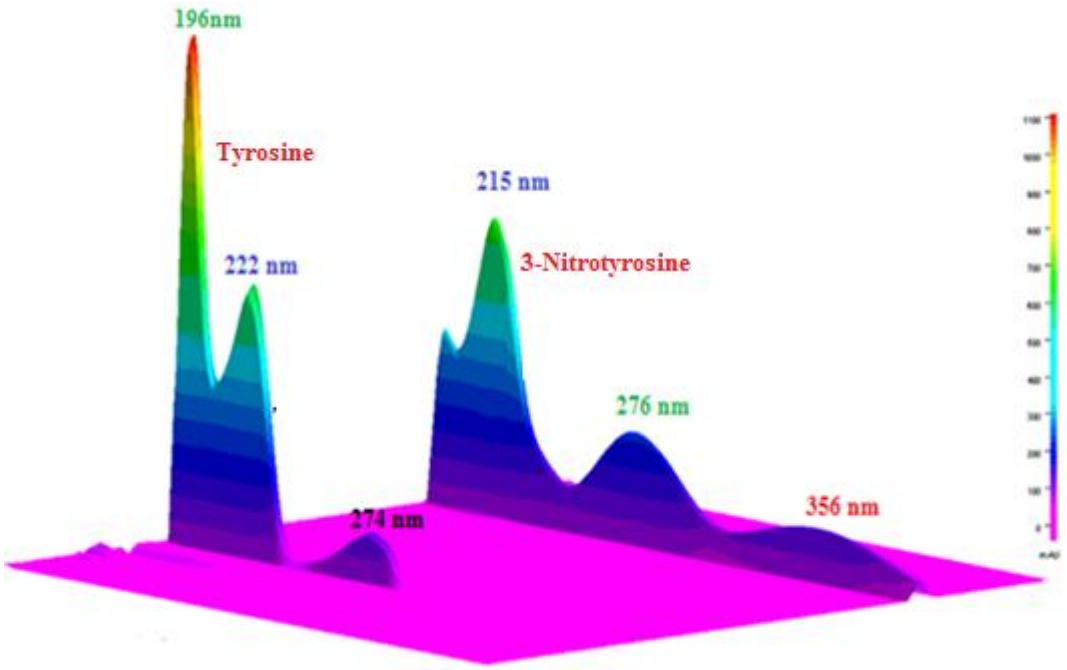


Figure 6. 3-NT and Tyr absorption spectra, obtained by DAD.

2.3.1. Mobile phase: KH_2PO_4

The first step in our method development process was the selection of the mobile phase to be used in our assays. F. Khan et al. (2006) used potassium phosphate buffer (500 mM, pH 3.5) with 10% MeOH (v/v), as mobile phase for detection and quantification of 3-NT, claiming good results. As a first approach, we also tested this mobile phase, which has revealed quite satisfactory results, well-defined peaks (Figure 7), short time of analysis, as well as LoD and LoQ on the order of tens of nanogram per mililiters (Table VIII). The LoD and LoQ were established by evaluating the minimum level at which the analyte could be readily detected and quantified accurately, respectively (Thakkar, Saravaia, Ambasana, Kaila, & Shah, 2011).

However, this mobile phase has also revealed a worrisome problem: the precipitation of potassium phosphate within the HPLC tubing system, leading to increased pressure and column clogging. Consequently, the results of the last samples were pretty difficult to analyse due to column obstruction.

Table VIII. Description of the assays performed, and respective retention time (mean ± standard deviation), LoD and LoQ values; n/a not applicable

Mobile phase	Composition	Assay
KH_2PO_4	500mM KH_2PO_4, 10% MeOH	A
	50mM KH_2PO_4	B
	25mM KH_2PO_4	C and D
0.5%CH_3COOH:MeOH:H_2O	29:1:70	E and F
	30:0:70	G and H
	15:15:70	I and J

(A)	Retention time (min)	LoD (ng/mL)	LoQ (ng/mL)	Flow (mL/min)	Column oven temperature (ºC)	Analysis time (min)
3-NT (220nm)	9.959 ± 0.820	24.22	73.40			
3-NT (276nm)	9.973 ± 0.818	40.38	122.37			
3-NT (356nm)	9.947 ± 0.806	87.33	264.63	0.800	35	15
Tyr (220nm)	3.741 ± 0.120	21.40	64.84			
Tyr (276nm)	3.593 ± 0.224	37.15	112.58			
(B)						
3-NT (220nm)	n/a	n/a	n/a			
3-NT (276nm)	n/a	n/a	n/a			
3-NT (356nm)	n/a	n/a	n/a	1.000	25	15
Tyr (220nm)	7.423 ± 0.058	0.06	0.17			
Tyr (276nm)	7.425 ± 0.055	0.33	1.01			
(C)						
3-NT (220nm)	n/a	n/a	n/a			
3-NT (276nm)	n/a	n/a	n/a			
3-NT (356nm)	n/a	n/a	n/a	1.000	25	25
Tyr (220nm)	7.247 ± 0.011	0.17	0.51			
Tyr (276nm)	7.244 ± 0.023	0.86	2.62			
(E)						
3-NT (220nm)	28.683 ± 0.128	6.91	20.93			
3-NT (276nm)	28.644 ± 0.110	1.76	5.32			
3-NT (356nm)	28.583 ± 0.220	71.32	216.11	1.000	25	40
Tyr (220nm)	8.170 ± 0.031	0.45	1.36			
Tyr (276nm)	8.153 ± 0.043	0.76	2.29			
(F)						
3-NT (220nm)	18.819 ± 0.053	0.26	0.80			
3-NT (276nm)	18.813 ± 0.055	0.44	1.33			
3-NT (356nm)	18.805 ± 0.082	44.05	133.48	1.000	40	30
Tyr (220nm)	5.870 ± 0.017	0.17	0.52			
Tyr (276nm)	5.874 ± 0.021	60.85	184.39			

Table VIII. (continued)

(G)	Retention time (min)	LoD (ng/mL)	LoQ (ng/mL)	Flow (ml/min)	Column oven temperature (ºC)	Analysis time (min)
3-NT (220nm)	34.178 ± 0.107	10.60	32.13			
3-NT (276nm)	34.198 ± 0.118	36.97	112.02			
3-NT (356nm)	34.184 ± 0.161	36.15	109.53	1.000	25	40
Tyr (220nm)	8.929 ± 0.049	0.72	2.18			
Tyr (276nm)	8.926 ± 0.064	66.82	202.50			
(H)						
3-NT (220nm)	21.801 ± 0.119	2.90	9.67			
3-NT (276nm)	22.885 ± 0.125	44.35	134.40			
3-NT (356nm)	22.884 ± 0.148	8.46	25.65	1.000	40	35
Tyr (220nm)	6.734 ± 0.051	0.61	1.84			
Tyr (276nm)	6.734 ± 0.050	1.37	4.15			
(I)						
3-NT (220nm)	7.133 ± 0.041	0.34	1.04			
3-NT (276nm)	7.133 ± 0.043	0.85	2.56			
3-NT (356nm)	7.133 ± 0.042	1.22	3.71	1.000	25	15
Tyr (220nm)	3.479 ± 0.019	0.10	0.29			
Tyr (276nm)	3.480 ± 0.019	0.26	0.78			
(J)						
3-NT(220nm)	9.878 ± 0.080	6.09	18.47			
3-NT(276nm)	9.878 ± 0.079	0.93	2.81			
3-NT(356nm)	9.878 ± 0.081	5.11	15.49	1.000	25	15
Tyr (220nm)	4.145 ± 0.023	1.81	5.49			
Tyr (276nm)	4.146 ± 0.022	0.53	1.60			

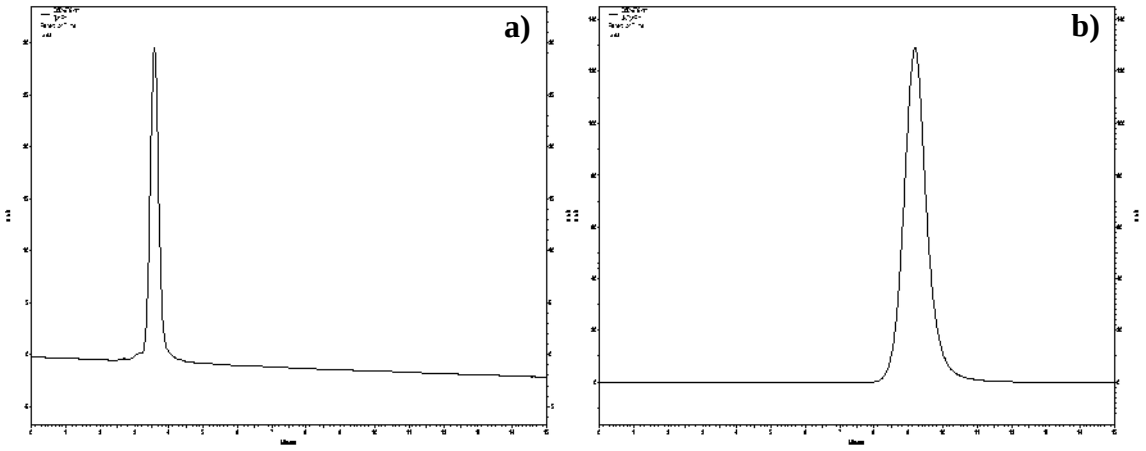


Figure 7. Representative chromatograms of (a) Tyr and (b) 3-NT analysis using mobile phase A (500mM KH_2PO_4, 10%MeOH).

In order to wash the HPLC system the following steps were carried out: (i) the column was removed and washed with ultrapure water between 40-50°C for about 30 min. (time required to remove phosphate precipitates); (ii) the column was put back and washed again with ultrapure water between 40-50°C during 10 min.; (iii) the steps (i) and (ii) were repeated until the pressure dropped down; (iv) once the pressure was stabilized, the column was washed with a mixture of MeOH:ultrapure water 50:50 for 10 minutes, and then washed with 100% MeOH. This last step was carried out after carefully ensuring that no precipitate remained in the system, otherwise the use of 100% MeOH would worsen the precipitate formation problem. Furthermore, caution was taken in order to minimize the contact time between the column and 100% ultrapure water.

Common factors favouring buffer precipitation are (i) high phosphate buffer concentrations, and (ii) reaction between phosphate and the organic solvent. According to the literature, phosphate buffer concentration should be about 10-50 mM for analysis of small molecules, since this concentration range provides the required buffering capacity (Ahuja & Dong, 2005; D. D. Joshi, 2012). In order to overcome the precipitation issue, we performed a 1:10 dilution of the mobile phase (50mM KH_2PO_4) and removed the organic solvent.

Reducing phosphate buffer concentration did not however solved the precipitation problem, thus a 25mM concentration was further tested. Chromatograms obtained with this mobile phase are shown in Figure 9. Unfortunately, and despite partially solving the precipitation problem, the resolution of the peaks was compromised (Figure 7 and Figure 9). Using this mobile phase, and testing standard solutions containing both compounds (assay D), a co-elution phenomenon between 3-NT and Tyr was observed, hindering the analysis of this assay.

The linearity of the methods using KH_2PO_4 as mobile phase was calculated through linear regression and correlation coefficient (Figure 8). The correlation coefficient is used to assess the strength of a linear relationship between pairs of variables (Mukaka, 2012). Overall, linear regression analysis of the calibration data showed good linear relationship, with correlation coefficients extremely close to 1.

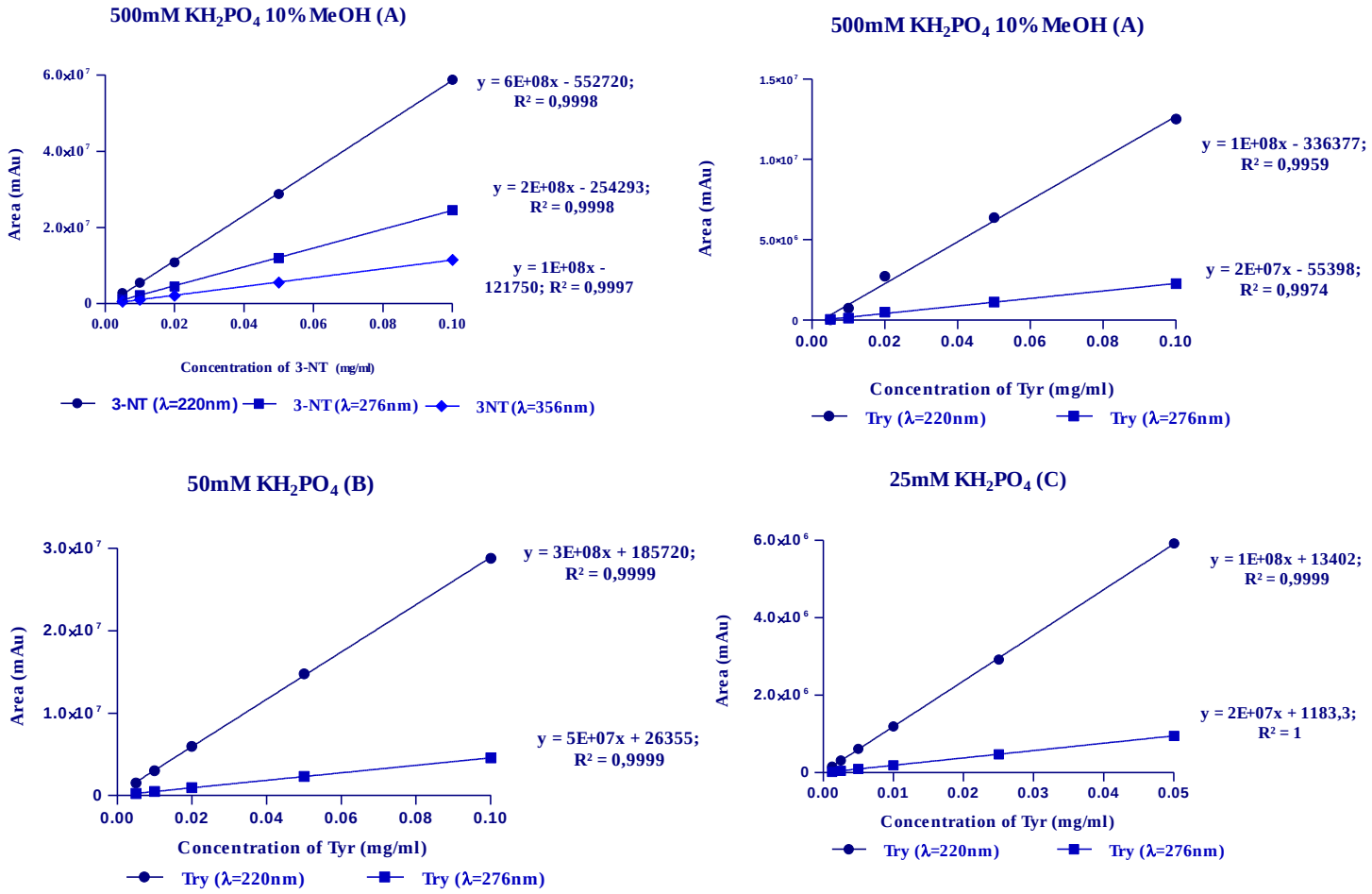


Figure 8. Calibration curves obtained using linear regression analysis for mobile phases composed by KH_2PO_4.

