



Cite this: *RSC Adv.*, 2022, 12, 4536

# A highly selective and sensitive spectrofluorimetric method for the assessment of 3-nitrotyrosine in serum using (Eu(TTA)<sub>3</sub>Phen) photo probe

Mohmed S. Attia,<sup>1</sup> Ahmed O. Youssef,<sup>1</sup> Nour A. Abdel-Sattar,<sup>1</sup> Mohammed A. Amin,<sup>2</sup> Sarah Alharthi,<sup>2</sup> Ekram H. Mohamed,<sup>3</sup> Safwat A. Mahmoud<sup>4</sup> and Mona N. Abou-Omar<sup>5</sup>

Received 2nd October 2021  
 Accepted 22nd January 2022

DOI: 10.1039/d1ra07351f

[rsc.li/rsc-advances](http://rsc.li/rsc-advances)

A simple, accurate and fast method was developed for the assessment of 3-nitrotyrosine as a biomarker for the early diagnosis of liver cirrhosis with minimal hepatic encephalopathy (MHE) using a (Eu(TTA)<sub>3</sub>Phen) photo probe. 3-Nitrotyrosine can remarkably quench the luminescence intensity of the (Eu(TTA)<sub>3</sub>Phen) complex in DMSO at pH = 9 and  $\lambda_{em} = 617$  nm. The quenching of the luminescence intensity of (Eu(TTA)<sub>3</sub>Phen) complex particularly the electrical emission band at  $\lambda_{em} = 617$  nm is used for the assessment of 3-nitrotyrosine in different serum samples of patients with liver cirrhosis.

## 1. Introduction

3-Nitrotyrosine has an IUPAC name ((2*S*)-2-amino-3-(4-hydroxy-nitrophenyl)propanoic acid), Fig. 1a. Hepatic encephalopathy is a brain dysfunction caused by acute or chronic liver disease. It is of two types: overt and covert type. The overt type is characterized by bedside characteristic clinical features and does not need sophisticated investigations for diagnosis.<sup>1</sup> Covert or minimal hepatic encephalopathy (MHE) is characterized by an examination of normal mental and neurological status. It can be diagnosed by sophisticated psychometric tests, *e.g.*, paper-and-pencil psychometric tests, inhibitory control test, critical flicker frequency and the stroop smartphone application.<sup>2,3</sup> Up to 80% of patients with cirrhosis have MHE. Its presence is associated with poor quality of life, inability to drive, traffic violation and accidents. Within 3 years, ~50% may develop overt hepatic encephalopathy.<sup>1,4</sup> MHE is commonly found with advanced liver disease, history of overt hepatic encephalopathy, esophageal varices and alcohol abuse as etiology of liver cirrhosis.<sup>5</sup>

It is probable that MHE is a marker of advanced liver failure because it is associated with a shorter survival time, particularly among patients with high concentrations of ammonia after oral glutamine load.<sup>6,7</sup> For this reason, MHE has been proposed as

an indication for liver transplantation. Currently, the “gold standard” for the diagnosis of MHE is the psychometric hepatic encephalopathy score (PHES).<sup>8</sup> However, “gold standard” method is time consuming and needs adjusting for time. Therefore, MHE is not routinely diagnosed in most clinical settings because of lack of simple procedures, and most patients with MHE remain undiagnosed and untreated. Hence,

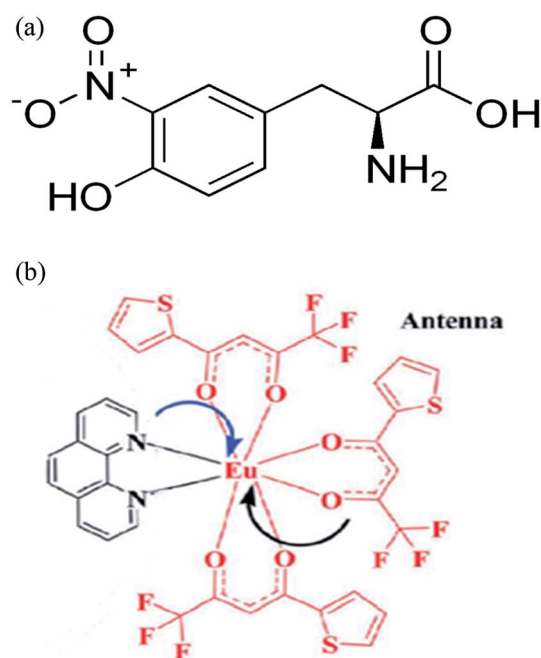


Fig. 1 (a) Structure of 3-nitrotyrosine. (b) Structure of Eu(TTA)<sub>3</sub>phen. (TTA = thenoyl trifluoro acetone, phen = 1,10-phenanthroline).

