Development of a new HPLC-based method for 3-nitrotyrosine quantification in different biological matrices

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A B S T R A C T

Background: The nitration of tyrosine residues in proteins is associated with nitrosative stress, resulting in the formation of 3-nitrotyrosine (3-NT).[1] 3-NT levels in biological samples have been associated with numerous physiological and pathological conditions. Hence several attempts have been made in order to develop methods that accurately quantify 3-NT in these matrices. The aim of this study was to develop a simple, rapid, low-cost and sensitive high-performance liquid chromatography (HPLC)-based 3-NT quantification method.
Methods: All experiments were performed on an Hitachi LaChrom Elite® HPLC system. The method was validated according to International Conference on Harmonisation (ICH) guidelines for serum samples. Additionally, other biological matrices were tested, namely 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 mobile phase, with detection at wavelengths 215, 276 and 356 nm, at 25 °C, and using a flow rate of 1 mL min^{-1}. By using this protocol, it was possible to obtain a linear calibration curve, limits of detection and quantification in the order of μg L^{-1}, and a short analysis time (<15 min *per* sample). The developed protocol allowed the successful detection and quantification of 3-NT in all biological matrices tested, with detection at 356 nm.
Conclusion: This method, successfully developed and validated for 3-NT quantification, is simple, cheap and fast. These features render this method a suitable option for analysis of a wide range of biological matrices, being a promising useful tool for both research and diagnosis activities.

Keywords: 3-Nitrotyrosine Nitrosative stress HPLC-DAD Quantification methods

[1] 3-NT – 3-nitrotyrosine; ICH – International Conference on Harmonisation; ROS – reactive oxygen species; Try – tyrosine; RNS – reactive-nitrogen species; PYCC – Portuguese Yeast Culture Collection; MEM – Minimal Essential Medium; TSA – Trypticase Soy Agar; YEPD – yeast extract peptone dextrose.

1. Introduction

Molecules modified by interactions with reactive oxygen species (ROS) in the microenvironment, and those changed in response to increased redox stress, are considered biomarkers of oxidative stress [1]. The nitration of tyrosine (Tyr) residues in proteins is associated with nitrosative stress. L-Tyr and protein-associated Tyr are the target of various reactive-nitrogen species (RNS), resulting in the formation of free 3-nitrotyrosine (3-NT) [(2-amino-3-(4-hydroxy-3-nitrophenyl) propanoic acid)] and protein-associated 3-NT [2–4]. 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 [2,4]. Recently, a study suggested that 3-NT is likely to have a deleterious effect on protein function and less likely to be important in normal cellular function [5].

The nitration of proteins is a common process that occurs under physiological conditions [3,6,7] and the concentration of 3-NT in plasma of healthy humans is on the threshold of the nM-to-pM range [3].

A significant increase in the extent of this process results in increased 3-NT levels in biological samples and has been associated with a wide range of diseases [3,6]. Among these there are cardiovascular diseases [8,9], diseases associated with immunological

reactions [10,11], neurological diseases and psychiatric disorders [12,13]. Other diseases have also been associated with increased protein nitration, such as Fabry disease [14], diabetes mellitus [15,16], diabetic vascular dysfunction [17], Chagas disease [18], systemic lupus erythematosus [19], erectile dysfunction [20] among others. Measurement of 3-NT in biological samples can be used as a biomarker of nitrosative stress, since it is very stable and suitable for analysis.

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 [21]. 3-NT has been detected in several biological matrices and fluids including plasma, serum, urine, cerebrospinal fluid, synovial fluid, tissue sample and other biological samples [22].

Among the techniques used for 3-NT detection and quantification, chromatographic methods have been shown to exhibit better performance. The different chromatographic methods described for the detection and quantification of this molecule include: (i) liquid chromatography, namely high-performance liquid chromatography (HPLC)-based methods that use electrochemical (ECD) and diode array (DAD) detection, liquid chromatography-mass spectrometry (LC–MS) and liquid chromatography-tandem mass spectrometry (LC–MS/MS); (ii) gas chromatography, such as gas chromatography-mass spectrometry (GC–MS) and gas chromatography-tandem mass spectrometry (GC–MS/MS) [23,24]. These methods have been developed during the last years, all of them presenting pros and cons, although it is evident that chromatography-based methods present good sensitivity and specificity. GC-based methods exhibit the highest sensibility in the quantification of 3-NT [25–27]. Nevertheless, and owing to 3-NT chemical properties, a derivatization step prior to analysis is required, which ends up being time-consuming for the analyst [28]. Moreover, derivatization reactions often induce artefacts formation, which may further influence the final analysis. Conversely, HPLC does not require such derivatization step and is cheaper, despite not being as accurate as GC [7,29].