The correlation coefficient determined for Tyr analysis using 500mM KH_2PO_4, 10% MeOH (assay A) was slightly lower in comparison with assays B and C. This fact may be partially explained by the aforementioned buffer precipitation issue, rendering the results at the end of the run not reliable

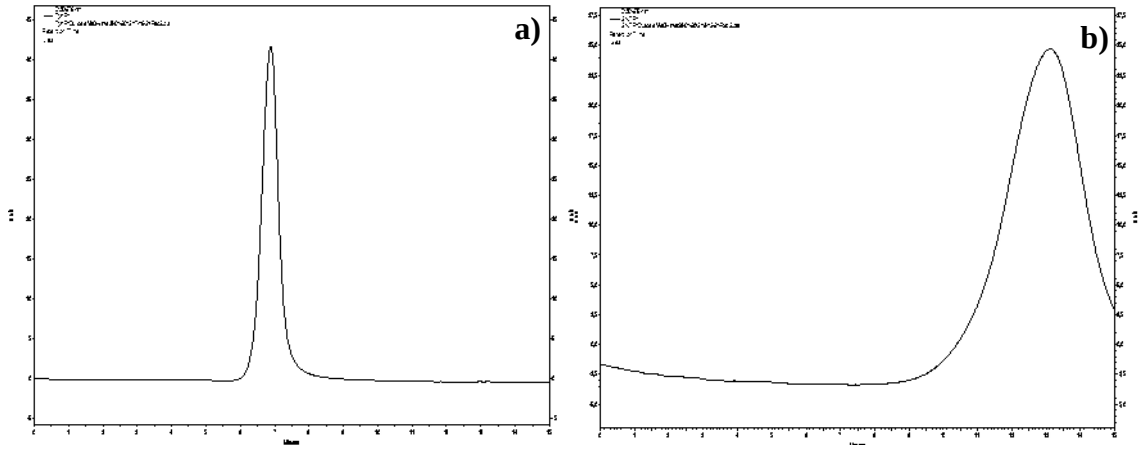


Figure 9. Representative chromatograms of (a) Tyr and (b) 3-NT using mobile phase C (25mM KH_2PO_4).

2.3.2. Mobile phase: 0.5% CH_3COOH:MeOH:H_2O

For mobile phases based on 0.5% CH_3COOH:MeOH:H_2O, three different proportions of these reagents were tested, as shown in Table VIII. The advantage of this mobile phase in relation to the previous one is the absence of phosphate buffer on its composition.

Firstly, we tested 0.5% CH_3COOH:MeOH:H_2O (29:1:70, v/v) (assay E), which has already been used before for 3-NT and Tyr determination by (Kikugawa et al., 2004), although using different experimental conditions. Since the analysis time for assay E was about 40 minutes, we then proceeded to increase the oven temperature. A temperature increase leads to an increase in solute solubility and diffusivity, as well as a decrease in the viscosity of the mobile phase. This significantly improves the partition process kinetics and, consequently, the peak shape and the column efficiency (Clark, 2004; Roses, Subirats, & Bosch, 2009; Zhu, Goodall, & Wren, 2005). Temperature has also a large effect on the thermodynamics of the retention process that can leads to a reduction in the running time and selectivity (Roses et al., 2009). Running assay F with a temperature of 40ºC allowed to reduce the analysis time from 40 to 30 min. Furthermore, it also allowed to obtain an increased efficiency, since peaks showed better resolution using these new conditions (Figure 10).

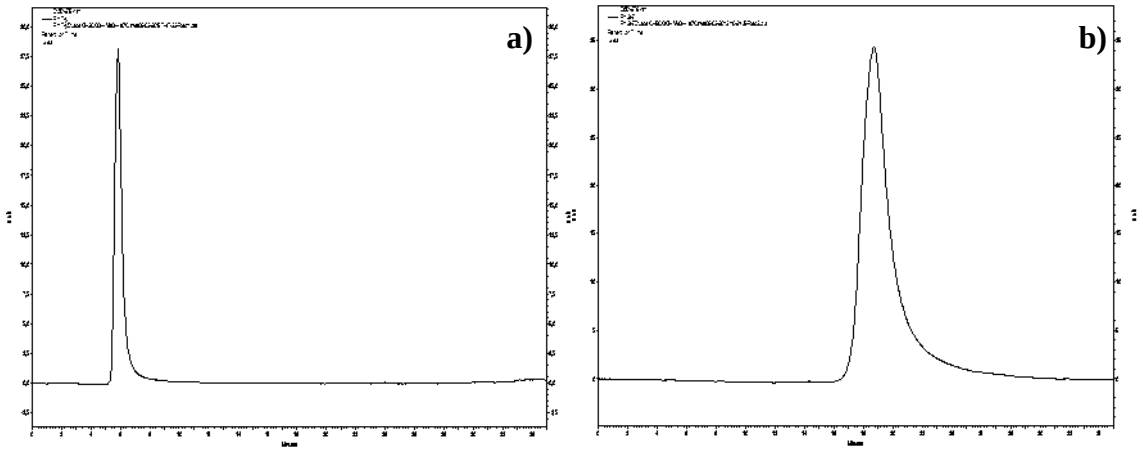


Figure 10. Representative chromatograms of (a) Tyr and (b) 3-NT using mobile phase F (0.5% CH_3COOH:MeOH:H_2O (29:1:70, v/v)).

Afterwards, we tested 0.5% CH_3COOH:MeOH:H_2O (30:0:70, v/v) (assays G and H). This mobile phase has the advantage of not making use of an organic solvent, rendering it an example of "green" liquid chromatographic analysis. Other researchers have already chosen similar strategies, namely mobile phases containing the nonionic surfactant Brij-35 instead of using an organic solvent (Fernandez-Navarro, Ruiz-Angel, & Garcia-Alvarez-Coque, 2012)**.** Since the analysis time of assay G was 40 minutes, we decided to follow the previous approach and proceed to increase the oven temperature to 40ºC (assay H). The analysis time was reduced to 35 minutes, although the resolution of the 3-NT peaks was the lowest among all tested proportions (Figure 11).

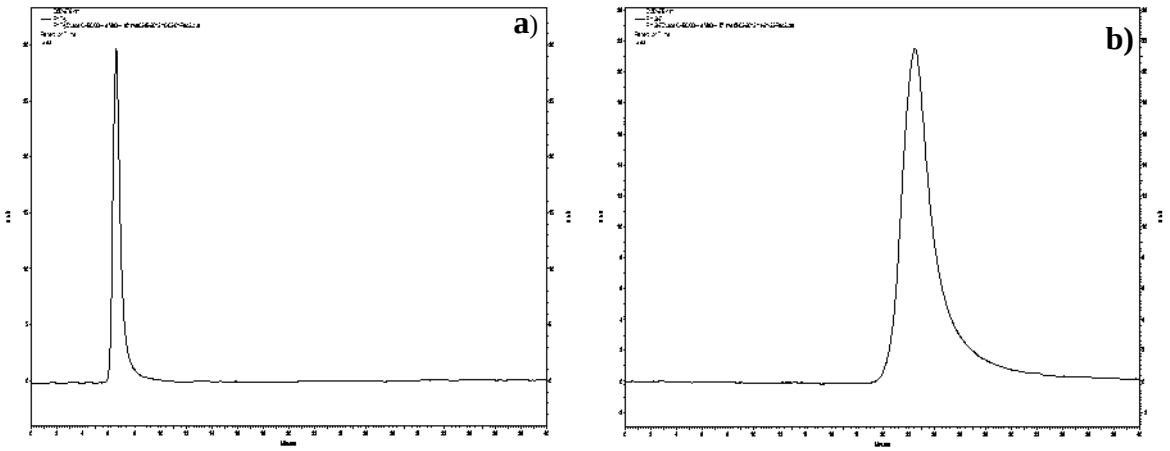


Figure 11. Representative chromatogram of (a) Tyr and (b) 3-NT analysis using mobile phase H (0.5% CH_3COOH:MeOH:H_2O (30:0:70,v/v)).

The other proportion tested was 0.5% CH_3COOH:MeOH:H_2O (15:15:70, v/v) (assays I and J) which analysis time was 15 minutes. Thus reduced analysis time was obtained owing to an increase in methanol concentration, which is known to decrease the retention time of solutes (Q. Zhang, Feng, Yan, & Da, 1999)**.** Therefore, this reduced analysis time was a significant advantage of this mobile phase composition over all the others. On the other hand, assay J was performed in a new column, which might have accounted for the increased retention time of both 3-NT and Tyr (Table VIII) comparatively with assay I. The increased retention times obtained with the new column was likely the result of an unstable column packing. Regarding the LoD and LoQ, this proportion exhibited great results, in the order of units of nanogram per mililiters, and the lowest time analysis as well (Table VIII). In Figure 12 is shown a representative chromatogram obtained with assay J, where is possible to observe that the peaks shape is narrower in comparison with the other proportions tested.

According to 96/23/EC directive, retention time in liquid chromatography should not vary 0.10 minutes within technical replicates (Bartesaghi et al., 2007; European Commission, 2002), which was the case when these proportions were tested (Table VIII)

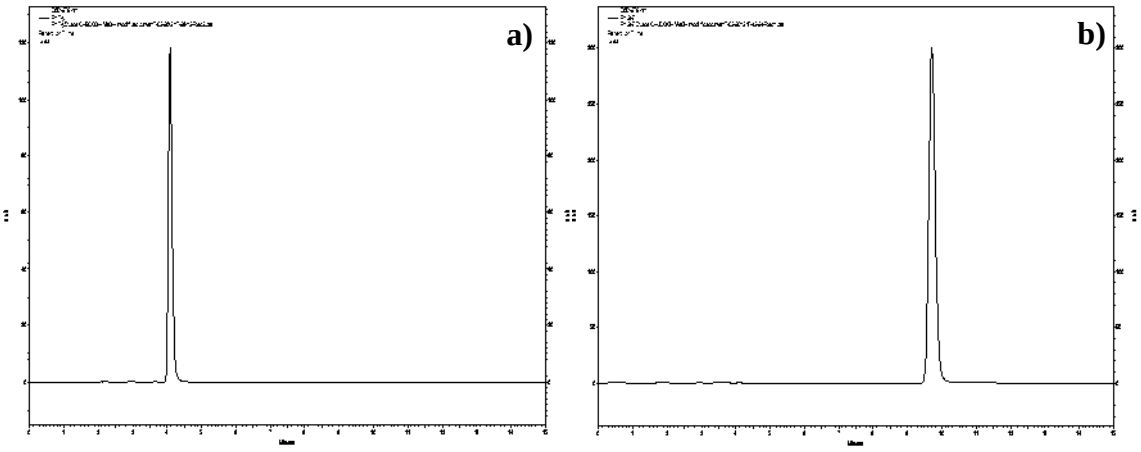


Figure 12. Representative chromatograms of (a) Tyr and (b) 3-NT analysis obtained in assay J (0.5% CH_3COOH:MeOH:H_2O (15:15:70,v/v).

Linear regression analysis of the methods using 0.5% CH_3COOH:MeOH:H_2O as mobile phase also showed good linear relationship, with correlation coefficients very close to 1 (Figure 13). Nevertheless, the proportion 30:0:70 (v/v) was the one which exhibited the lowest correlation coefficient for 3-NT.

2.3.3. Mobile phases comparison and selection

All mobile phases tested for 3-NT detection and quantification exhibited pros and cons.

Briefly, and as previously mentioned, KH_2PO_4-based mobile phases presented as major disadvantage the precipitation of potassium phosphate within the HPLC tubing system. For this reason, we considered this mobile phase to be not suitable for our purpose. Nevertheless, this kind of mobile phases had revealed satisfactory results regarding other parameters (Table VIII).

Conversely, 0.5% CH_3COOH:MeOH:H_2O mobile phase was not affected by any kind of precipitation issue. Regarding the different proportions and oven temperatures tested, the proportions 29:1:70 (v/v) and 30:0:70 (v/v) at 25ºC exhibited a long analysis time, as well as 3-NT broad peaks. On the other hand, the proportion 15:15:70 showed the best results with regards to the different parameters evaluated, as can be seen from Table IX to Table XIII. Besides, it also exhibited a good resolution, with narrow peaks for both Tyr and 3-NT. However, the larger concentration of organic solvent used in this proportion could be regarded as a disadvantage from an environmental point of view. As a result, and taking into account all the pros and cons, the mobile phase 0.5% CH_3COOH:MeOH:H_2O (15:15:70 (v/v)) was regarded as the most suitable for our purposes.