<sup>1</sup>Chemistry Department, Faculty of Science, Ain Shams University, Cairo 11566, Egypt. E-mail: Mohamed\_sam@yahoo.com

<sup>2</sup>Department of Chemistry, College of Science, Taif University, P.O. Box 11099, Taif 21944, Saudi Arabia

<sup>3</sup>Pharmaceutical Chemistry Department, Faculty of Pharmacy, The British University in Egypt, El Sherouk City, Cairo, Egypt

<sup>4</sup>Physics Department, Faculty of Science, Northern Border University, Arar, Saudi Arabia. E-mail: samahmoud2002@yahoo.com

<sup>5</sup>Department of Chemistry, Faculty of Women for Arts, Science and Education, Ain Shams University, Cairo, Egypt. E-mail: mona.na66@yahoo.com



there is a need for a simple diagnostic test that can be performed routinely in the laboratory to detect MHE in patients with liver cirrhosis. It would be useful to have some peripheral biomarkers that could be measured in blood samples and reflect the presence of MHE in cirrhotic patients in clinical practice. In the last decade, 3-nitrotyrosine has been used as a marker for MHE.<sup>7</sup> There are many different methods for the determination of 3-nitrotyrosine such as, gas chromatography-mass spectrometry (GC-MS)<sup>9–12</sup> or LC-MS<sup>13–18</sup> analysis or on affinity approaches (immunoassays).<sup>19,20</sup> For a non-derivative analysis, either LC-MS or affinity approaches can be used. However, both techniques have their limitations as well. Most LC-MS methods have an additional step of purifying obtained extracts using a solid-phase extraction (SPE) technique,<sup>13–16</sup> hence increasing costs and time required for sample preparation. Regarding immunoassays, the biggest concern is their insufficient selectivity and specificity due to cross-reactivity with 3-nitrotyrosine and other structurally similar metabolites,<sup>21,22</sup> but the immunoassay sensitivity is not optimal either.<sup>23</sup> These shortcomings make it difficult to compare the immunoassay results obtained in different studies.<sup>23</sup> In this study, 3-nitrotyrosine was determined by the quenching of the luminescence intensity of the Eu(TTA)<sub>3</sub>Phen photo probe at  $\lambda_{em} = 617$  nm in the presence of different concentrations of 3-nitrotyrosine in the blood serum samples of patients with MHE.

## 2. Experimental

### 2.1. Apparatus

The absorption spectra were recorded on a double beam PerkinElmer Lambda 25 UV-visible spectrophotometer fitted with a tungsten halogen lamp for operation in the visible range and a deuterium lamp for operation in the UV range. All luminescence measurements were recorded on a Meslo-PN (222-263000) Thermo Scientific Lumina fluorescence spectrometer in the range of 190–900 nm. The pH was measured using a pHs-JAN-WAY 3040 research pH meter. The separation of serum samples was carried out by centrifuging the sample for 15 min at 4000 rpm.

### 2.2. Materials

Pure standards, 3-nitrotyrosine, phenylalanine, valine, tyrosine, tryptophan, citrulline, albumin, glucose, urea and methionine were purchased from Sigma Aldrich. Human serum samples were collected from two hospitals, namely Al-Kasr-EL-Aini new teaching hospital (Cairo, Egypt) and Ain Shams Specialized Hospital (Cairo, Egypt). Sample collection was performed in accordance with WHO (World Health Organization) approved protocol for human specimen collection. All experiments were performed in accordance with the Guidelines “Ministry of Health and Population, Egypt” and approved by the ethics committee at “Ain Shams” university. Informed consents were obtained from the human participants of this study.