Currently, 3-NT has raised great interest concerning its potential as biological tool for the therapeutic monitoring of various diseases involved in nitrosative stress. In this sense, our study aimed to develop a new chromatographic method, which is simple, cheap and user-friendly, without compromising the sensitivity and specificity levels required for quantification of 3-NT. The ultimate goal was to apply this method to a myriad of biological samples, so that it can be used both in research and medical laboratories. The results obtained were validated and interpreted in accordance with the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use guidelines for serum samples [30]. Additionally, and in order to provide a proof-of-concept for our method, other biological matrices were tested, namely whole blood, urine, B16 F-10 melanoma cell line, growth medium conditioned with the same cell line, bacterial and yeast suspensions. In the future, validation for these matrices should be performed as well.

2. Material and methods

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, L-2200 LaChrom Elite autosampler, L-2300 LaChrom Elite column heater and L-2455 LaChrom Elite photo DAD. EZChrom Elite Compact Software Version 3.3.2. (Agilent Technologies, Inc., Santa Clara, CA, United States) was used for data collection and analysis.

2.2. Chemicals, 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 0.2 μm (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 0.45 μm, 47 mm, were purchased from Advantec®, Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). Puradisc™, 0.2 μm, 25 mm sterile and endotoxin free filters were purchased from Whatman™ (GE Healthcare UK Limited, Buckinghamshire, UK). 2 mL syringes were purchased from Terumo® Medical Corporation (Leuven, Belgium).

2.3. Biological fluids and cellular models

The biological fluids' samples tested in this study are described in Supporting information Table 1. B16 F10 murine (ATCC, No CRL®-6475) melanoma cell line was obtained from American Type Culture Collection (ATCC). Gram-positive *Staphylococcus aureus* (ATCC® 25923) and gram-negative *Escherichia coli* (ATCC® 25922) bacteria, as well as the yeast *Saccharomyces cerevisiae* [PYCC (Portuguese Yeast Culture Collection) 4072].

2.4. Analytical procedure

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

0.5% CH_3COOH:MeOH:H_2O solutions were prepared according to the following proportions: 30:0:70 and 15:15:70. All mobile phases were filtered through a 0.45 μm membrane.

2.4.2. Calibration standards

0.5 gL^{-1} 3-NT and Tyr stock solutions were prepared 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 (50,000; 25,000; 10,000; 5000.0 2500.0; 1250.0; 625.00 and 312.50 $\mu g\,L^{-1}$). Standard solutions were prepared by diluting the respective stock solution into the desired mobile phase. These standard solutions were used for calibration purposes.

2.4.3. Chromatographic conditions

The chromatographic conditions used in all assays were as follows: flow rate of 1 $mL\,min^{-1}$, detection in the range 190–400 nm, volume of injection of 25 μL and oven temperature of 25 °C.

2.5. Method optimization

2.5.1. Temperature optimization

In order to establish the best operating temperature, a wide range of temperatures (15, 20, 35, 45, 55 and 65 °C) were assayed using two standard solutions (50,000 $\mu g\,L^{-1}$ and 25,000 $\mu g\,L^{-1}$) and two serum samples.

2.6. Method validation

All the methods tested were validated according to ICH guidelines for validation of analytical procedures [30].

2.6.1. Specificity

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

2.6.2. Linearity

Six to eight standard solution concentrations comprising the range between 312.5–50,000 $\mu g\,L^{-1}$ were assayed. Calibration curves were constructed by plotting average peak area versus concentration. The linearity was evaluated using regression analysis.

2.6.3. Accuracy

Accuracy was determined by measuring recovery in 3-NT-spiked serum samples (three concentration levels: 25000, 1000.0 and 50.000 $\mu g\,L^{-1}$) and in standard solutions (50000, 25000, 10000, 5000.0, 2500.0, 1250.0, 625.00, 312.50 $\mu g\,L^{-1}$).

2.6.4. Precision

Precision was determined by means of repeatability (intraday precision). The repeatability was evaluated by analysing standard solutions (eight concentration levels: 50 000, 25 000, 10 000, 5000.0, 2500.0, 1250.0, 625.00, 312.50 $\mu g\,L^{-1}$) and 3-NT-spiked serum samples (three different concentration levels: 25000, 1000.0, 50.000 $\mu g\,L^{-1}$).

2.6.5. LoD and LoQ

LoD and LoQ were determined using the following equations: (i) $LoD = 3.3 \times (SD/s)$; (ii) $LoQ = 10 \times (SD/s)$, where *SD* is the standard deviation of the response and *s* is the slope retrieved from the calibration curve data.

2.7. Sample preparation

2.7.1. Serum, urine, and whole blood

All samples were initially submitted to a previous established protocol in order to acid hydrolysis. Briefly, 1 mL of 15% trifluoroacetic acid (TFA) was added to 1 mL of each sample, and then vortexed for 20 s. Afterwards, samples were centrifuged at 8000 rcf during 10 min. The supernatant was then extracted and a new centrifugation step under the same conditions was performed in order to pellet any remaining cellular debris. Lastly, the obtained supernatants were filtered through a filter membrane device (0.2 μm), and spiked with three different concentration levels of either 3-NT or Tyr (50.000, 1000.0, and 25000 $\mu g\,L^{-1}$). Non-spiked supernatants were tested in simultaneous.