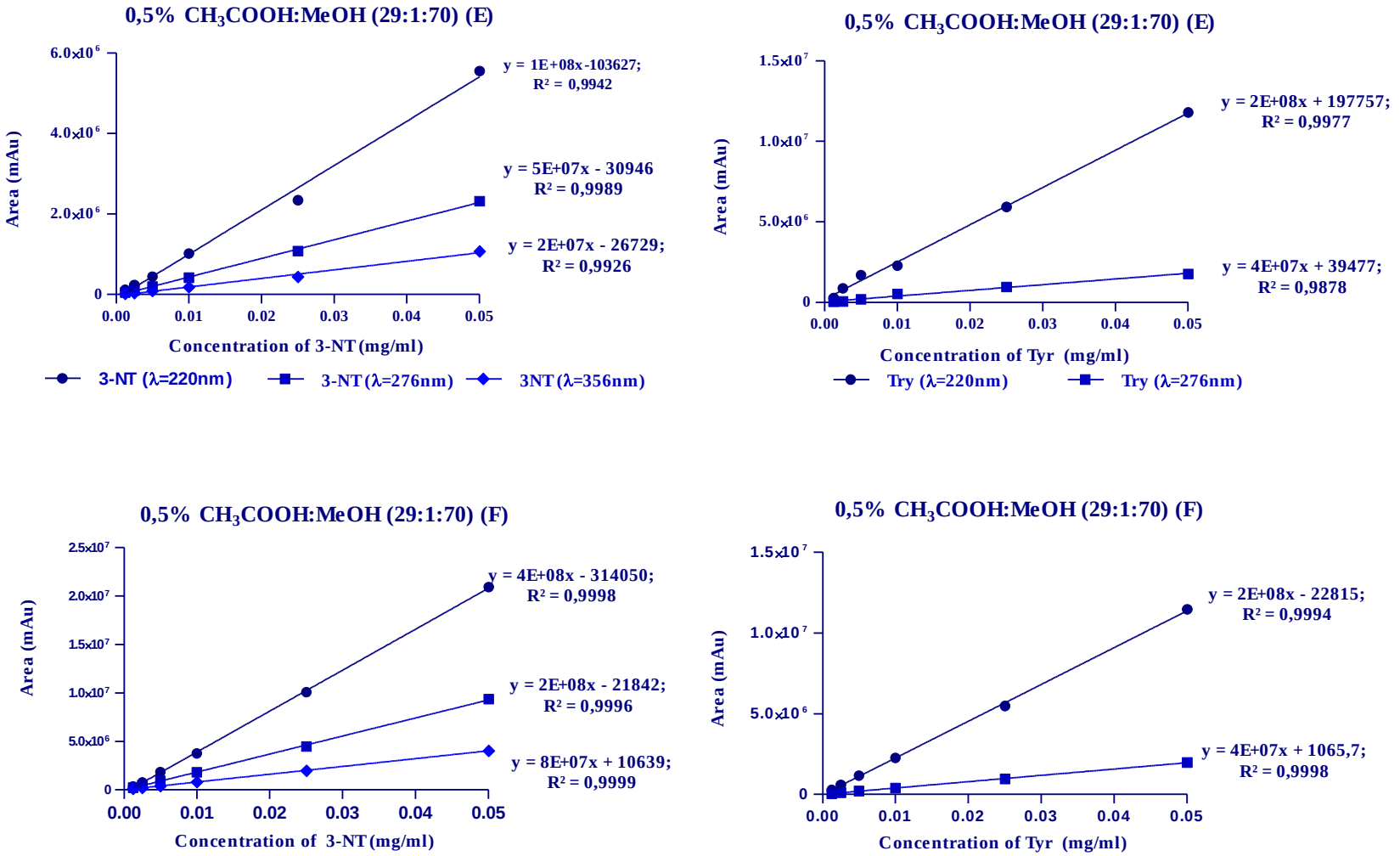
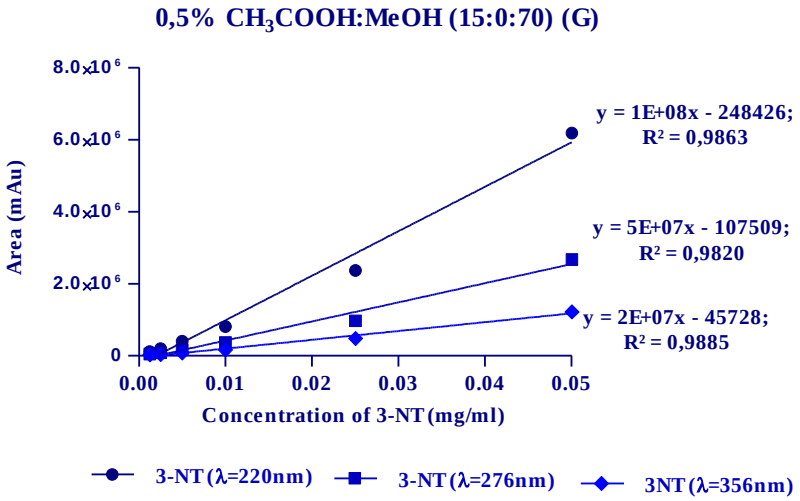
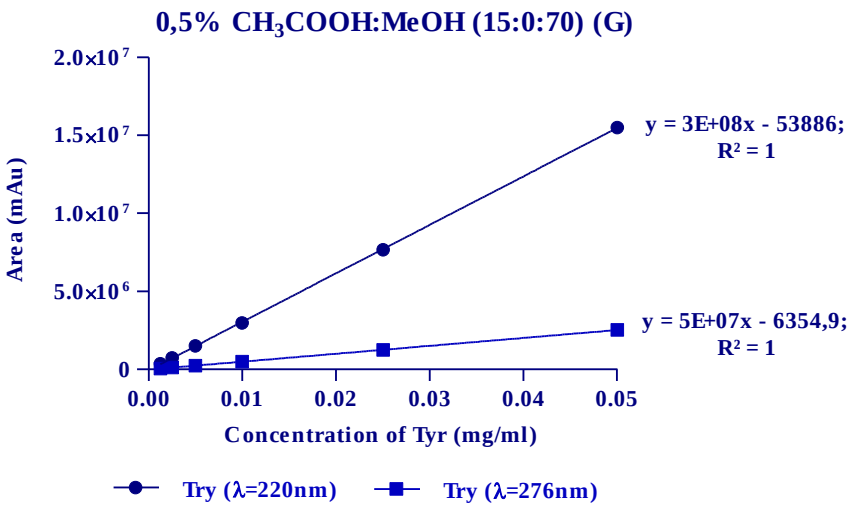
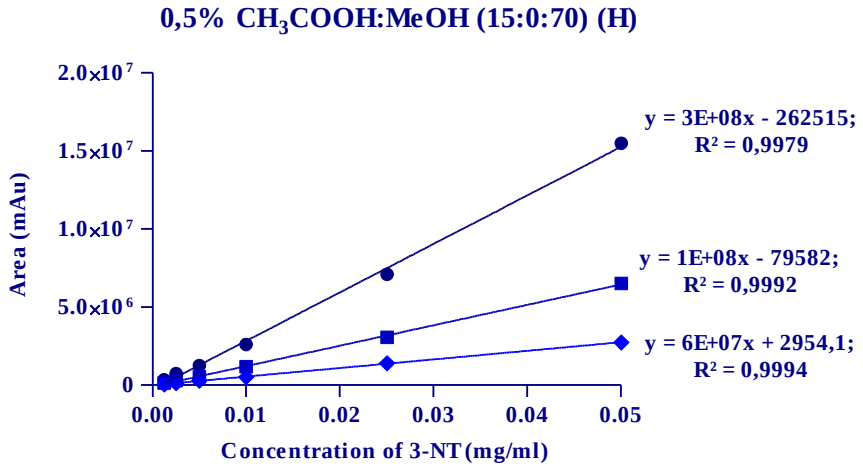


Figure 13. Calibration curves obtained using linear regression analysis for mobile phases composed by 0.5% CH_3COOH:MeOH:H_2O.







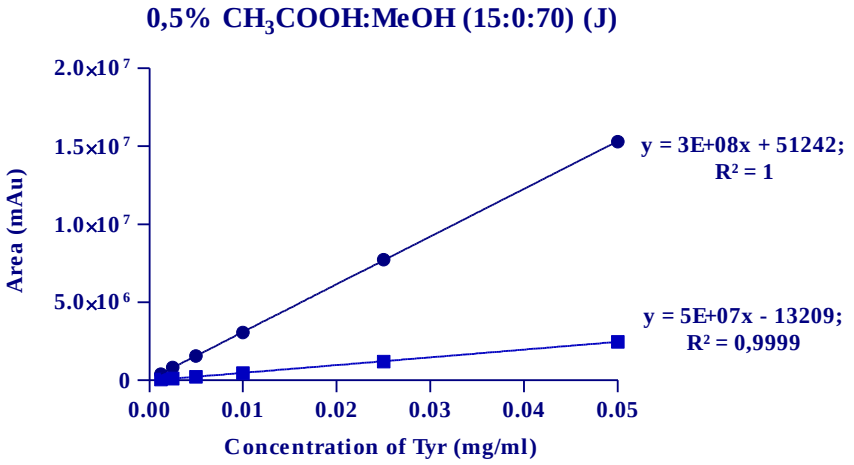


Figure 13. (continued).

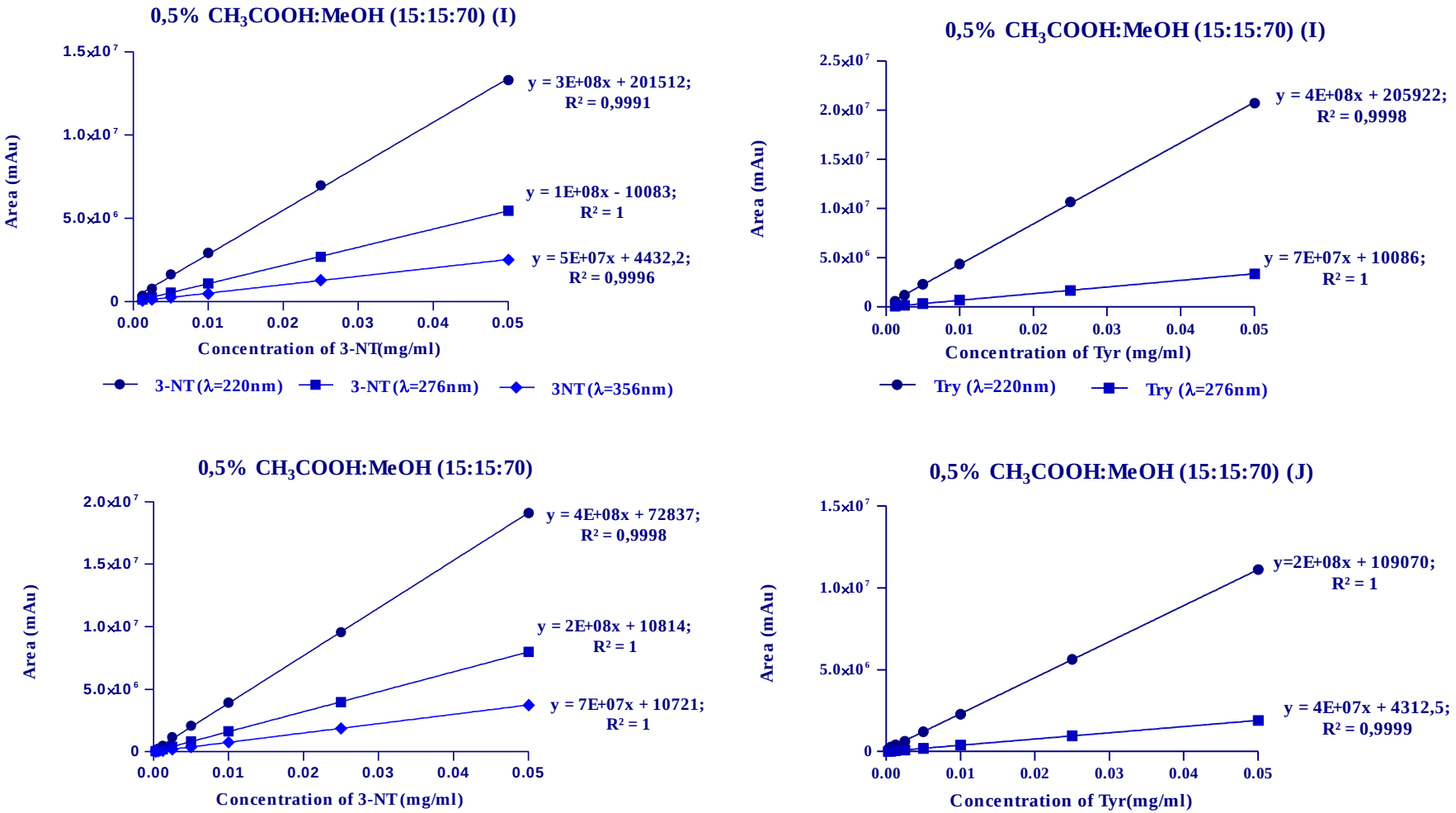


Figure 13. (continued).