### 2.3. Preparation of standard solutions

A stock solution of 3-nitrotyrosine ( $1.0 \times 10^{-3}$  mol L<sup>-1</sup>) was freshly prepared by dissolving 5.60 mg in 25 mL pure DMSO. A

more diluted solution ( $1.0 \times 10^{-5}$ ,  $1.0 \times 10^{-7}$  mol L<sup>-1</sup>) was prepared by appropriate dilution with DMSO. Stock and working solutions are stored at 4 °C when not in use. An Eu<sup>3+</sup> ion stock solution ( $1.0 \times 10^{-4}$  mol L<sup>-1</sup>) was prepared by dissolving 2.47 mg Eu(TTA)<sub>3</sub>Phen in a small amount of deionized water and the volume was completed to the mark in a 25.0 mL measuring flask. The pH was adjusted to 8.24 using a phosphate buffer solution. The above-mentioned working solutions were used for the subsequent measurements of absorption spectra, emission spectra, effect of pH and solvents. The fluorescence intensity was measured at  $\lambda_{ex}/\lambda_{em} = 375/617$  nm.

### 2.4. Calibration curve

After the preparation of the solution of 3-nitrotyrosine in DMSO as described above, 1.0 mL of the photo probe Eu(TTA)<sub>3</sub>Phen was put in the cell and different concentrations of 3-nitrotyrosine were added to the cell of the spectrofluorimetric device, followed by recording the luminescence spectra at the selected excitation wavelength  $\lambda_{em} = 617$  nm.

### 2.5. Proposed method

The blood was allowed to clot by leaving it 20 min undisturbed at room temperature. The clot was removed by centrifugation at 4000 rpm for 15 min and then the supernatant was decanted, which was designated as serum. The serum was transferred into a clean tube and then kept at –20 °C. Furthermore, 100  $\mu$ L of the serum was added to 1.00 mL of Eu<sup>3+</sup> stock solution ( $1.0 \times 10^{-5}$  mol L<sup>-1</sup>) in DMSO and the pH was adjusted to 8.24 in the cell of the spectrofluorimetric device. The luminescence intensity of the Eu<sup>3+</sup> photo probe was measured before and after the addition of the serum solution. The change in the luminescence intensity was used for the determination of 3-nitrotyrosine in the serum sample.

### 2.6. Standard method

**2.6.1 Assay principle.** A 3-nitrotyrosine quantitation kit used was a competitive ELISA. The unknown protein nitrotyrosine sample or nitrated BSA standards were first added to a nitrated BSA preabsorbed EIA plate. After a brief incubation, an anti-nitrotyrosine antibody was added, followed by an HRP conjugated secondary antibody. The protein nitrotyrosine content in an unknown sample was determined by comparing with a standard curve that was prepared from predetermined nitrated BSA standards.

**2.6.2 Assay protocol.** All reagents were prepared and mixed thoroughly before use. Each protein sample including nitrated BSA and blank were assayed in duplicate. 50.0  $\mu$ L of the unknown protein sample or nitrated BSA standard were added to the wells of the EIA plate. The samples were incubated at room temperature for 10 min on an orbital shaker. 50  $\mu$ L of the diluted anti-nitrotyrosine antibody was added to each well and incubated at room temperature for 1.0 hours on an orbital shaker. Micro well strips were washed 3 times with 250  $\mu$ L Wash Buffer per well with thorough aspiration between each wash. After the last wash, the wells were emptied and tapped micro well strips on an absorbent pad or paper towel to remove excess



Wash Buffer. 100  $\mu\text{L}$  of the diluted secondary antibody–enzyme conjugate was added to all the wells. The wells were incubated at room temperature for 1 h on an orbital shaker. Micro well strips were washed 3 times according to step 4 above, and we proceeded immediately to the next step. The substrate solution should be warmed to 30  $^{\circ}\text{C}$ . 100  $\mu\text{L}$  of the substrate solution was added to each well, including the blank wells. The wells were incubated at room temperature on an orbital shaker. Actual incubation time varied from 2.0–30 min. The enzyme reaction was stopped by adding 100  $\mu\text{L}$  of the Stop Solution into each well, including the blank wells. Results were read immediately (color will fade over time). The absorbance of each micro well was read on a spectrophotometer using 450 nm as the primary wavelength.