2.7.2. B16 F10 melanoma cell line and conditioned growth medium

B16 F10 murine (ATCC, No CRL®-6475) melanoma cell line was cultured in Minimal Essential Medium (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 37 °C in a humidified 5% CO_2 atmosphere. Cell density was assessed using a haemocytometer and trypan blue exclusion test, and a final concentration of 1×10^4 cells mL^{-1} was tested. Cells stored at −80 °C 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 2.7.1. 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 was also tested for 3-NT quantification, thus the same protocols for acid hydrolysis were also applied. Samples spiked with 25,000 $\mu g\,L^{-1}$ of 3-NT and Tyr were also tested, according to the protocol described above.

2.7.3. Bacterial and yeast suspensions

Gram-positive *S.aureus* and gram-negative *E.coli* bacteria, as well as the yeast *S.cerevisiae* were tested for 3-NT detection and quantification. Bacteria were inoculated into Trypticase Soy Agar (TSA, VWR Chemicals Prolabo, Portugal), whereas yeast was inoculated into yeast extract peptone dextrose (YEPD, VWR). Cultures were then incubated overnight at 37 °C and adjusted with sterile ultra pure water to an optical density at 620 nm equivalent to $\sim 2 \times 10^8$ cells mL^{-1}. Subsequently, cells were sonicated for 10 min (Silent Crusher S at 47–63 Hz). Afterwards, acid hydrolysis protocol has been applied as previously described in 2.7.1. Samples spiked with 25,000 $\mu g\,L^{-1}$ of 3-NT and Tyr were also tested.

2.7.4. Recovery rate—influence sample preparation protocol on recovery rates

In order to assess whether the acid hydrolysis protocol affects the recovery rate of the developed method, a random serum sample was spiked with 25,000 $\mu g\,L^{-1}$ of 3-NT prior and after the acid hydrolysis protocol has been applied. A non-spiked serum sample was used as control. Assays were performed twice, with technical triplicates.

2.8. Statistical analysis

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

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 features [31]. Our major objective was to develop a suitable method for use in most clinical and research laboratories. Therefore, and taking into account all the positive and negative features of the methods, we defined as major prerequisites i) availability of the required equipment in a wide range of laboratory facilities, ii) reduced pre-analysis steps and analysis time, and iii) cost-effective analysis. After a bibliographic analysis, we found that an HPLC-based method would perfectly meet the previous pre-requisites.

The development and validation of an HPLC-based method is, 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 [30].

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 C18 column.

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. Accordingly, we defined 215, 276 and 356 nm as the optimal wavelengths for detection in our subsequent assays.

Regarding the composition of the mobile phases, they were prepared with different methanol concentrations. Such compositions were selected based on the information shown in Table 1.

Table 1
Description of the assays performed, and respective retention time (mean ± standard deviation), LoD and LoQ values.

Mobile phase		Composition		Assay	
0.5%CH_3COOH:MeOH:H_2O		30:0:70 15:15:70		A and B C	
(A)	Retention time (min)	LoD ($\mu g\,L^{-1}$)	LoQ ($\mu g\,L^{-1}$)	Column oven temperature (°C)	Analysis time (min)
3-NT (215 nm)	34.186 ± 0.129.	10.60.	32.13.	25.	40.
3-NT (276 nm)	34.186 ± 0.129.	36.97	112.02		
3-NT (356 nm)	34.186 ± 0.129	36.15	109.53		
Tyr (215 nm)	8.928 ± 0.056	0.72	2.18		
Tyr (276 nm)	8.928 ± 0.056	66.82	202.50		
(B)					
3-NT (215 nm)	22.883 ± 0.131	3.19	9.67	40	35
3-NT (276 nm)	22.883 ± 0.131	44.35	134.40		
3-NT (356 nm)	22.883 ± 0.131	8.46	25.65		
Tyr (215 nm)	6.734 ± 0.050	0.61	1.84		
Tyr (276 nm)	6.734 ± 0.050	1.37	4.15		
(C)					
3-NT(215 nm)	9.878 ± 0.080	6.09	18.47	25	15
3-NT(276 nm)	9.878 ± 0.080	0.93	2.81		
3-NT(356 nm)	9.878 ± 0.080	5.11	15.49		
Tyr (215 nm)	4.146 ± 0.022	1.81	5.49		
Tyr (276 nm)	4.146 ± 0.022	0.53	1.60		

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

For mobile phases based on 0.5% CH_3COOH:MeOH:H_2O, two different proportions of these reagents were tested, as shown in Table 1.