Table IX. Validation results for 3-NT quantification at 276 nm

Validation experiment	**(A)**	**(E)**	**(F)**	**(G)**	**(H)**	**(I)**	**(J)**
Specificity	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak
LoD (ng/mL)	40.38	1.76	0.44	36.97	44.35	0.85	0.93
LoQ (ng/mL)	122.37	5.32	1.33	112.02	134.40	2.56	2.81
Linearity							
Co-relation coefficient	0.9998	0.9989	0.9996	0.9820	0.9992	1.000	1.000
Regression equation	**$y=2.470x10^8X-254293$**	**$y= 4.638x10^8X-30946$**	**$y=1.8635x10^8X-21842$**	**$y=1.8635x10^8X-21842$**	**$y=1.30677x10^8X-79582$**	**$y=1.307x10^8X-1008$**	**$Y=1.595x10^8X+10814$**
Analysis time	15	40	30	40	35	15	15

Table X. Validation results for 3-NT quantification at 356 nm

Validation experiment	**(A)**	**(E)**	**(F)**	**(G)**	**(H)**	**(I)**	**(J)**
Specificity	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak
LoD(ng/mL)	87.33	71.32	44.05	36.15	8.46	1.22	5.11
LoQ(ng/mL)	264.63	216.11	133.48	109.53	25.65	3.71	15.49
Linearity							
Co-relation coefficient	0.9997	0.9999	0.9999	0.9885	0.9994	0.9996	1.000
Regression equation	**$y=1.155x10^{8}X-121751$**	**$y=2.132x10^{7}X-26729$**	**$y=8.059x10^{7}X+10638$**	**$y=2.459x10^{7}X-45728$**	**$y=5.508x10^{7}X-2954,1$**	**$y=5.056x10^{7}X+4432$**	**$y=7.463\ x10^{7}X+10721$**
Analysis time	15	40	30	40	35	15	15

Table XI. Validation results for 3-NT quantification at 215nm

Validation experiment	**(A)**	**(E)**	**(F)**	**(G)**	**(H)**	**(I)**	**(J)**
Specificity	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak
LoD(ng/mL)	24.22	6.91	0.26	10.60	2.90	0.34	6.09
LoQ(ng/mL)	73.40	20.93	0.80	32.13	9.67	1.04	18.47
Linearity							
Co-relation coefficient	0.9998	0.9942	0.9998	0.9863	0.9979	0.9991	0.9998
Regression equation	**$y = 5.9142x10^8X - 552720$**	**$y = 1.1026x10^8X - 103627$**	**$y = 4.2281x10^8X - 314\ 050$**	**$y = 1.2349\ x10^8X - 248426$**	**$y = 3.1054x10^8X - 262515$**	**$y = 2.6398x10^8X + 201512$**	**$y = 3.8061x10^8X + 72837$**
Analysis time	15	40	30	40	35	**15**	**15**

Table XII. Validation results for Tyr quantification at 215 nm

Validation experiment	(A)	(E)	(F)	(G)	(H)	(I)	(J)
Specificity	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak
LoD (ng/mL)	21.40	0.45	0.17	0.72	0.61	0.10	1.81
LoQ (ng/mL)	64.84	1.36	0.52	2.18	1.84	0.29	5.49
Linearity							
Co-relation coefficient	0.99594	0.9977	0.9994	1.000	1.000	0.9998	1.000
Regression equation	**$y=1.3020x10^8X-336377$**	**$y = 2.3130x10^8X+197757$**	**$y =2.2779x10^8X+22815$**	**$y = 3.1070x10^8X - 53886$**	**$y = 3.0565x10^8X+51242$**	**$y = 4.1258x10^8X+205922$**	**$y = 2.1995x10^8+109\ 070$**
Total analysis time	15	40	30	40	35	15	15

Table XIII. Validation results for Tyr quantification at 276 nm

Validation experiment	**(A)**	**(E)**	**(F)**	**(G)**	**(H)**	**(I)**	**(J)**
Specificity	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak	No interference with analyte peak
LoD (ng/mL)	37.15	0.76	60.85	66.82	1.37	0.26	0.53
LoQ (ng/mL)	112.58	2.29	184.39	202.50	4.15	0.78	1.60
Linearity							
Co-relation coefficient	0.9974	0.9878	0.9998	1.000	0.9999	1.000	0.9999
Regression equation	**$y = 2.3391x10^7X - 55398$**	**$y = 3.5431x10^7X + 39477$**	**$y = 3.9245x10^7X + 1065.7$**	**$y = 5.0731x10^7X - 6355$**	**$y= 4.9759x10^7X - 13209$**	**$y = 6.7530x10^7X + 10086$**	**$y = 3.7935x10^7X +4312,5$**
Total analysis time	15	40	30	40	35	15	15

2.3.4. Optimization method

2.3.4.1. Optimization of Tyr standard preparation

One trouble that was present throughout the study was the dissolution of Tyr. For that purpose, we tested four different solvents, as shown in Figure 14 . Dissolving Tyr in the selected mobile phase (0.5% CH_3COOH:MeOH:H_2O 15:15:70, v/v) using a water bath between 40-50ºC for 15 min ended up being the most effective way. Conversely, and regarding the acidified water, some chromatograms obtained showed nonspecific peaks. Moreover, the use of ultrapure water was not efficient since insoluble crystalline particles were observed after dissolution. Lastly, the main drawback of using PBS is that its pH (7.35) is different from the pH of the selected mobile phase (3.10).

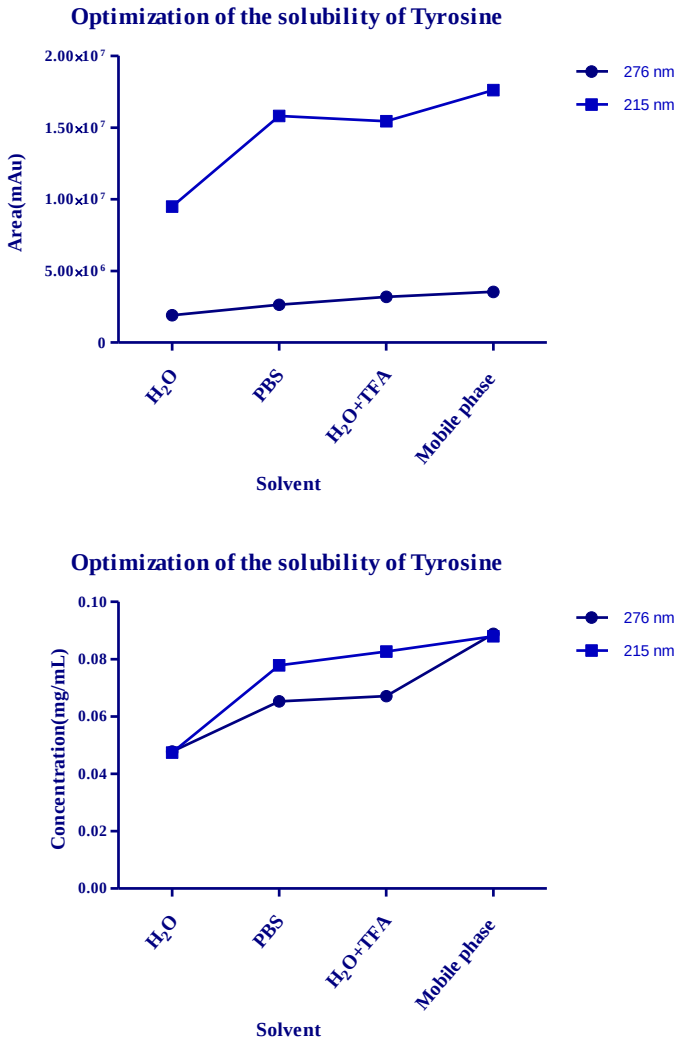


Figure 14. Comparison of the solvents using for Tyr dissolved.

2.3.4.2. Optimization of the temperature

Regarding the temperature of the method, a wide range (15, 20, 25, 35, 45, 55 and 65ºC) was assayed while maintaining the conditions of other parameters. When temperatures from 35 to 65ºC, with detection at 215 and 276 nm, were tested, a 3-NT non-specific peak was obtained. A similar phenomenon was observed when testing at 15ºC. Temperatures between 20 and 25ºC showed to be specific for 3-NT, with detection at 276 nm and 356 nm. Furthermore, and in order to obtain a higher peak resolution, running the assays at 20ºC with a flow rate of 1.2 mL/min is advisable.

2.3.5. Method Validation

2.3.5.1. Specificity

A specific 3-NT quantification method should have the ability to detect 3-NT unequivocally without any interference from tyrosine or other close structural relatives (Tsikas & Duncan, 2014). In order to determine the specificity of the developed method in all assays, 3-NT and Tyr were spiked into different biological matrices (more details can be found in Chapter III). The method was specific for 3-NT with detection at 356 nm for all biological matrices. However, when quantification was carried out at 215 or 276 nm in whole blood, other unknown molecules were also present in the 3-NT peak. Consequently, these wavelengths are not recommended for 3-NT quantification in whole blood using this method.

2.3.5.2. Linearity, LoD and LoQ

Table XIV lists the linearity parameters of the calibration curves for 3-NT (and Tyr). The LoD and LoQ are also given in this table.

Table XIV. Linearity, retention time, LoD and LoQ

	3-NT (ʎ=215nm)	3-NT (ʎ=276nm)	3-NT (ʎ=356nm)	Tyr (ʎ=215nm)	Tyr (ʎ=276nm)
LoD (ng/mL)	**0.655**	**0.367**	**1.862**	**0.213**	**1.380**
LoQ (ng/mL)	**2.184**	**1.113**	**5.642**	**0.645**	**4.182**
Retention Time (min ± sd)	**10.164 ± 0.054**	**10.164 ± 0.053**	**10.165 ± 0.053**	**4.278 ± 0.020**	**4.278 ± 0.020**
Linearity					
Co-relation coefficient	**1.000**	**1.000**	**0.999**	**1.000**	**0.999**
Regression equation	**$y = 7.0805x10^8x - 7475.6$**	**$y = 2.5134x10^8x + 25707$**	**$y = 1.1970x10^8x + 10814$**	**$y = 1x10^8x + 7175.6$**	**$y = 3x10^7x + 586.64$**

2.3.5.3. Accuracy - recovery rate

The accuracy of an analytical method is defined as the proximity of the mean concentration determined by the analytical method to the true concentration of the analyte (Hui et al., 2012). Table XV presents the results of accuracy determined from standard solutions. The inaccuracy varied from 0.007 to -6.26%. Samples with the highest or lowest 3-NT concentrations (close to LoQ) were those which exhibited a higher relative error (RE). According to the literature, the RE mean value should be within 15% of the nominal value (Sonawane et al., 2014), which was the case of this method.

Table XV. Analytical results of accuracy test by standard solutions

Wavelength (nm)	**Concentration** (ng/mL)	**RE**[a] (%)	**No. of samples**
356	50000	0.007	6
	25000	-3.49	6
	10000	0.51	6
	5000	-0.52	6
	2500	0.72	6
	1250	0.16	6
	625	-2.32	6
	312.5	-0.83	6
276	50000	-0.08	6
	25000	-3.05	6
	10000	0.24	6
	5000	-0.82	6
	2500	0.16	6
	1250	0.30	6
	625	-2.79	6
	312.5	-3.82	6
215	50000	0.12	6
	25000	-3.33	6
	10000	0.44	6
	5000	0.28	6
	2500	-0.03	6
	1250	1.88	6
	625	-3.31	6
	312.5	-6.26	6

a)Relative error (RE) is derived by using the following equation: Relative error % = ((Mean cal. conc. - True conc.)/True conc.) × 100%.

Table XVI shows the results expressed as percent recoveries of 3-NT in serum samples. Regarding detection at 356 nm and 276 nm, the recovery value ranged from 90.92 to 113.12 %, and RE values varied between 0.99 to 13.12%. The concentrations obtained were relatively close to the known concentrations, with RE or inaccuracy not greater than 13.12% in all cases, which is in accordance with the previous mentioned RE limit (<15%) (Sonawane

et al., 2014). Concerning detection at 215 nm, inaccuracy was greater than 15% (79.62%), thereby not validated for this parameter.

Table XVI. Analytical results for accuracy test from serum sample

Wavelength (nm)	**Spiked Concentration** (ng/mL)	**Measured Concentration** (ng/mL)	**RE**[a] (%)	**Recovery** (%)	**No. of samples***
356	25000	23067,3	-7,73	92,27	6
	1000	910,7	-8,93	91,07	6
	50	50,5	0,99	100,99	6
276	25000	24519,8	-1,92	98,08	6
	1000	909,2	-9,08	90,92	6
	50	56,6	13,12	113,12	6
215	25000	21360	-14,56	85,44	6
	1000	848	-15,20	84,80	6
	50	90	79,62	179,62	6

a)Relative error (RE) is derived by using equation: Relative error % =([Mean cal. conc.] – [True conc.])/[True conc.] × 100%.

*Each sample performed in triplicated.

2.3.5.4. Precision

Intra-day precision was assessed by standard solutions (50000, 25000, 10000, 5000, 2500, 12500, 625, 312.5 ng/mL) as well as by spiking different commercial human serum samples with three different 3-NT concentrations (25000, 1000, 50 ng/mL). The precision of a bioanalytical method for each concentration level should be < 15% of relative standard deviation (RSD) or coefficient of variation (Sonawane et al., 2014). The precision of our method, which was determined using FDA guidelines for bioanalytical method validation (FDA, 2013), was 2.23% and 6.75% below the RSD for all standard solutions and serum samples, respectively (Table XVII and Table XVIII).

Table XVII. Analytical results for repeatability (intraday test) from standard solutions

Wavelength (nm)	**Concentration** (ng/mL)	**RSD** (%)	**No. of samples**
356	50000	0.7	6
	25000	0.23	6
	10000	0.76	6
	5000	0.97	6
	2500	1.87	6
	1250	0.72	6
	625	2.23	6
	312.5	1.37	6

Table XVII. (continued)

Wavelength (nm)	**Concentration** (ng/mL)	**RSD** (%)	**No. of samples**
276	50000	1.26	6
	25000	0.16	6
	10000	0.56	6
	5000	0.54	6
	2500	0.62	6
	1250	0.88	6
	625	1.14	6
	312.5	2.03	6
215	50000	0.87	6
	25000	0.76	6
	10000	0.58	6
	5000	1.08	6
	2500	0.54	6
	1250	1.38	6
	625	1.36	6
	312.5	0.72	6

Table XVIII. Analytical results for repeatability (intraday test) from serum samples

Wavelength (nm)	**Concentration** (ng/mL)	**RSD (%)**[a]	**No. of samples***
356	25000	0.40	6
	1000	2.15	6
	50	2.93	6
276	25000	0.97	6
	1000	1.15	6
	50	6.75	6
215	25000	1,22	6
	1000	3,60	6
	50	4,75	6

a Relative standard deviation is derived by using equation: RDS % = (standard deviation)/(mean peak area) × 100%.
*Each sample performed in triplicated.

2.4. Conclusions

After a deep analysis of the results obtained with all the mobile phases tested, the best results were obtained using 0.5% CH_3COOH:MeOH (15:15:70 (v/v)), at 25ºC, flow rate of 1 mL/min, and with detection at wavelengths 215, 276 and 356 nm. By using this protocol, it was possible to obtain a linear calibration curve (correlation coefficient = 1), L_OD/ L_OQ in

the order of units ng/ml, and a reduced analysis time *per* sample (15 minutes). Moreover, and most importantly, the developed method exhibited a good specificity, with no interference observed with 3-NT structural relatives, namely Tyr. Lastly, the method revealed good precision and accuracy.