## 3. Results and discussion

### 3.1. Absorption and emission spectra

The absorption spectra of  $\text{Eu}(\text{TTA})_3\text{Phen}$  complex, 3-nitrotyrosine and 3-nitrotyrosine in the presence of the photo probe are shown in Fig. 2. The absorption spectrum of ( $1.0 \times 10^{-5} \text{ mol L}^{-1}$ ) [ $\text{Eu}(\text{TTA})_3\text{Phen}$ ] shows ( $\pi \rightarrow \pi^*$ ) transition band at 345 nm. Upon the addition of ( $1.0 \times 10^{-5} \text{ mol L}^{-1}$ ) of 3-nitrotyrosine, no shift was observed in the band. This indicates that no complexation between the photo probe and 3-nitrotyrosine in the ground state occurred. The characteristic peaks of  $\text{Eu}^{3+}$  ion appear at ( $^5\text{D}_0 \rightarrow ^7\text{F}_0 = 580 \text{ nm}$ ,  $^5\text{D}_0 \rightarrow ^7\text{F}_1 = 590 \text{ nm}$ ,  $^5\text{D}_0 \rightarrow ^7\text{F}_2 = 617 \text{ nm}$ ,  $^5\text{D}_0 \rightarrow ^7\text{F}_3 = 650 \text{ nm}$ ,  $^5\text{D}_0 \rightarrow ^7\text{F}_4 = 690 \text{ nm}$  and  $^5\text{D}_0 \rightarrow ^7\text{F}_5 = 710$ ), as shown in Fig. 3.<sup>24–29</sup> The effect of the solvent on the luminescence intensity of the photo probe [ $\text{Eu}(\text{TTA})_3\text{Phen}$ ] was studied under the conditions optimized above. The high luminescence intensity of the photo probe was observed in aprotic solvents such as DMSO and DMF than in protic solvents such as ethanol. This could be attributed to the efficient quenching of the excited state of the photo probe by interactions with high-energy vibration oscillators such as O–H groups, as shown in Fig. 3.<sup>30–36</sup> The pH of the medium has a great effect on the luminescence intensity of the [ $\text{Eu}(\text{TTA})_3\text{Phen}$ ] complex. The pH meter has been adjusted using the phosphate buffer solution ( $\text{Na}_2\text{HPO}_4$ ). The pH has been

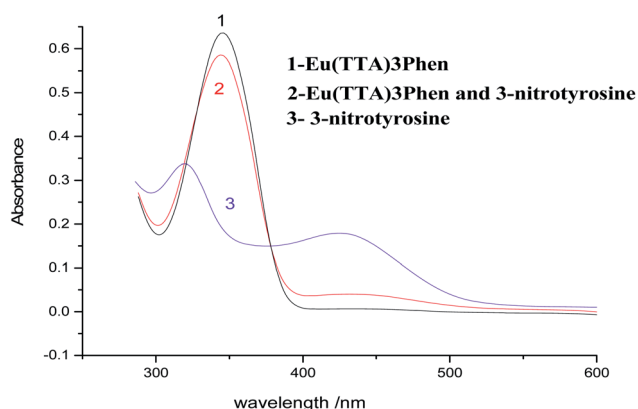


Fig. 2 Absorption spectra of ( $1.0 \times 10^{-5} \text{ mol L}^{-1}$ ) photo probe and 3-nitrotyrosine.

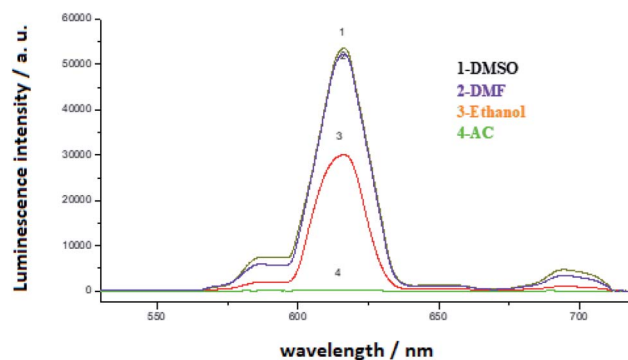


Fig. 3 Luminescence emission spectra of ( $1.0 \times 10^{-5} \text{ mol L}^{-1}$ )  $\text{Eu}(\text{TTA})_3\text{phen}$  in different solvents.