Firstly, we tested 0.5% CH_3COOH:MeOH:H_2O (30:0:70, v/v) (assays A and B). This mobile phase composition has the advantage of not making use of an organic solvent, rendering it a good 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 [32]. Since the analysis time of assay A was 40 min, we decided to follow the previous approach and proceed to increase the oven temperature to 40 °C (assay B – Fig. 1A). The analysis time was reduced to 35 min, although the resolution of the 3-NT peaks was the lowest among all tested proportions. It is known that 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 [33,34]. Temperature has also a large effect on the thermodynamics of the retention process that can lead to a reduction in the running time and selectivity [35].

The last proportion tested was 0.5% CH_3COOH:MeOH:H_2O (15:15:70, v/v) at 25 °C (assay C –Fig. 1B), which allowed a reduced analysis time (15 min). This drop in the analysis time was likely the result of an increase in methanol concentration, which is known to decrease the retention time of solutes [36]. This reduced analysis time was a significant advantage of this mobile phase composition over all the others, thus no further increase in the operating temperature was tested. Regarding the limited of detection (LoD) and quantification (LoQ), this proportion exhibited great results, in the order of units of $\mu g\,L^{-1}$, as we as the lowest time analysis (Table 1). In Fig. 1B is shown a representative chromatogram obtained with assay C, 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 min within technical replicates [37], which was verified in the tested conditions.

Linear regression analysis for all the mobile phases tested showed good linear relationship, with correlation coefficients very close to 1.

3.1.1. Mobile phases comparison and selection

Overall, all mobile phases tested exhibited pros and cons. Regarding the proportion 30:0:70 (v/v) at 25 °C 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 (see Table 2 from Supporting information). 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 positive and negative aspects, the mobile phase 0.5% CH_3COOH:MeOH:H_2O (15:15:70 (v/v)) was regarded as the most suitable for our purposes, and was therefore selected for our further optimization steps.

3.2. Optimization steps

3.2.1. Optimization of the temperature

Regarding the operating temperature, a wide range (15, 20, 25, 35, 45, 55 and 65 °C) was assayed while maintaining the other parameters. When temperatures from 35 to 65 °C (with detection at 215 and 276 nm), were tested, co-elution of 3-NT with other molecules was observed. A similar phenomenon was observed when testing at 15 °C. Temperatures between 20 and 25 °C showed to be specific for 3-NT, with detection at 276 nm and 356 nm.

3.3. Method validation

3.3.1. Specificity

A specific 3-NT quantification method should have the ability to detect 3-NT unequivocally without any interference from Tyr or other close structural relatives [29]. In order to determine the specificity of the developed method in all assays, 3-NT and Tyr were spiked into different biological matrices. The method presented it self as specific for 3-NT with detection at 356 nm for all biological matrices. However, when detection 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.