Chapter III

Applicability of the developed method for 3-nitrotyrosine quantification

3.1. Introduction

Most of 3-NT quantification methods developed throughout the last years have been applied in several biological matrices, such as biological tissues and fluids (plasma, serum, urine, cerebrospinal fluid, synovial fluid, etc.) (Radabaugh et al., 2008; Ryberg & Caidahl, 2007).

The human plasma and serum are of special interest since they are widely used among clinical and biological studies (Yu et al., 2011). Furthermore, serum has been reported has showing higher sensitivity in biomarker detection, which is likely explained by the globally higher metabolite concentrations in serum than in plasma (Yu et al., 2011).

On the other hand, urine has also been tested in biomarker research. Although it offers the possibility of a non-invasive measurement, these biological matrices are often referred to as giving rise to unreliable results (Ryberg & Caidahl, 2007). Regarding this, Misko et al. (2013) measured 3-NT levels in different biological matrices (urine, plasma and synovial fluid), demonstrating that urine exhibited different results in relation to the others samples tested.

3-NT is ubiquitous in a wide range of biological samples, although each type of biological sample has different basal concentrations (Tsikas & Duncan, 2014). Depending on the biological sample, and according to the literature, the normal concentrations found in healthy individuals may vary. Table XIX shows the 3-NT concentration range usually found in both healthy and pathological states, as determined by different methods and biological samples.

3.1.1. Aim

Chapter III briefly describes the various biological samples that can be used for 3-NT quantification. The aim was to evaluate the performance of an HPLC-based protocol for 3-NT quantification (described in Chapter II) in a wide range of biological matrices, namely (i) serum, (ii) urine, (iii) whole blood, (iv) B16 F-10 melanoma cell line, (v) growth medium conditioned with the same cell line, (vi) gram-negative (*Escherichia coli*) and (vii) gram positive (*Staphylococcus aureus*) bacterial and (viii) yeast (*Saccharomyces cerevisiae*) suspensions.

Table XIX. 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. (2003)	12 healthy non-smoking volunteers (43±9)	Plasma	GC/NCI-MS/MS	0.74 ± 0.31 nM*
Tsikas et al. (2003)	10 healthy volunteers (51±10)	Plasma	GC tandem MS	1.149 ± 0.73 nM*
	6 healthy volunteers (25±3)	Plasma	GC tandem MS	2.677 ± 1.540nM*
	10 healthy volunteers (51±10)	Plasma	GC-MS	4.46 ± 4.49 nM*
	6 healthy volunteers (25±3)	Plasma	GC-MS	5.447 ± 2.783nM*
W.-Z. Zhang et al. (2007)	20 healthy non-smoking volunteers (41.2)	Plasma	LC-MS/MS	4.54 ± 2.75 nM*
	18 healthy smokers (42.0)	Plasma	LC-MS/MS	17.42 ± 11.6 nM*
Radabaugh et al. (2008)	40 healthy volunteers	Plasma	LC–MS/MS	224–962 pg/mL (0.99-4.2 nM)[#]
Misko et al. (2013)	143 healthy volunteers	Plasma	Immunoaffinity two-dimensional LC-MS/MS	536.4 pg/mL (2.49nM)[#]
	174 osteoarthritis patients	Plasma	Immunoaffinity two-dimensional LC-MS/MS	704.1 pg/mL (3.11nM) [#]
Pourfarzam et al. (2013)	50 patients with no history of cardiac diseases (58.9 ± 10.3)	Plasma	HPLC-fluorescence detector 470 nm	4.4 ± 1.8 nM*
	50 stable CAD patients (61.2 ± 11.23)	Plasma	HPLC-fluorescence detector 470 nm	12.8 ± 3.9 nM*
	50 unstable CAD patients (59.9 ± 10.45)	Plasma	HPLC-fluorescence detector 470 nm	14.8 ± 4.8 nM*
Sun et al. (2007)	70 healthy Chinese volunteers	Plasma	Sandwich ELISA	7.9±7 nmol/L[#]

Table XIX. (continued)

Reference	Sample (mean age ± years)	Biological sample	Method	Concentration 3-NT
Radabaugh et al. (2008)	131 healthy volunteers	Serum	LC–MS/MS	179–1540 pg/mL (0.79 - 6.8 nM)[#]
F. Khan et al. (2006)	25 non-smoking healthy female volunteers (age 24–50)	Serum	Sandwich ELISA	1.1 ± 0.81 µM[#]
	24 systemic lupus erythematosus patients (age 18–50)	Serum	Sandwich ELISA	96.52 ± 21.12 µM[#]
Tsikas et al. (2005)	10 healthy volunteers (36.5 ± 7.2)	Urine	GC–tandem MS	8.4 ± 10.4 nM
Radabaugh et al. (2008)	8 healthy volunteers	Urine	LC–MS/MS	63.5–751 pg/mL (0.28-3.32nM)[#]
Ryberg et al. (2004)	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*
	17 Alzheimer's disease patients	Cerebrospinal fluid	GC/NCI-MS/MS	0.44 ± 0.031 nM*
	14 amyotrophic lateral sclerosis patients	Cerebrospinal fluid	GC/NCI-MS/MS	0.38± 0.034 nM*
Radabaugh et al. (2008)	35 healthy volunteers	Cerebrospinal fluid	LC–MS/MS	335–5730 pg/mL (1.48-25.33nM)[#]
Seven, Aslan, Incir, and Altintas (2013)	15 volunteers with normal pressure hydrocephalia	Cerebrospinal fluid	ELISA based on the sandwich	573.54 ± 142.86 nM
Seven et al. (2013)	20 relapsing remitting multiple sclerosis patients before methyl prednisolone therapy	Cerebrospinal fluid	ELISA based on the sandwich	927.89 ± 244.84 nM

Table XIX. (continued)

Reference	**Sample** (mean age ± years)	**Biological sample**	**Method**	**Concentration 3-NT**
Lärstad et al. (2005)	10 healthy subjects (46 ± 2.6)	Exhaled breath condensate	GC/NICI/ tandem MS	31 pM*
	8 asthma patients (49 ± 5.1)	Exhaled breath condensate	GC/NICI/ tandem MS	31pM*
Radabaugh et al. (2008)	40 healthy volunteers	Synovial fluid	LC–MS/MS	54.4–822 pg/mL (0.24-3.63nM)[#]
Ueshima et al. (2007)	5 chronic obstructive pulmonary disease patients (71.6 ± 2.4)	Sputum	HPLC-ECD	0.55 ± 0.15 pmol/mL[#]
	5 chronic obstructive pulmonary disease patients (71.6 ± 2.4)	Saliva	HPLC-ECD	0.02 ± 0.01 pmol/mL[#]

* Free 3-NT; [#] Total 3-NT

3.2. Material and Methods

3.2.1. Instrument and software

As described in 2.2.1.

3.2.2. Reagents and consumables

As described in 2.2.2.

3.2.3. Analytical procedure

3.2.3.1. Mobile phase

0.5% CH_3COOH:MeOH:H_2O solutions were prepared according to the proportion 15:15:70 and filtered through a 0.45 µm membrane.

3.2.3.2. Calibration standards

0.5 mg/mL 3-NT and Tyr stock solutions were made using the mobile phase previously mentioned. All stock solutions were filtered through a filter membrane device. The first assays were performed using separate 3-NT and Tyr standard solutions in the following concentrations (50000; 25000; 10000; 5000; 2500; 1250; 625 and 312,5 ng/mL). Standard solutions were prepared by diluting the respective stock solution into the mobile phase. When required, warm was used in order to better dissolve the reagents. These standard solutions were used for calibration purposes.

3.2.3.3. Sample preparation: serum, urine, and whole blood

The biological samples tested in this chapter are described in Table XX. All samples were initially submitted to a previously published protocol in order to precipitate proteins (Peixoto, 2012). Briefly, 1mL of 15% TFA (freshly prepared) was added to 1mL of each sample, and then vortexed for 20 seconds. Afterwards, samples were centrifuged at 8000 rcf during 10 min. The supernatant was then extracted and a new centrifugation was performed in order to pellet any remaining protein precipitates. Lastly, the obtained supernatants were filtered through a filter membrane device, and spiked with three different concentration levels of either 3-NT or Tyr (50, 1000, and 25000 ng/mL). Non-spiked supernatants were simultaneously tested.

Table XX. List of the biological matrices used to evaluate the applicability of the developed method

Sample ID	Company	Reference	Designation	Biological sample
A	Cormay	5-172	Cormay serum HN	Human serum
B	Cormay	5-173	Cormay serum HP	Human serum
C	Human	13951	Serodo Universal control serum for Clinical Chemistry assayed	Human serum
D	Cormay	5-174	Cormay multicalibrator level 1	Human serum
E	Randox	UE 1557	Human precision control - level 2	Human serum
F	Randox	UE 1558	Human precision control - level 3	Human serum
G	HOSLAB	n/a	HD normal control	Human serum
H	Cormay	5-161	Cormay urine control level 1	Human urine
I	Cormay	5-162	Cormay urine control level 2	Human urine
J	Randox	SD 126	RANSOD	Bovine-based whole blood
K	Randox	SC 692	RANDEL	Bovine-based whole blood

n/a, not applicable

3.2.3.4. Sample preparation: B16 F10 melanoma cell line and conditioned growth medium

B16 F10 murine (ATCC, No CRL®-6475) melanoma cell line was obtained from American Type Culture Collection (ATCC) and cultured in MEM supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Portugal), 1% penicillin/ streptomycin (Gibco) and 1% non-essential aminoacids (Gibco). Cells were grown at 37ºC in a humidified 5% CO_2 atmosphere. B16 cells were tested at concentration $1x10^4$ cells/mL. Cells stored at -80ºC were initially submitted to a defrosting/freezing/defrosting cycle (15 min. each), followed by sonication for 10 min using two different systems: (A) Silent Crusher S (Heidolph Instruments GmbH & Co. KG, Germany), and (B) Bandelin Sonorex RK 100 (Sigma-Aldrich®, Portugal), at 47-63 Hz and 35 kHz, respectively. Afterwards, samples were prepared as previously described in 3.2.3.3. Regarding this, 15% TFA was added prior (A1 and B1) or after (A2 and B2) the sonication step in order to assess the most suitable protocol. The medium used for cell growth (MEM, conditioned medium) was also tested for 3-NT quantification, thus the same protocols for protein precipitation were also applied. Samples spiked with 25000 ng/mL of 3-NT were also tested.

3.2.3.5. Sample preparation: bacterial and yeast suspensions

Gram-positive *S. aureus* (ATCC® 25923) and gram-negative *E. coli (*ATCC® 25922) bacteria, as well as the yeast *S. cerevisiae* were tested. Bacteria were inoculated into trypticase soy agar (TSA,VWR Chemicals Prolabo, Portugal), whereas yeast was inoculated into yeast extract peptone dextrose (YPD, VWR). Cultures were then incubated overnight at 37ºC and adjusted to an optical density (OD) at 620 nm of 0.2 using sterile ultrapure water. Subsequently, cells were sonicated for 10 min (Silent Crusher S at 47-63 Hz). Afterwards, proteins were precipitated as described in 3.2.3.3. Samples spiked with 25000 ng/mL of 3-NT were also tested.

3.2.3.6. Recovery rate

In order to assess whether the protein precipitation protocol affects the recovery rate of the developed method, a random serum sample was spiked with 25000 ng/mL of 3-NT prior or after the protein precipitation protocol has been applied. A non-spiked serum sample was use as control. Assays were performed twice, with technical triplicates.

3.2.3.7. Chromatographic conditions

The chromatographic conditions used were the same as described in 2.2.3.4.

3.2.4. Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.02 (La Jolla, CA, USA). Calibration curves/ equations were obtained using linear regression analysis.

3.3. Results and discussion

The purpose of this chapter was to assess which biological matrices are suitable for 3-NT quantification with our developed method, producing reliable results. Hence, a wide variety of samples was tested, ranging from human-associated matrices to microbial suspensions.

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 (Delatour et al., 2007; Radabaugh et al., 2008; Tsikas, 2012). Delatour et al. (2007) 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. (2008) pointed out that a prior acid hydrolysis step is more prone to artifact formation in comparison with enzymatic digestion. In fact, a concern

about 3-NT quantification residues in proteins is that the measurement of this Tyr nitration product may be subjected to artifacts and/or incomplete digestion of proteins. Vivekanandan-Giri, Byun, and Pennathur (2011) applied a protein precipitation protocol based on the addition of 10% trichloroacetic acid (final concentration) followed by a centrifugation step (3000*g* for 10 min), which is similar to the protocol used in this work. The protocol used in this work for protein precipitation was previously optimized by our group (Peixoto, 2012), providing evidence that 15% TFA is the optimum concentration for this kind of application. Moreover, the TFA solution should be prepared right before analysis, since older solutions may lead to erroneous results.

3.3.1. Serum, urine and whole blood

In this phase, several biological matrices from quality control samples were tested, such as serum, urine and whole blood. The linearity was determined for all the assays (Table XXI). Each sample was spiked with three different 3-NT concentrations and a quantitative analysis was performed.

Table XXI. Linearity results obtained for all assays

Linearity	3-NT (ʎ=215nm)	3-NT (ʎ=276nm)	3-NT (ʎ=356nm)	Try (ʎ=215nm)	Try (ʎ=276nm)
Co-relation coefficient	1.000	0.9997	0.9998	0.9997	0.9999
Regression equation	y =6.0545x10^8x -9563.5	y =2.33 x10^8x + 7823.4	y =1.10x10^8x - 2769,7	y =1.78x10^8x - 9205.5	y = 3.89x10^7x +8569.7

3.3.1.1. Serum

As stated above, serum is one of the most tested biological matrices in routine diagnosis (Yu et al., 2011). The results drawn from this study revealed that 3-NT can be reliably quantified in serum samples, even when present at very high or low concentrations (Table XXII).