adjusted using  $\text{NH}_4\text{OH}$  and  $\text{HCl}$  solutions. The optimum pH value where the peak at  $\lambda_{\text{em}} = 617 \text{ nm}$  has the highest intensity was obtained at  $\text{pH} = 8.24$ , as presented in Fig. 4. The validity and selectivity photo probe  $\text{Eu}(\text{TTA})_3\text{Phen}$  to 3-nitrotyrosine was tested by studying the influence of a series of interfering species such as,  $\text{NaCl}$ ,  $\text{KCl}$  ( $2.0 \times 10^{-3} \text{ mol L}^{-1}$ ), albumin ( $0.7 \text{ g L}^{-1}$ ), uric acid ( $0.08 \text{ g L}^{-1}$ ), urea ( $0.06 \text{ g L}^{-1}$ ), total protein ( $0.01 \text{ g L}^{-1}$ ), ( $0.06 \text{ g L}^{-1}$ ) triglyceride, glucose ( $0.08 \text{ g L}^{-1}$ ) and ( $1.0 \times 10^{-4} \text{ mol L}^{-1}$ ) of each amino acids such as, tyrosine, tryptophan, phenylalanine, valine, citrulline and methionine on the luminescence spectrum of  $\text{Eu}(\text{TTA})_3\text{Phen}$  in the presence of ( $1.0 \times 10^{-5} \text{ mol L}^{-1}$ ) 3-nitrotyrosine. The tolerable limit was defined as the concentration of the individually added species causing a deviation less than 5.0% of the luminescence intensity of the  $\text{Eu}(\text{TTA})_3\text{Phen}$  photo probe at the optimum conditions. The results indicated no significant change in the luminescence intensity of the  $\text{Eu}(\text{TTA})_3\text{Phen}$  photo probe, as shown in Fig. 5. The luminescence emission spectrum of the photo probe exhibits a strong luminescence intensity band at 617 nm with a broad and structureless shape. The emission spectra of [ $\text{Eu}(\text{TTA})_3\text{Phen}$ ] complex in different concentrations of 3-nitrotyrosine is shown in Fig. 6. After the addition of different concentrations of 3-nitrotyrosine to [ $\text{Eu}(\text{TTA})_3\text{Phen}$ ] in DMSO, the intensity of the characteristic peak at  $\lambda_{\text{em}} = 617 \text{ nm}$  of  $\text{Eu}^{3+}$  was quenched, which indicates that 3-nitrotyrosine is a good

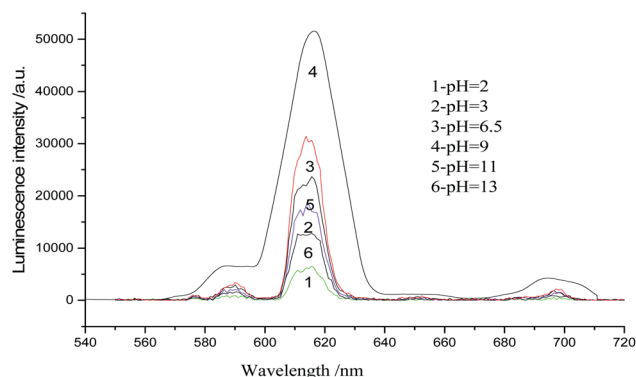


Fig. 4 Luminescence emission spectra of ( $1.0 \times 10^{-5} \text{ mol L}^{-1}$ )  $\text{Eu}(\text{TTA})_3\text{phen}$  in DMSO at different pH values.