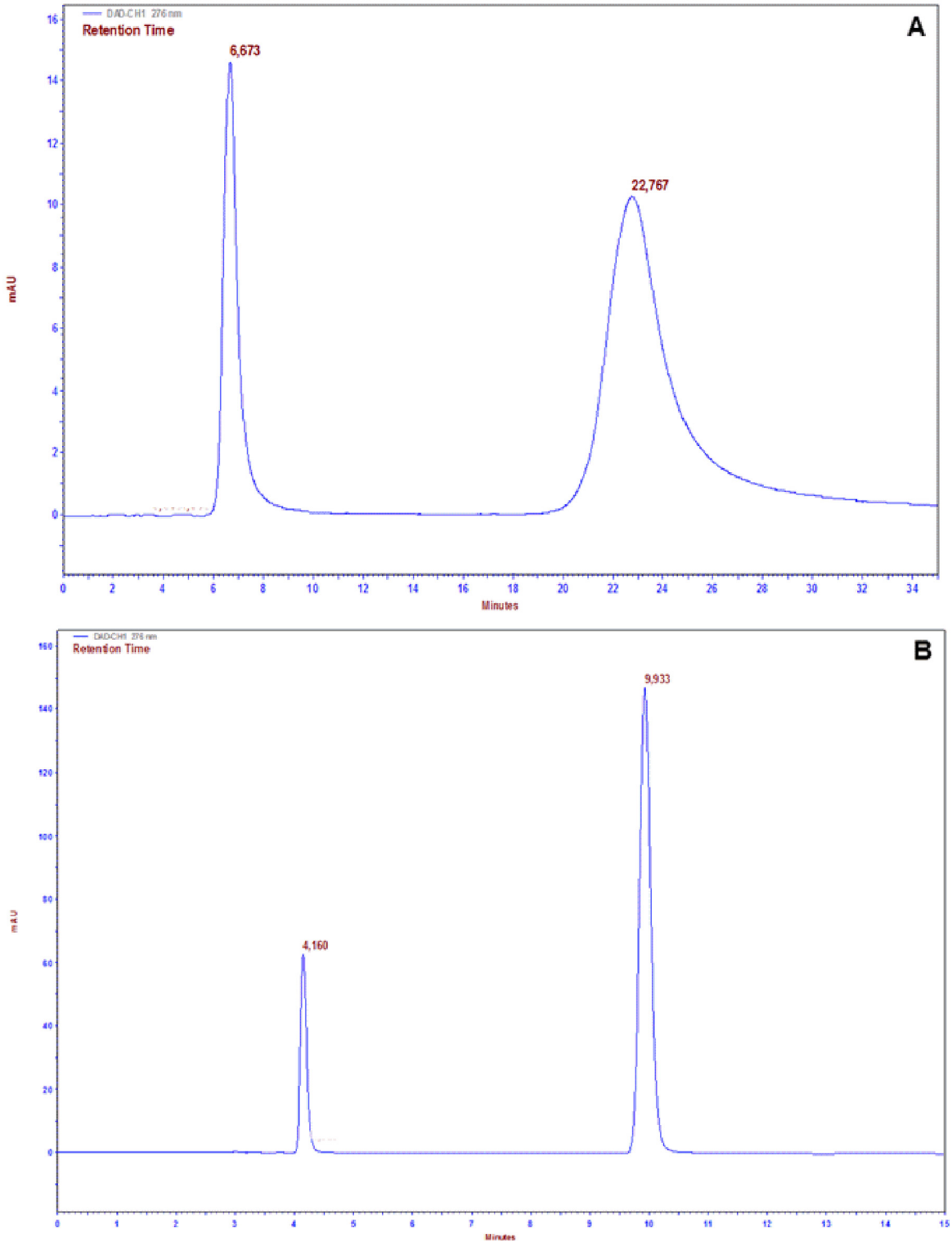

Fig. 1. Representative chromatograms of Tyr and 3-NT analysis obtained in assay B (0.5% CH_3COOH:MeOH:H_2O (30:0:70,v/v)) [A] and assay C (0.5% CH_3COOH:MeOH:H_2O (15:15:70,v/v)) [B].

Table 2
Linearity, retention time, LoD and LoQ.

	3-NT (λ = 215 nm)	3-NT (λ = 276 nm)	3-NT (λ = 356 nm)
LoD ($\mu g\,L^{-1}$)	0.721	0.367	1.862
LoQ ($\mu g\,L^{-1}$)	2.184	1.113	5.642
Retention Time (min ± sd)	10.164 ± 0.054	10.164 ± 0.053	10.165 ± 0.053
Correlation coefficient	1.000	1.000	0.999
Regression equation	$y = 7.08 \times 10^8 \times -7.48 \times 10^3$	$y = 2.51 \times 10^8 x + 2.57 \times 10^4$	$y = 1.20 \times 10^8 x + 1.08 \times 10^4$