Table XXII. 3-NT concentrations obtained in serum spiked-samples

Sample_spiked concentration (ng/mL)	3-NT concentration ng/mL (356nm)	3-NT concentration ng/mL (276nm)	3-NT concentration ng/mL (215nm)
A_25000	22328	26070	22151
A_1000	1035	897	1014
A_50	84	84	216
A_0	28	39	16
B_25000	24403	24151	28619
B_1000	799	973	1796
B_50	83	198	1078
B_0	29	39	17
C_25000	22536	24324	20737
C_1000	955	975	847
C_50	82	75	94
C_0	31	41	16
D_25000	23258	24566	21509
D_1000	911	943	812
D_50	65	78	55
D_0	43	54	30
E_25000	23412	25026	21582
E_1000	990	1017	843
E_50	96	88	140
E_0	32	40	19
F_25000	22658	23538	20598
F_1000	966	958	833
F_50	80	66	46
F_0	29	40	25
G_25000	23512	24723	21704
G_1000	939	961	860
G_50	74	73	82
G_0	29	39	16

Moreover, it was possible to detect 3-NT at the three different wavelengths (215, 276 and 356 nm) in all serums samples. As previously stated in Chapter II, 3-NT quantification at 215 nm showed not good accuracy (RE >15%). With regards to the extraction efficiency in serum samples, the mean recovery rates were 94.31±4.43% at 356 nm, 100.46±9.81% at 276 nm, and 214.92±209.76% at 215 nm. Figure 15 exhibits a representative chromatogram of a serum sample.

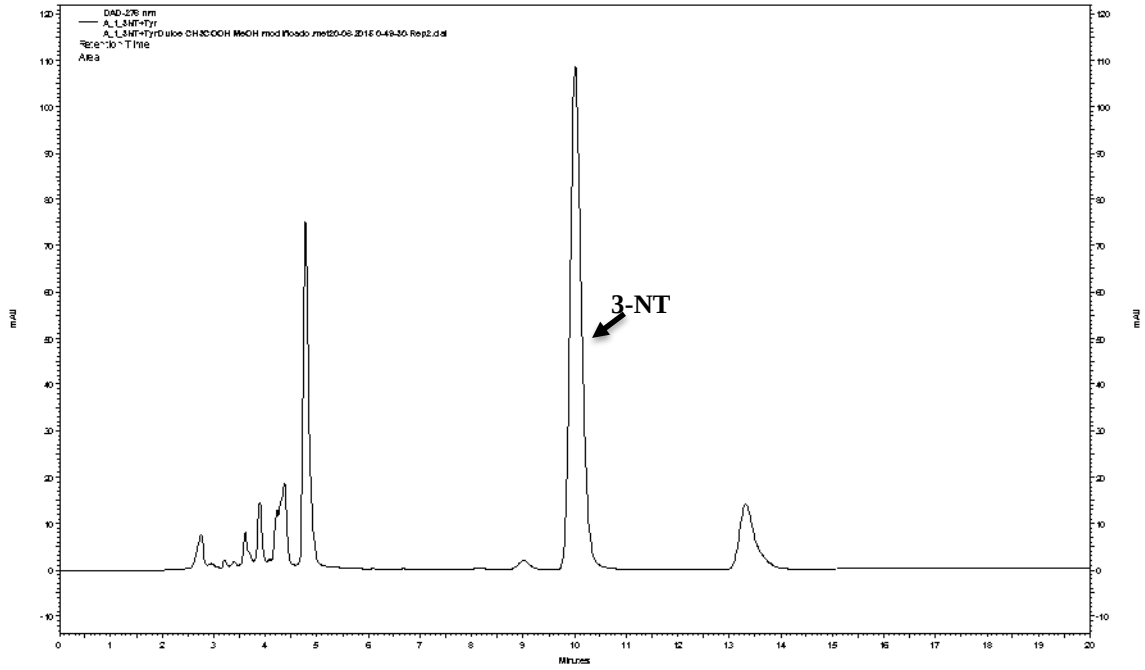


Figure 15. Representative chromatogram of a 3-NT-spiked serum sample (detection 276 nm).

3.3.1.2. Urine

Similarly to that observed for serum, a reliable 3-NT quantification was also possible in urine samples (Table XXIII). Urine is a biological sample with special characteristics, particularly its acidic nature, which is associated with 3-NT artefact formation (Mergola et al., 2013). Nevertheless, Mergola et al. (2013) have found a good 3-NT recovery rate (95%), especially in urine samples from patients with neurological diseases where low concentrations are usually found. Concerning the extraction efficiency in urine samples, the mean recovery rates were 95.03±5.12% at 356 nm, 97.93±23.51% at 276 nm, and 198.32±195.32 at 215 nm. The recovery rate at 215 nm was due to the extremely high recovery rate observed for spiked-samples with 50 ng/mL 3-NT (423.65±373.12%). Representative chromatograms of a 3-NT-spiked urine sample with detection at 276 and 356 nm are shown in Figure 16 and Figure 17 , respectively.

Overall, the recovery rates observed for serum and urine were > 90% (356 and 276nm).

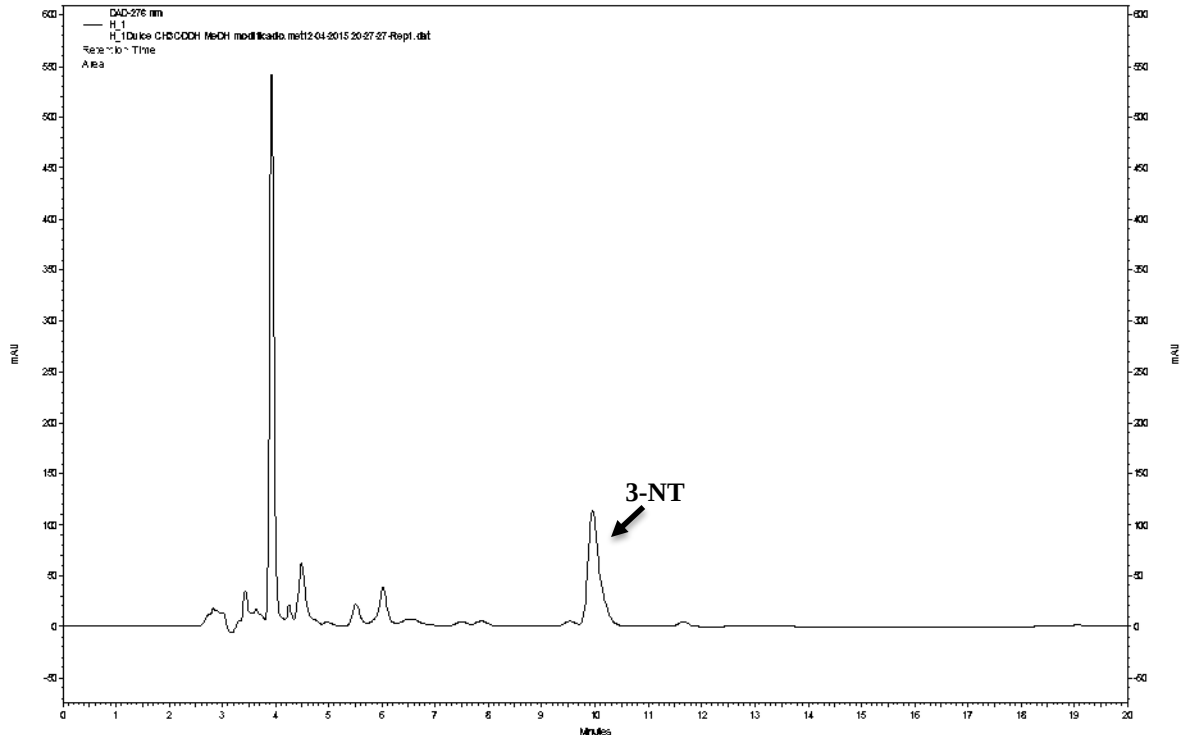


Figure 16. Representative chromatogram of a 3-NT-spiked urine sample (detection at 276 nm).

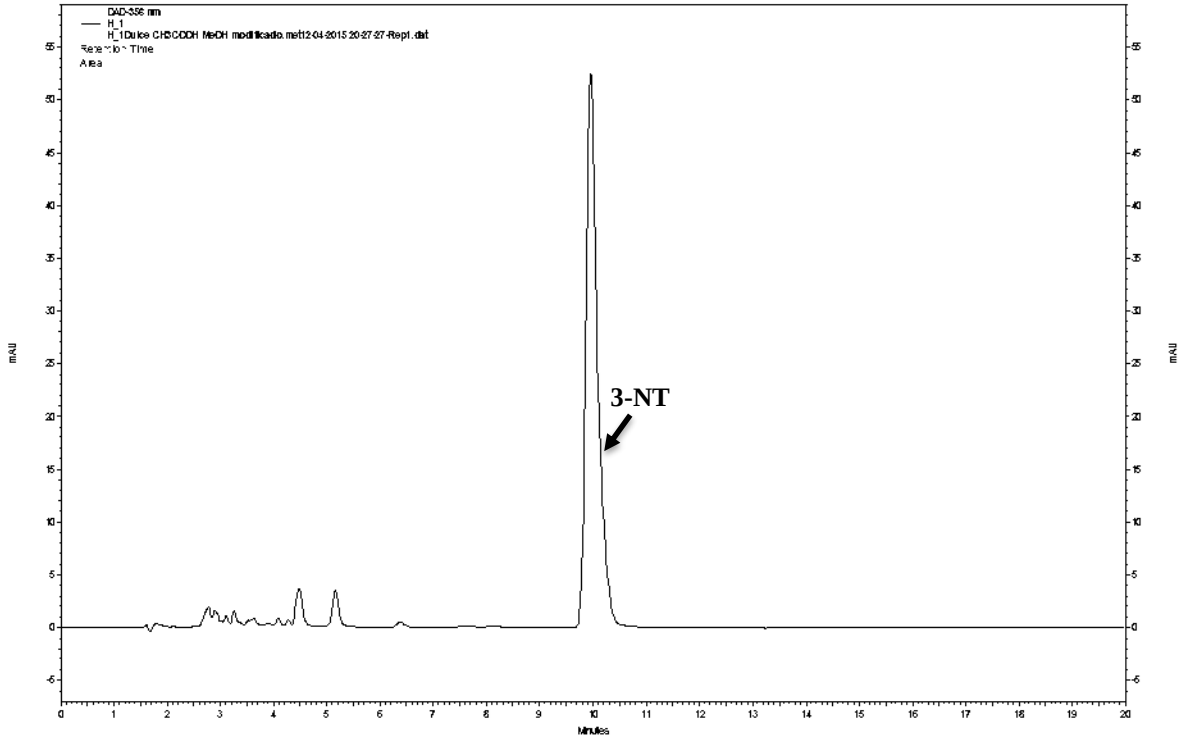


Figure 17. Representative chromatogram of a 3-NT-spiked urine sample (detection at 356 nm).

Table XXIII. 3-NT concentrations obtained in urine spiked-samples

Sample_spiked concentration (ng/mL)	3-NT concentration ng/mL (356nm)	3-NT concentration ng/mL (276nm)	3-NT concentration ng/mL (215nm)
H_25000	22794	23132	20437
H_1000	937	771	762
H_50	74	46	96
H_0	31	39	16
I_25000	23151	23042	20669
I_1000	974	864	1050
I_50	90	156	361
I_0	33	39	17

3.3.1.3. Whole blood

Besides serum and urine, this study has also shown that 3-NT quantification is possible in a biological matrix like whole blood (Table XXIV). However, and contrary to that observed for all serum and urine samples, which detection was possible at three different wavelengths, detection in whole blood was possible only at 356 nm (Figure 18). Nevertheless, and as previously reported by Herce-Pagliai et al. (1998), 356 nm is the most specific wavelength for 3-NT detection (Figure 18 – c) and d)). Therefore, the mobile phase developed and validated in this study is suitable for 3-NT quantification in the three different sample types (serum, urine, and whole blood), with detection at 356 nm. On the other hand, Tyr quantification in whole blood was not possible, since an impure chromatographic peak for Tyr was observed in most cases.

Regarding whole blood, the recovery rates at 356nm were 88.82±7.47% for 25000ng/mL, 87.19±2.08% for 1000ng/mL, and 64.43±0.87% for 50ng/mL.

Table XXIV. 3-NT concentrations obtained in whole blood spiked-samples; n/a not applicable

Sample_spiked concentration (ng/mL)	3-NT concentration ng/mL (356nm)	3-NT concentration ng/mL (276nm)	3-NT concentration ng/mL (215nm)
J_25000	23556	n/a	n/a.
J_1000	916	n/a.	n/a.
J_50	62	n/a.	n/a.
J_0	29	n/a.	n/a.
K_25000	20914	n/a.	n/a.
K_1000	887	n/a.	n/a.
K_50	62	n/a.	n/a.
K_0	30	n/a.	n/a.