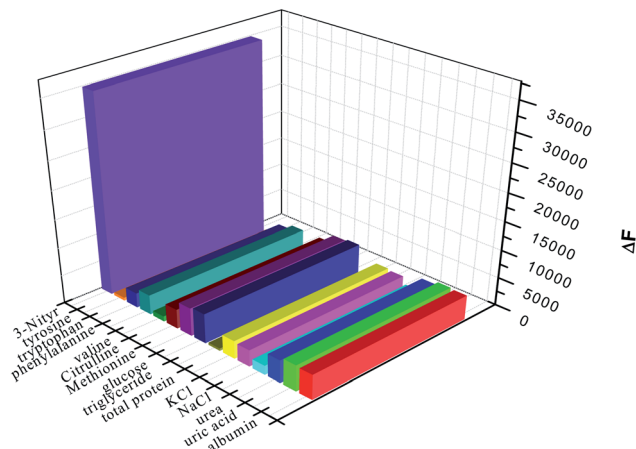


Fig. 5 Effect of foreign species on the selectivity of the photo probe  $\text{Eu}(\text{TTA})_3\text{Phen}$  to 3-nitrotyrosine.

quencher of the luminescent  $\text{Eu}^{3+}$  complex. A correlation between the emission intensity of the photo probe  $[\text{Eu}(\text{TTA})_3\text{Phen}]$  complex at  $\lambda_{\text{em}} 617 \text{ nm}$  and 3-nitrotyrosin was obtained by applying the Stern–Völmer plot in with  $(F_0/F - 1)$  versus  $[\text{3-nitrotyrosine}]$ ,<sup>37</sup> where,  $\kappa_{\text{sv}}$  is the Stern–Völmer constant, which is determined from the slope of the fitted data and equals to  $0.0148 \text{ L mol}^{-1}$ . One over  $\kappa_{\text{sv}}$  equals to  $C_{1/2}$  (half quenching concentration),  $C_{1/2} = 1/\kappa_{\text{sv}} = 67.56 \text{ mol L}^{-1}$ .  $R_0$  is the critical transfer distance, which is the average distance between the donor and the acceptor at which the probability of fluorescence quenching is just equal to 0.5 excited state,  $R_0 = 7.35/(C_{1/2})^{1/3} = 1.81 \text{ \AA}$ , indicating the electron transfer mechanism of quenching (Fig. 7). A linear correlation was found between  $(F_0/F - 1)$  at  $\lambda_{\text{em}} = 617 \text{ nm}$ , and the concentration of 3-nitrotyrosine in the ranges given in Table 1 and the regression parameters are summarized in Table 1. The limit of detection (LOD) and quantitation (LOQ) were also calculated according to ICH guidelines<sup>38–42</sup> and presented in Table 1.

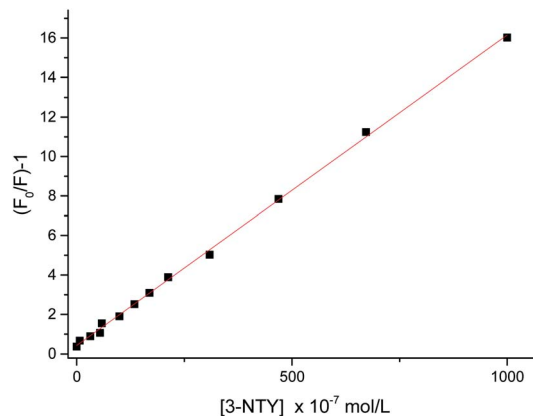


Fig. 7 Linear relationship between  $(F_0/F) - 1$  and the concentration of 3-nitrotyrosine  $\lambda_{\text{em}} = 617 \text{ nm}$ .

Table 1 Sensitivity and regression parameters for the photo probe

Parameter	Values
$\lambda_{\text{em}} \text{ nm}$	617
Linear range, $\text{mol L}^{-1}$	$2.3 \times 10^{-4}$ to $1.0 \times 10^{-9}$
Limit of detection (LOD), $\text{mol L}^{-1}$	$2.6 \times 10^{-10}$
Limit of quantification (LOQ), $\text{mol L}^{-1}$	$7.8 \times 10^{-10}$
Intercept ( $a$ )	$0.626 \pm 0.071$
Slope ( $b$ ) $\times 10^{-7}$	$0.0152 \pm 1.84742 \times 10^{-4}$
Standard deviation	0.431
Variance ( $\text{sa}^2$ )	0.186
Regression coefficient ( $r^2$ )	0.998