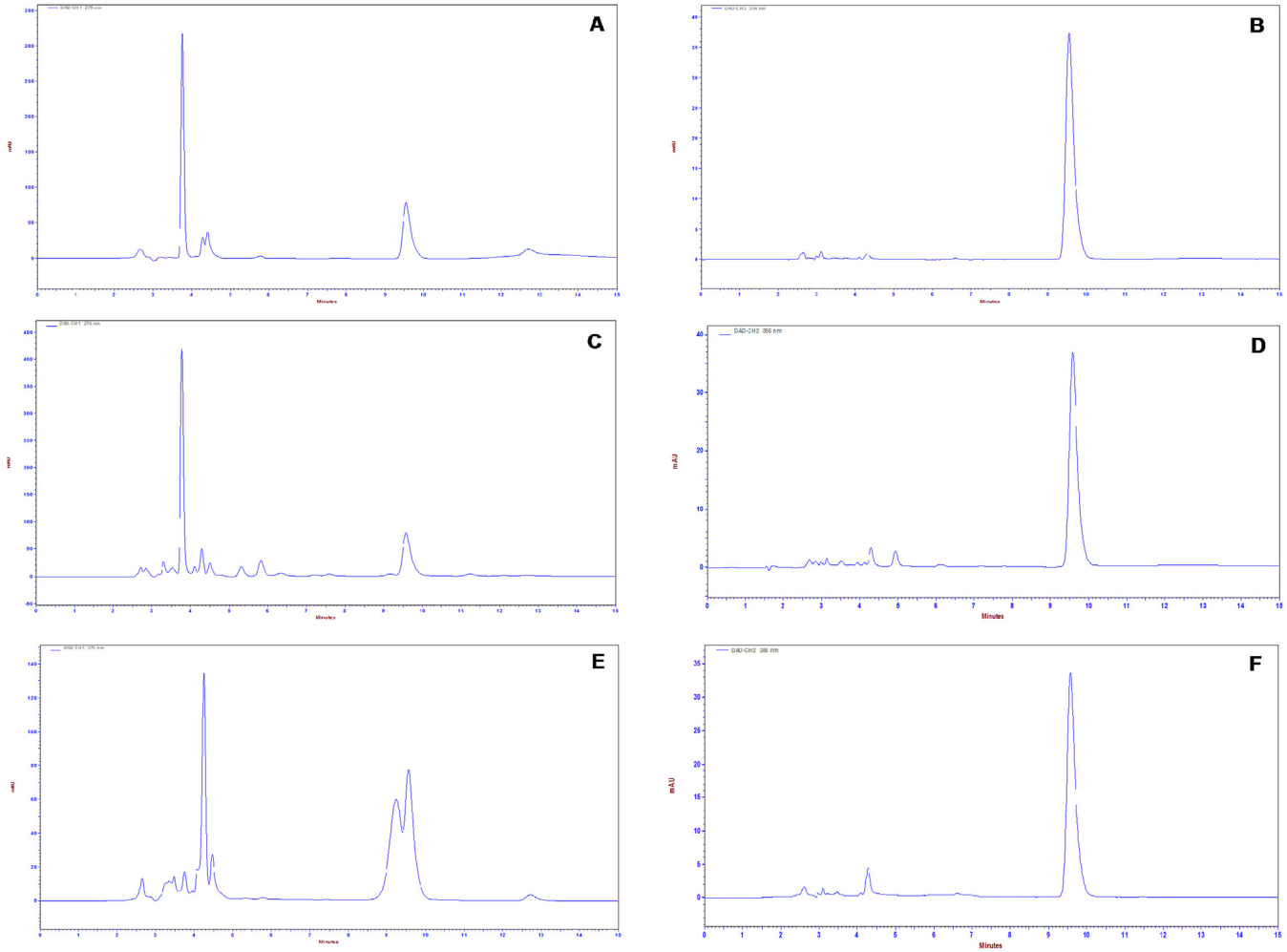

Fig. 2. Representative chromatogram of a 3-NT-spiked (25,000 μg L^{-1}) serum sample at 276 nm [A] and 356 nm [B]; urine sample at 276 nm [C] and 356 nm [D]; and whole blood at 276 nm [E] and 356 nm [F].

3.3.2. *Linearity, LoD and LoQ*

Table 2 lists the linearity parameters of the calibration curves for 3-NT (and Tyr). The LoD and LoQ are also given in Table 2.

3.3.3. *Accuracy – recovery rate*

Table 3 shows the results of accuracy determined from standard solutions. The inaccuracy varied from −6.26 to 0.007%. In most cases, 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 [38], which was also observed for this method.

Table 3 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 −9.08 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%) [38]. Concerning detection at 215 nm detection, inaccuracy was greater than 15% (79.62%), thereby not validated for this parameter.

3.3.4. *Precision*

Intra-day precision was assessed by testing standard solutions (50 000, 25 000, 10 000, 5000.0, 2500.0, 1250.0, 625.00, 312.50 μg L^{-1}) as well as by spiking different commercial human serum samples with three different 3-NT concentrations (25000, 1000.0, 50.000 μg L^{-1}). The precision of a bioanalytical method for each concentration level should be <15% of relative standard deviation (RSD) or coefficient of variation [38,39]. The precision of our method was 2.23% and 6.75% below the RSD for all standard solutions and serum samples, respectively (Table 3).

3.4. *Applicability of the developed method for 3-nitrotyrosine quantification*

Since one of the main goals of the present study was to apply the developed method to a myriad of biological samples, a wide variety of samples was tested, ranging from human-associated matrices to microbial suspensions.

A common approach for 3-NT quantification in biological specimens 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 [3,22,40]. This protocol, which was previously optimized by our group (unpublished data), 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.