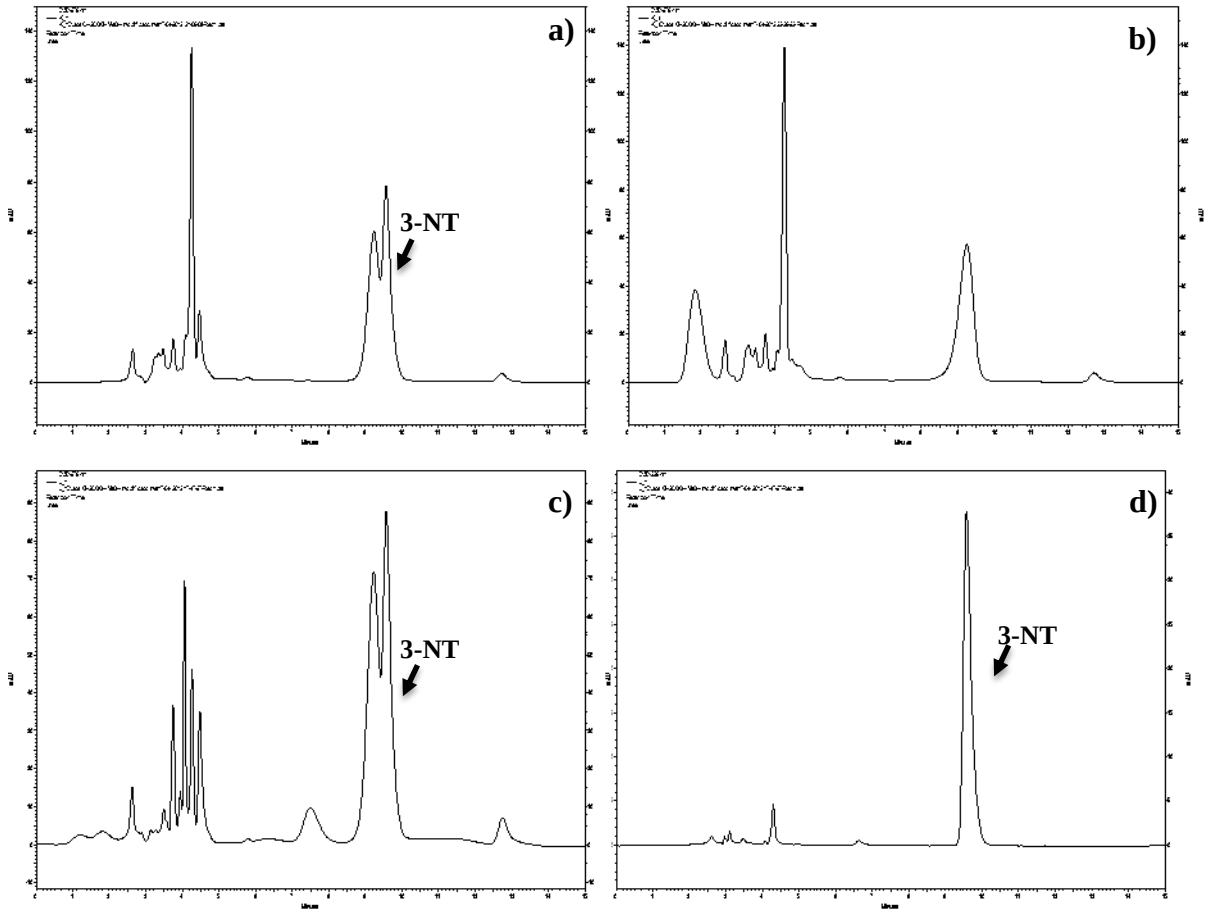


Figure 18. Representative chromatograms of a 3-NT-spiked whole blood sample with detection at a) and c) 276 nm and b) 356 nm and d) non-spiked whole blood sample (detection at 276 nm)

3.3.2. Other biological matrices

In order to evaluate the applicability of the developed method in other biological matrices, we evaluated 3-NT detection/quantification in i) B16 F-10 melanoma cell line, ii) growth medium conditioned with the same cell line, iii) gram-negative (*E. coli*) and gram positive (*S. aureus*) bacterial and iv) yeast (*S. cerevisiae*) suspensions.

3.3.2.1. Melanoma cell line and growth medium conditioned

Human melanoma cells express iNOS which is responsible for induced NO-based immune response. The expression of this molecule is associated with poor survival of patients with melanoma, thereby iNOS is a molecular marker of poor prognosis and a possible target for therapy (De Sanctis et al., 2014; Grimm, Ellerhorst, & Ekmekcioglu, 2008). On the other hand, a significant association between iNOS expression and 3-NT production has been

found in melanoma patients, using immunohistochemistry detection (Grimm et al., 2008; Winkler, Koedel, Kastenbauer, & Pfister, 2001). Therefore, the applicability of the developed method in these type of cell lines was also evaluated.

In this study, B16 F10 melanoma cell line (Figure 19) was tested, since it is widely used in a variety of studies, and is also associated with high metastatic activity (Herlyn & Fukunaga-Kalabis, 2010). This cell line (at a concentration of ~10^4 cells/mL), as well as the medium used for cell growth (Figure 20), were spiked with the same 3-NT concentration (25000 ng/mL).

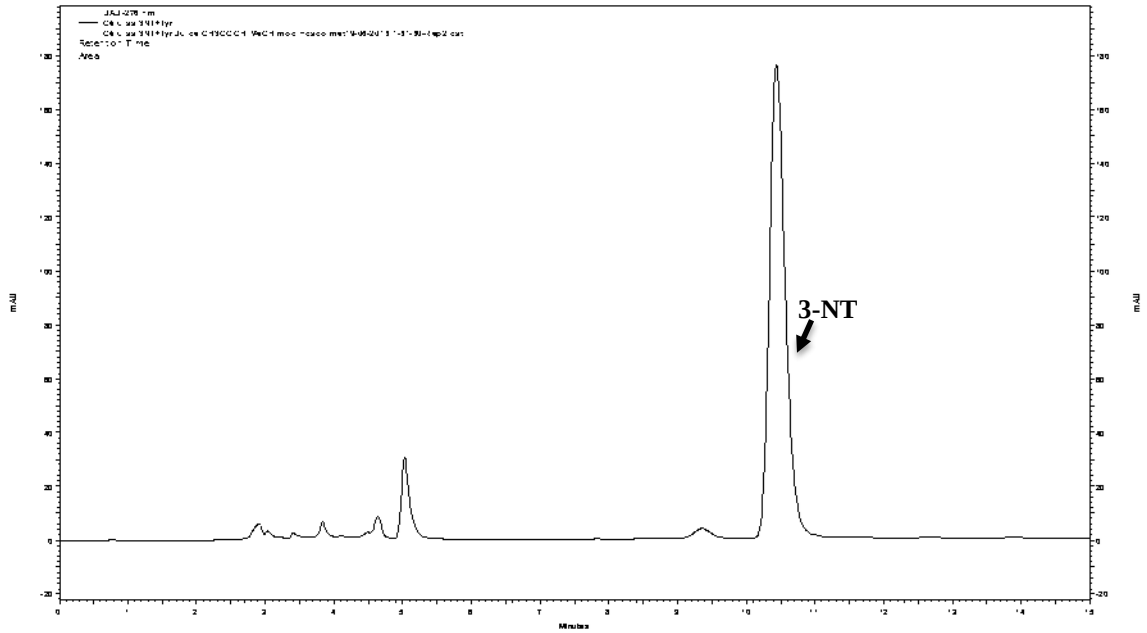


Figure 19. Representative chromatogram of a 3-NT-spiked B16 F10 melanoma cell line sample (detection at 276 nm).

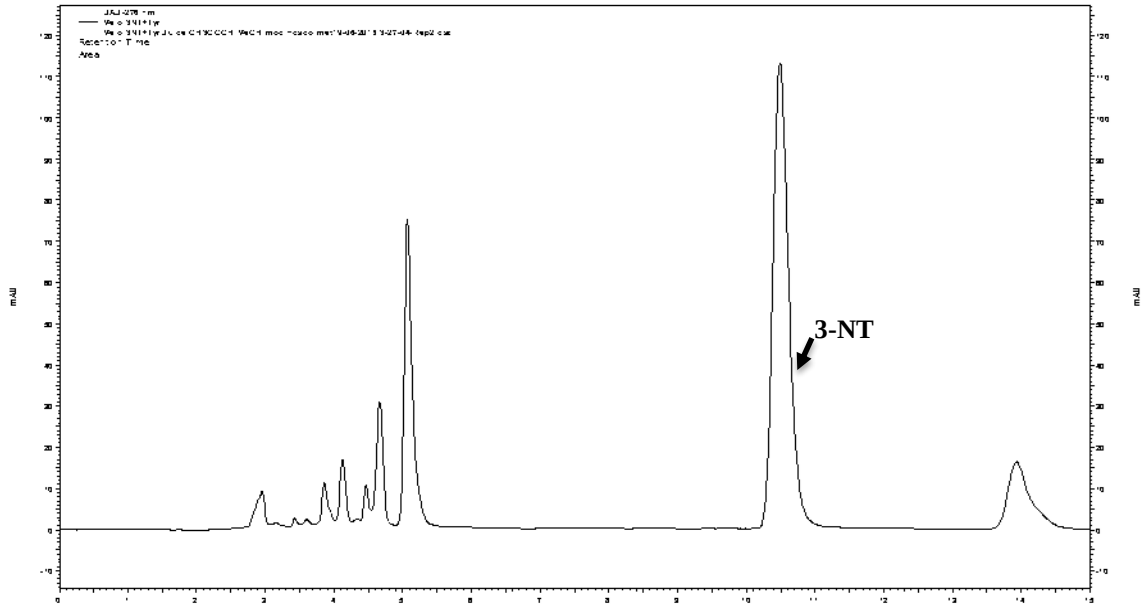


Figure 20. Representative chromatogram of a 3-NT-spiked growth medium conditioned sample (detection at 276 nm).

3.3.2.1.1. Optimization of cell lysis and protein precipitation

Since human cells are difficult to disrupt, four different sonication-based protocols for cell lysis and protein precipitation were applied to B16 F-10 melanoma cell line, as well as to the medium used for their growth (conditioned medium). Cellular lysis through sonication is an effective way for the recovery of periplasmic, membrane-bound, or insoluble recombinant proteins (Bystryak, Santockyte, & Peshkovsky, 2015). Moreover, sonication systems are usually rapid and easy-to-use. Overall, protocols B1 and B2 provided better results, although protocol B2 (sonication with ultrasound bath followed by addition of 15% TFA) was superior (Figure 21). The differences between the results originated by the two sonication systems was likely to be due to the frequency used.

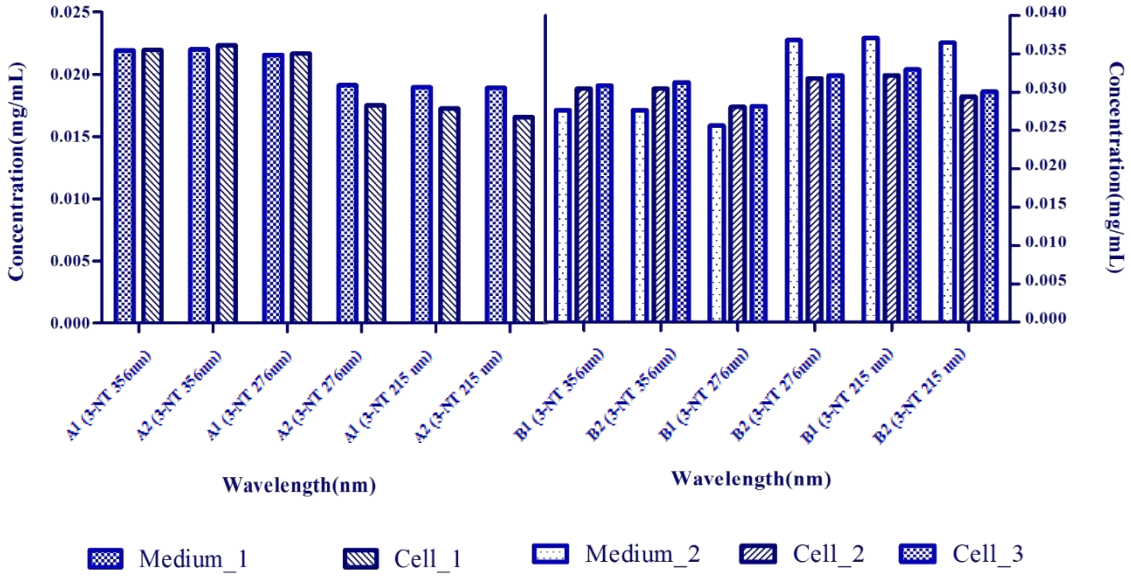


Figure 21. Results obtained using the different cell lysis and protein precipitation protocols.

3.3.2.2. Bacterial and yeast suspensions

Besides human biological matrices, the developed protocol was also tested for 3-NT quantification in bacterial and yeast suspensions, which was also successful (Figure 22 and 23). Therefore, this study provides evidence that the developed method may also be applied to different fields other than human biology, for instance in microbial research.

The same protocol used for B16 F-10 melanoma cell line and growth medium (sonication-based method) was also applied to bacterial and yeast suspensions, since several studies has claimed that sonication for 10 min is effective to achieve bacterial cells lysis (Joyce, Al-Hashimi, & Mason, 2011; Monsen, Lövgren, Widerström, & Wallinder, 2009).

Furthermore, bacterial and yeast suspensions were made with ultrapure water, allowing cell wall disruption. Figure 24, 25 and 26 show representative chromatograms of 3-NT-spiked bacterial and yeast suspensions.

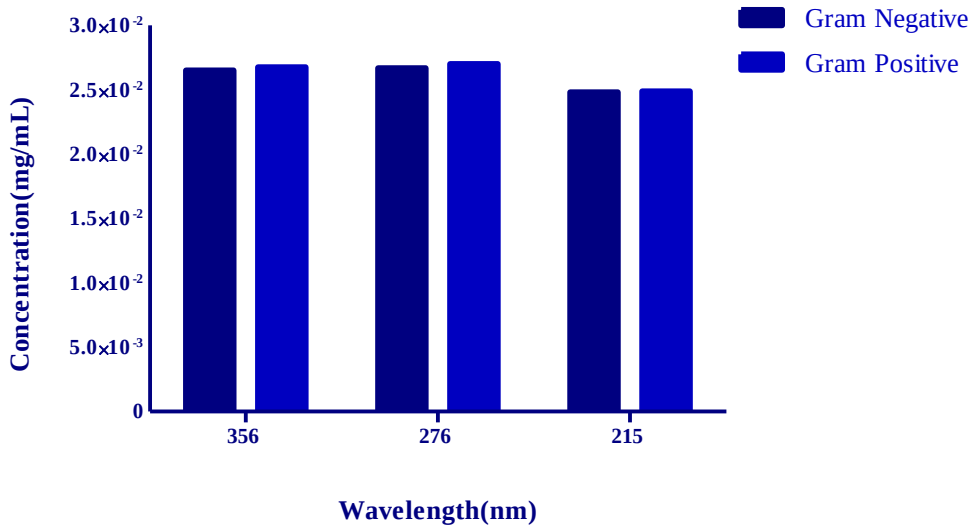


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Figure 22. 3-NT quantification in 3-NT spiked bacterial suspensions.