## 4. Accuracy and precision

To compute the accuracy and precision, the assays described under general procedures were repeated three times within a day to determine the repeatability (intraday precision) and three times on different days to determine the intermediate precision (inter day precision) of the method. These assays were performed for three levels of the analyte. The results of this study are summarized in Table 2. The percentage relative standard deviation (% RSD) values were  $\leq 0.056$ – $0.1\%$  (intraday) and  $\leq 0.054$ – $0.097\%$  (inter day) for patients. The inter day values indicated high precision of the method. Accuracy was evaluated as percentage relative error (RE) between the measured mean concentrations and the taken concentrations of 3-nitrotyrosine. Bias {bias%} was calculated at each concentration, as presented in Table 2. Percent relative error (% RE) values of  $\leq 0.29$ – $4.60$  (intraday) and  $0.18$ – $2.80\%$  (inter day) for the patient states demonstrate the high accuracy of the proposed method. The obtained results are summarized in Table 2. The proposed spectrofluorimetric method was applied for the determination of 3-nitrotyrosine in 5 serum samples of healthy volunteers and 5 serum samples of patients with MHE. The results obtained are summarized in Table 2. There was good agreement between the average values obtained by the developed procedure ( $8.534 \pm 0.0089$ ) and the standard spectrophotometric method ( $8.57 \pm 0.004$ ) without recording any significant differences between

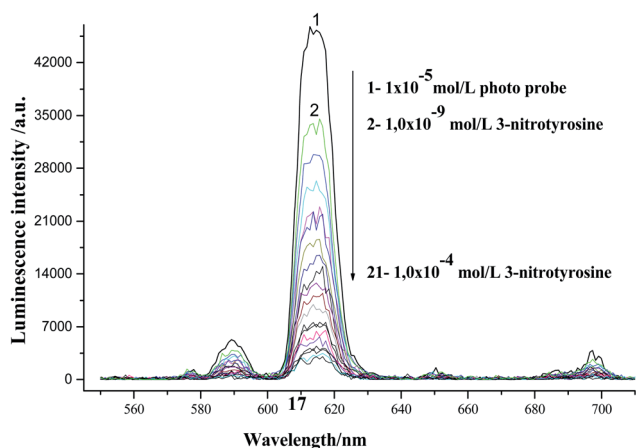


Fig. 6 Luminescence emission spectra of the  $[\text{Eu}(\text{TTA})_3\text{Phen}]$  complex in the presence of different concentrations of 3-nitrotyrosine in DMSO at  $\lambda_{\text{ex}} = 375 \text{ nm}$  and  $\text{pH} = 9.0$ .



Table 2 Evaluation of intra-day and inter-day accuracy and precision

Sample	Standard method average nM	Propose method					
		Intra-day accuracy and precision ( $n = 3$ )			Inter-day accuracy and precision ( $n = 3$ )		
		Average found nM <sup>a</sup> ± CL	% RE	% RSD	Average found nM <sup>a</sup> ± CL	% RE	% RSD
Health (1)	8.3	8.39 ± 0.89	1.08	0.072	8.3 ± 0.90	1.33	0.073
Health (2)	7.9	7.84 ± 0.92	0.76	0.077	7.9 ± 0.92	0.25	0.076
Health (3)	7.5	7.54 ± 0.94	0.53	0.08	7.5 ± 0.96	2.80	0.082
Health (4)	9.1	9.24 ± 0.85	1.54	0.065	9.1 ± 0.86	1.21	0.067
Health (5)	6.8	6.82 ± 0.99	0.29	0.088	6.8 ± 0.98	2.06	0.086
Patient (1)	177.8	177.66 ± 0.93	-0.07	1.78	178.8 ± 0.94	0.56	1.79
Patient (2)	109.9	109.74 ± 0.79	-0.14	1.56	110.9 ± 0.78	0.90	1.55
Patient (3)	206.3	206.01 ± 0.105	-0.14	1.1	207.3 ± 0.104	0.48	1.97
Patient (4)	111.1	111.8 ± 0.79	0.63	1.56	112.0 ± 0.77	0.81	1.54
Patient (5)	170.1	170.9 ± 0.80	0.47	1.05	171.1 ± 0.80	0.58	1.58

<sup>a</sup> CL, confidence limits:  $CL = \pm tS/(n)^{1/2}$ , the tabulated value of  $t$  is 4.303, at the 95% confidence level.  $S$ , standard deviation.  $N$ , number of measurements. % RE, percent relative error. % RE =  $[(\text{concentration proposed} - \text{concentration known})/(\text{concentration known})] \times 100$ . % RSD, relative standard deviation. % RSD =  $[S/(\text{average measurements})] \times 100$ .