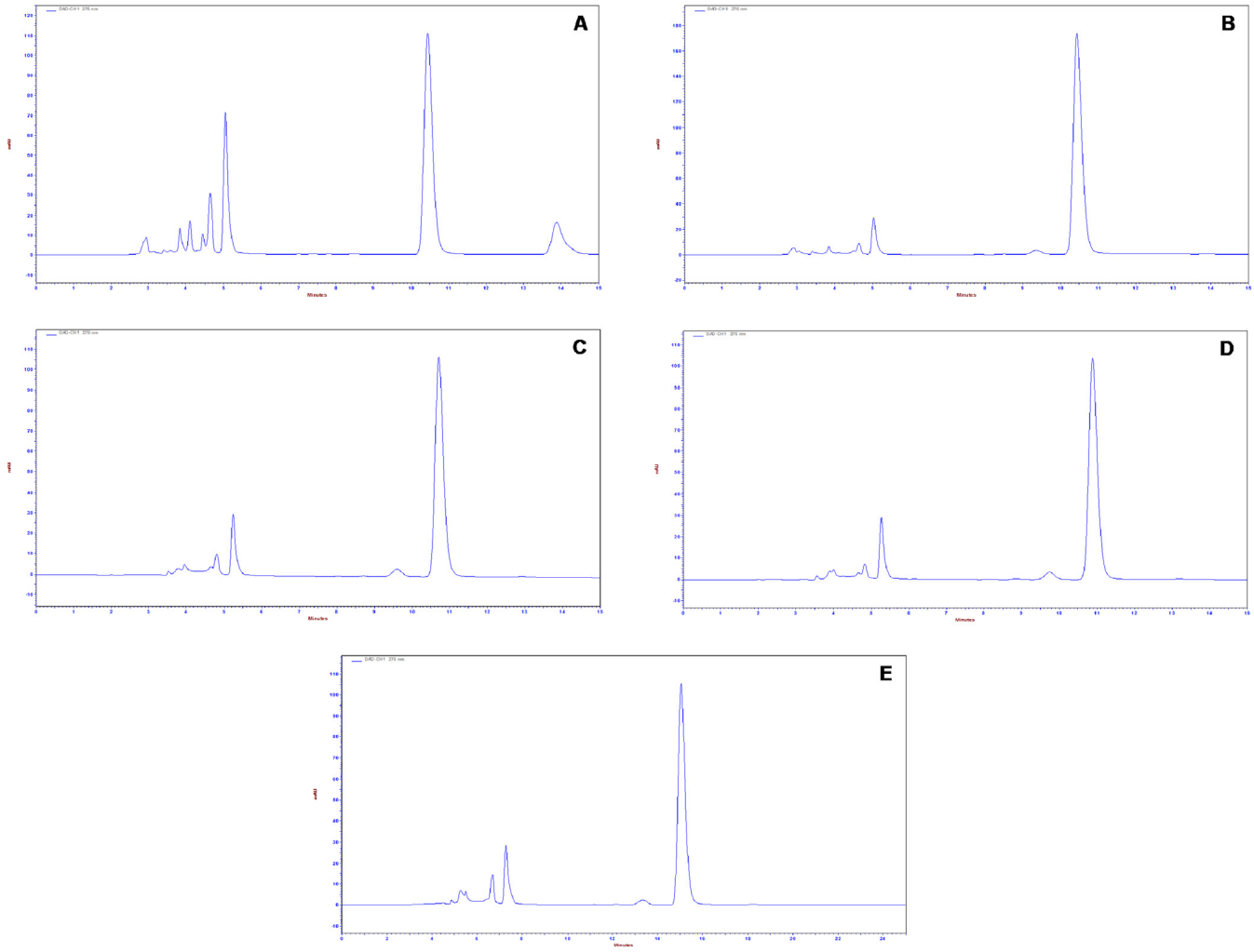

Fig. 3. Representative chromatogram of a 3-NT-spiked (25,000 μg L^{-1}) growth medium conditioned sample (276 nm) [A]; B16 F10 melanoma cell line sample (276 nm) [B]; *S.aureus* suspension (276 nm) [C]; *E.coli* suspension (276 nm) [D]; and *S.cerevisiae* suspension (276 nm) [E].

3.4.1. 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 (see Table 3 from Supporting information). Each sample was spiked with three different 3-NT concentrations and a quantitative analysis was performed. í

3.4.1.1. Serum. Serum is regarded as one of the most tested biological matrices in routine diagnosis [41]. 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. Moreover, it was possible to detect 3-NT at the three different wavelengths (215, 276 and 356 nm) in all serum samples. As previously stated, 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 94.78 ± 5.41% at 356 nm, 100.71 ± 11.33% at 276 nm, and 116.62 ± 54.56% at 215 nm. Fig. 2A and 2B exhibits a representative chromatogram of a serum sample.

3.4.1.1.1. Influence sample preparation protocol on recovery rates. The recovery rates using a random serum sample spiked with 25,000 μg L^{-1} of 3-NT were calculated in order to assess the degree of analyte loss when the acid hydrolysis protocol was applied. Overall, the recovery rates obtained for samples spiked before and after the protocol has been applied were 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 result.

3.4.1.2. Urine. Similarly, to that observed for serum, a reliable 3-NT quantification was also possible in urine samples (see Table 4 from Supporting information). Urine is a biological sample with special characteristics, particularly its acidic nature, which is associated with 3-NT artefact formation [42]. Nevertheless, Mergola, Scorrano, Del Sole, Lazzoi and Vasapollo [42] 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 results demonstrate that 215 nm is not a suitable wavelength for 3-NT quantification, since the respective extraction efficiency does not comply with the acceptable parameters for method validation. Regarding the precision, only quantification at 356 nm demonstrated compliable results.

Representative chromatograms of a 3-NT-spiked urine sample with detection at 276 and 356 nm are shown in Fig. 2C and D, respectively.

Table 3
Analytical results for accuracy test and repeatability (intraday test) from standard solutions and serum sample.