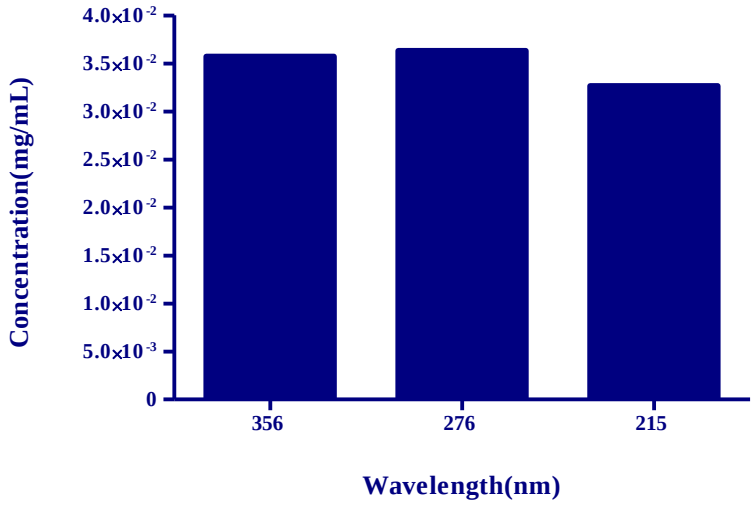

Figure 23. 3-NT quantification in 3-NT-spiked yeast suspensions.

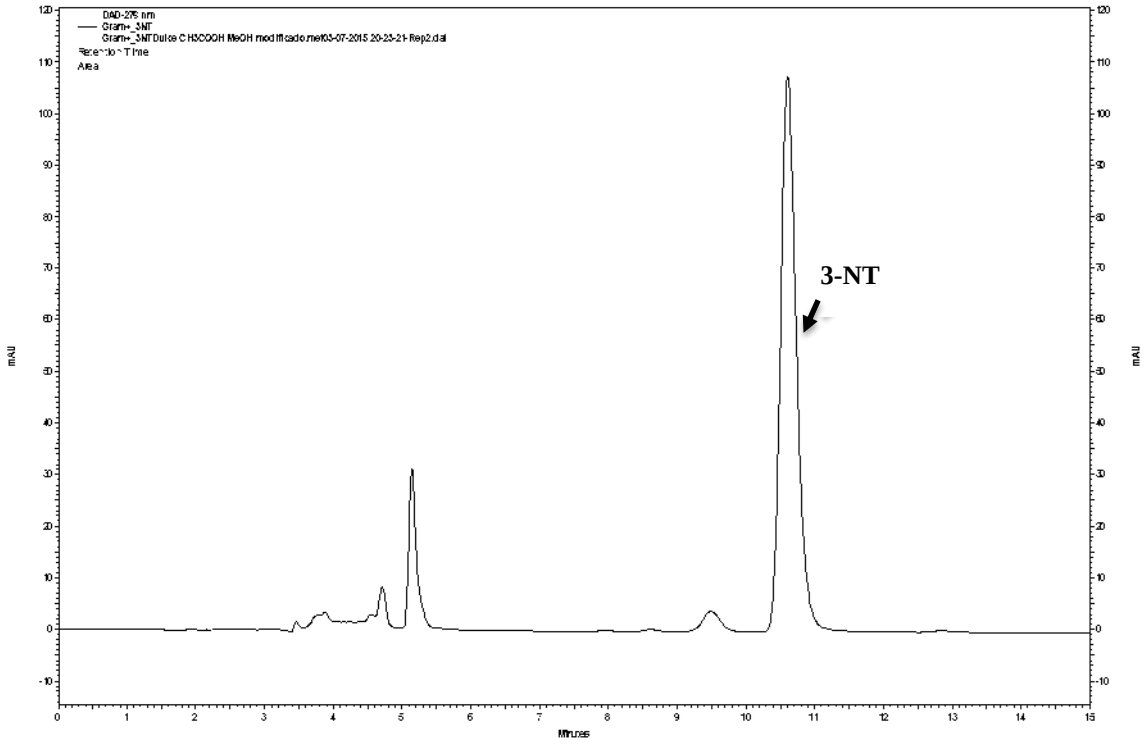

Figure 24. Representative chromatogram of a 3-NT-spiked gram positive bacterial suspensions sample (detection at 276 nm).

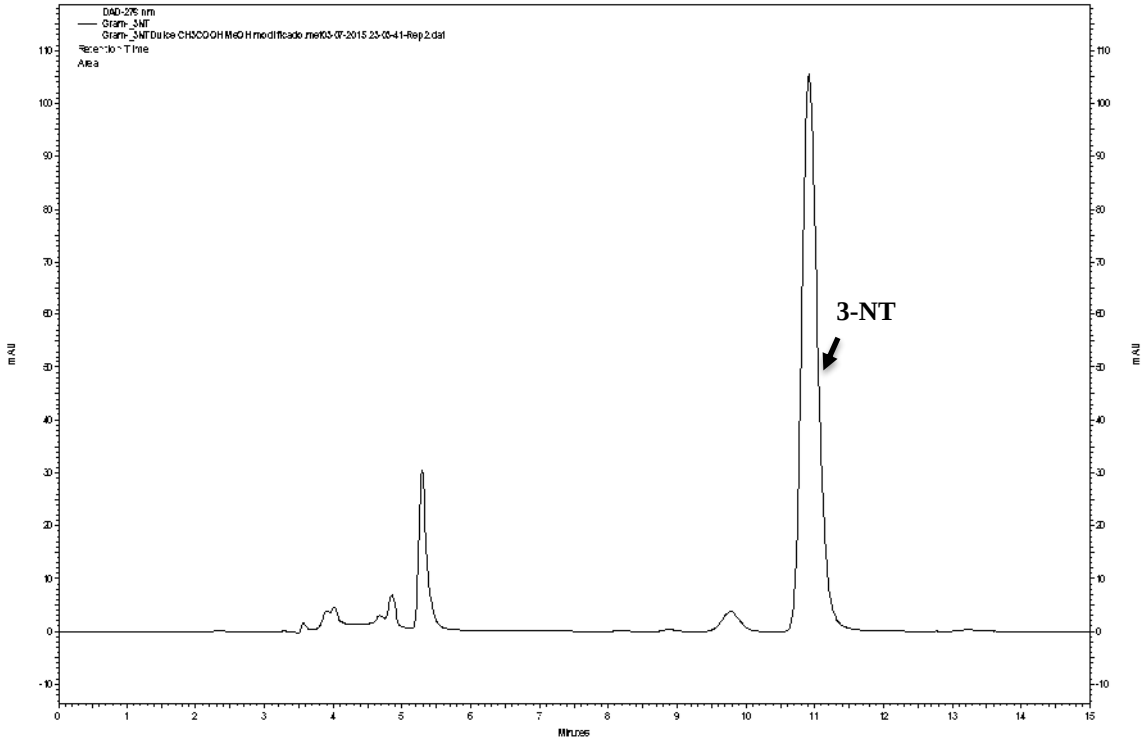

Figure 25. Representative chromatogram of a 3-NT-spiked gram negative bacterial suspensions sample (detection at 276 nm).

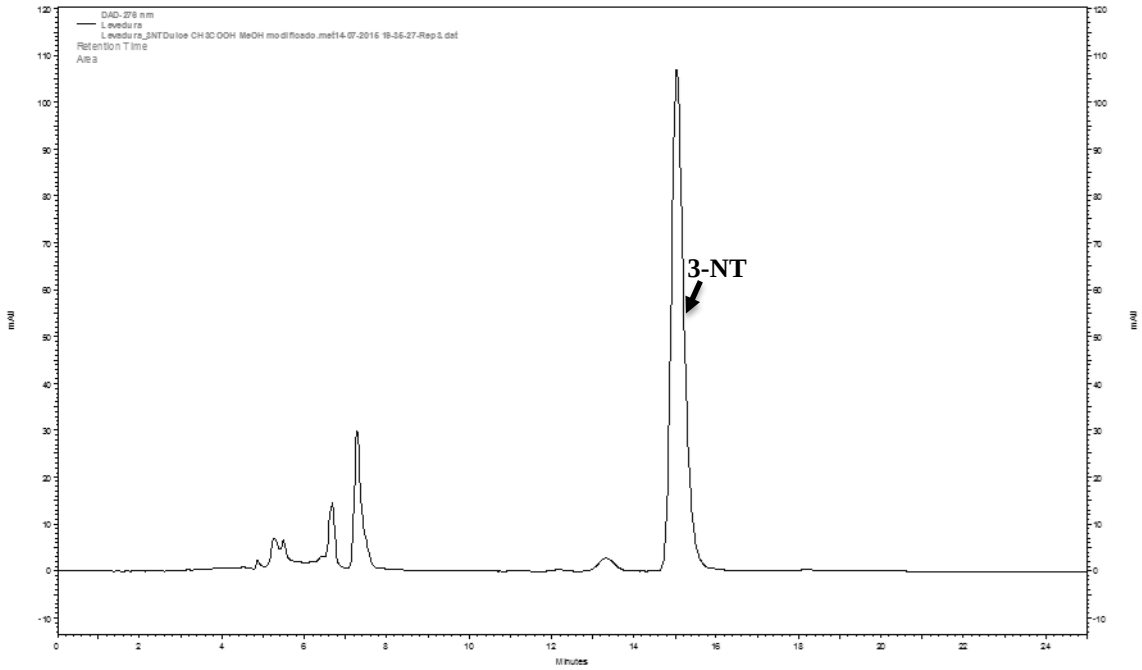

Figure 26. Representative chromatogram of a 3-NT-spiked yeast suspension sample (detection at 276 nm).

3.3.3. Influence of protein precipitation protocol on recovery rates

The recovery rates using a random serum sample spiked with 25000 ng/mL of 3-NT were calculated in order to assess the degree of analyte loss when the protein precipitation protocol is applied. Overall, the recovery rates obtained for samples spiked before and after the protocol has been applied were fairly similar (about 1% difference between them). This is indicative that such protocol does not lead to a significant loss of 3-NT and, as a result, does not compromise the accuracy of the results.

3.4. Conclusion

The protocol used (0.5% CH_3COOH:MeOH:H_2O 15:15:70 (v/v) as the mobile phase with detection at 356 nm), allowed the successful 3-NT detection and quantification in all biological matrices tested. Whole blood was the only biological matrix which 3-NT detection and quantification at 276 nm and 215 nm was not possible. Nevertheless, and unlike other previously described methods for 3-NT quantification, our HPLC-based method was successfully applied to a wide range of biological matrices, exhibiting a great performance in all of them.

Chapter IV

Main conclusions and future directions

4.1. Main conclusions

Research on 3-NT has been focusing on its use as biomarker of oxidative/nitrosative stress in wide range the pathologies. In another research line, 3-NT has also been proposed as a target for therapeutic interventions in pathologies involving oxidative stress (Joly & Grunfeld, 2014).

Recognising the potential use of 3-NT as a tool for diagnosis and/ or treatment monitoring of several pathological conditions, we developed a simple, fast, and cost-effective HPLC-based method, and validated it at the level of specificity, linearity, LoD and LoQ, accuracy and precision. Considering the availability of HPLC equipment in a wide range of laboratory settings nowadays, as is the case of clinical biochemistry laboratories, we chose this methodology as the base for our method development process.

The first step of this study was the evaluation of different mobile phases in the detection and quantification of 3-NT. The KH_2PO_4-based mobile phases have presented the disadvantage of precipitation of potassium phosphate as opposed to 0.5% CH_3COOH:MeOH:H_2O-based mobile phase. Regarding the three concentrations of 0.5% CH_3COOH:MeOH:H_2O tested, the proportion 15:15:70 showed the best results and chromatograms exhibited well defined peaks. In this sense, mobile phase 0.5% CH_3COOH:MeOH:H_2O (15:15:70 (v/v)) was regarded as the most suitable for our purposes.

The second step relied on the optimization and further validation of the selected method, which exhibited a good specificity, precision, accuracy and linearity. Moreover, L_OD/ L_OQ were in the order of units ng/mL, and the analysis time *per* sample was about 15 minutes.

The next step was to evaluate the applicability of such method in different biological samples. Firstly, we evaluated its performance in the most tested biological matrices in clinical biochemistry laboratories, namely serum, urine, and whole blood. Regarding this, the method allowed the successful detection and quantification of 3-NT at 356 nm, although accurate quantification in serum and urine was also possible at other wavelengths (215 and 276 nm). Additionally, and for the sake of increasing the applicability of the method, other biological matrices were tested, namely B16 F-10 melanoma cell line and respective conditioned medium, as well as bacterial and yeast suspensions. Again, detection and quantification of 3-NT was also successful at the aforementioned wavelengths. Up to date, and as far as we know, there is no published work describing 3-NT quantification methods the previous biological matrices by using HPLC or other chromatographic equipment.

Therefore, and besides the applicability of our method for clinical purposes, we gathered evidence in the present study that it might also be used for scientific research purposes, particularly, in the field of oxidative/ nitrosative stress.

4.2. Future directions

According to the main purpose of the present study, a major limitation of this study is the fact that real biological samples from both healthy volunteers and patients with different pathologies were not tested for our developed protocol. Therefore, it is pivotal in a near future to perform such analysis. If successful, the applicability of this method may be further evaluated, for instance, in the treatment monitoring of pathologies involving increased nitrosative stress state (e.g., CAD, neurodegenerative diseases, among others).

Regarding technical aspects, it would be interesting to assess the protein content of the biological samples tested in this study, thus the efficacy of the protocol for protein precipitation could be better assessed. Moreover, the performance of our method should be compared with the performance exhibited by other methods already described and validated. For instance, the samples used in the present study could be tested using commercially available ELISA kits for 3-NT quantification.

Moreover, it would be important to develop fast and easy protocols for sample concentration in order to obtain a more accurate quantification. Additionally, and given the current importance of the use of ecological methods, it would be interesting to find out whether more environment-friendly solvents, also called green solvents, could replace methanol in our developed method. Regarding this, ionic liquids might be a suitable alternative (Polyakova, Koo, & Row, 2006).

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