Standard solutions					
Wavelength (nm)	Concentration ($\mu g\,L^{-1}$)	RE[a] (%)	RSD (%)[b]		No. of samples
356	50,000	0.007	0.70		6
	25,000	−3.49	0.23		6
	10,000	0.51	0.76		6
	5000.0	−0.52	0.97		6
	2500.0	0.72	1.87		6
	1250.0	0.16	0.72		6
	625.00	−2.32	2.23		6
	312.50	−0.83	1.37		6
276	50,000	−0.08	1.26		6
	25,000	−3.05	0.16		6
	10,000	0.24	0.56		6
	5000.0	−0.82	0.54		6
	2500.0	0.16	0.62		6
	1250.0	0.30	0.88		6
	625.00	−2.79	1.14		6
	312.50	−3.82	2.03		6
215	50,000	0.12	0.87		6
	25,000	−3.33	0.76		6
	10,000	0.44	0.58		6
	5000.0	0.28	1.08		6
	2500.0	−0.03	0.54		6
	1250.0	1.88	1.38		6
	625.00	−3.31	1.36		6
	312.50	−6.26	0.72		6
Serum					
Wavelength (nm)	Spiked Concentration ($\mu g\,L^{-1}$)	Measured Concentration[a] ($\mu g\,L^{-1}$)	RE[b] (%)	RSD[c] (%)	No. of samples[d]
356	25,000	23067	−7.73	0.40	6
	1000.0	911	−8.93	2.15	6
	50.000	50	0.99	2.93	6
276	25,000	24520	−1.92	0.97	6
	1000.0	909	−9.08	1.15	6
	50.000	57	13.12	6.75	6
215	25,000	21360	−14.56	1.22	6
	1000.0	848	−15.20	3.60	6
	50.000	90	79.62	4.75	6

[a] Concentration was calculated as follows: Mean concentration (spiked sample) − Mean concentration (non-spiked sample); Mean concentration (non-spiked sample): 30 $\mu g\,L^{-1}$ at 356 nm; 42 $\mu g\,L^{-1}$ at 276 nm; 18 $\mu g\,L^{-1}$ at 215 nm.
[b] Relative error (RE) is derived by using equation: Relative error% = ([Mean cal. conc.] − [True conc.])/[True conc.] × 100%.
[c] Relative standard deviation is derived by using equation: RDS% = (standard deviation)/(mean peak area) × 100%.
[d] Each sample performed in triplicated.

3.4.1.3. Whole blood. Besides serum and urine, this study has also shown that 3-NT quantification is possible in a biological matrix like whole blood (see Table 4 from Supporting information). 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 (Fig. 2E and F). Nevertheless, and as previously reported by Herce-Pagliai, Kotecha and Shuker [43], 356 nm is the most specific wavelength for 3-NT detection. On the other hand, Tyr quantification in whole blood was not possible, since an impure chromatographic peak was observed in most cases. Regarding whole blood, the recovery rates for 3-NT at 356 nm were 88.82 ± 7.47% for 25000 $\mu g\,L^{-1}$, 87.19 ± 2.08% for 1000 $\mu g\,L^{-1}$, and 64.43 ± 0.87% for 50 $\mu g\,L^{-1}$. Concerning the latter concentration, the recovery rates were not within the accuracy limits.

3.4.2. Other biological matrices

3.4.2.1. 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 [44,45]. On the other hand, a significant association between iNOS expression and 3-NT production has been found in melanoma patients, using immunohistochemistry detection [44,46]. Therefore, the applicability of the developed method in these type of cell lines was also evaluated.

In this study, B16 F10 melanoma cell line (Fig. 3B) was tested, since it is widely used in a variety of studies, and is also associated with high metastatic activity [47]. This cell line (at a concentration of ~10^4 cells mL^{-1}), as well as the medium used for cell growth (Fig. 3A), were spiked with the same 3-NT concentration (25,000 $\mu g\,L^{-1}$).

Since human cells are difficult to disrupt, four different sonication-based protocols for cell lysis and acid hydrolysis were applied to B16 F-10 melanoma cell line. Cellular lysis through sonication is an effective way for the recovery of periplasmic, membrane-bound [48]. Overall, protocols B1 and B2 provided better results, although protocol B2 (sonication with ultrasound bath followed by addition of 15% TFA) was superior (data not shown). The differences between the results originated by the two sonication systems was likely to be due to the frequency used.

3.4.2.2. Bacterial and yeast suspensions. Besides human biological matrices, the developed protocol was also tested for 3-NT quantification in bacterial and yeast suspensions, and revealed to be also successful. The same lysis 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 in order to achieve bacterial cells lysis [49,50]. Furthermore, bacterial and yeast suspensions were prepared with ultrapure water, allowing cell wall disruption. Fig. 3C–E shows representative chromatograms of 3-NT-spiked bacterial and yeast suspensions. These results also

demonstrated that the developed method may also be applied to different fields other than human biology, for instance in microbial and biotechnological research.

4. Conclusion

After a thorough analysis of the results obtained with all the mobile phases and the operating conditions tested, the best results were obtained using 0.5% CH_3COOH:MeOH (15:15:70 (v/v)), at 25 °C, flow rate of 1 mL min^{-1}, 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 $\mu g L^{-1}$, and a reduced analysis time *per* sample (15 min). 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. Additionally, the same method, with detection at 356 nm, also allowed the successful detection and quantification of 3-NT in a wide variety of biological matrices. Therefore, 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 and allowing the effective quantification of 3-NT.

Conflicts of interest

The author declares no conflict of interest.

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