Table 3 Comparison of the spectrofluorimetric technique with some existing methods for the determination of 3-nitrotyrosine

Method	Detection limit	Reference
(GC/MS)	(1.73) nM	25
Isotope dilution liquid, chromatography-electro spray, ionization tandem, mass spectrometry	(1.83) nM	26
HPLC-UV method, combined with pre-column, cloud point extraction (CPE)	(5–15) nM	27
GC tandem MS	2.677 ± 1.540 nM	28
GC-MS	4.46 ± 4.49 nM	29
HPLC-fluorescence detector, 470 nm	4.4 ± 1.8 nM	30
ELISA based on the sandwich	573.54 ± 142.86 nM	31
Photo probe method, 3-nitrotyrosine-Eu(TTA) <sub>3</sub> Phen	1.6 ± 0.19 nM	The present work

the two methods for samples of healthy volunteers. Furthermore, there was a good agreement between the average values obtained by the developed procedure ( $158.5 \pm 0.89$ ) and the standard spectrophotometric method ( $156.4 \pm 0.4$ ) in case of the serum samples of patients with MHE. The average recovery and R.S.D for serum samples in our method were found to be ( $100.2 \pm 1.43\%$ ). The data obtained for the average recovery by the British Pharmacopoeia method were (99.99% and 99.75%) for healthy and patient samples, respectively. The RSD values for healthy and patient samples were 0.07% and 0.12%, respectively. These data have been shown for comparison and show good correlation with those obtained with the proposed method. The results obtained by the proposed method are agreed with the results of the reference method. The comparison of the proposed photo probe for the determination of 3-nitrotyrosine with other published methods<sup>43–49</sup> is summarized in Table 3. It was revealed that the developed method exhibited good stability, lower limit of detection ( $8.6 \times 10^{-10}$  mol L<sup>-1</sup>) and wide linear range ( $2.3 \times 10^{-4}$  to  $1.0 \times 10^{-9}$  mol L<sup>-1</sup>).

## 5. Conclusion

3-Nitrotyrosine has been determined by the quenching of the electric band of the [Eu(TTA)<sub>3</sub>Phen] complex at 617 nm. This method is very accurate and more sensitive compared with ELISA. The electron transfer mechanism of the quenching has

been identified from the Stern–Volmer plot. This method has been validated for health and patient sample of MHE.

## Conflicts of interest

There are no conflicts to declare.

## Acknowledgements

Authors extend their appreciation to the Deputyship for Research & Innovation, Ministry of Education in Saudi Arabia for funding this research work through the project number "IF\_2020\_NBU\_204". Authors thanks Taif University Researchers Supporting Project (TURSP-2020/200), Taif University, Taif, Saudi Arabia, for supporting this work.

## References

- H. Vilstrup, P. Amodio, J. Bajaj, J. Cordoba, P. Ferenci, K. D. Mullen, *et al.*, Hepatic encephalopathy in chronic liver disease: 2014 Practice Guideline by the American Association for the Study Of Liver Diseases and the European Association for the Study of the Liver, *Hepatology*, 2014, **60**, 715–735.
- K. Weissenborn, Diagnosis of minimal hepatic encephalopathy, *J. Clin. Exp. Hepatol.*, 2015, **5**, S54–S59.



- 3 S. L. Flamm, Covert hepatic encephalopathy, *Clin. Liver Dis.*, 2015, **19**, 473–485.
- 4 S. Agrawal, S. Umapathy and R. K. Dhiman, Minimal hepatic encephalopathy impairs quality of life, *J. Clin. Exp. Hepatol.*, 2015, **5**, S42.
- 5 M. Groeneweg, W. Moerland, J. C. Quero, W. C. Hop, P. F. Krabbe and S. W. Schalm, Screening of subclinical hepatic encephalopathy, *J. Hepatol.*, 2000, **32**, 748–753.
- 6 H. Ischiropoulos, L. Zhu, J. Chen, M. Tsai, J. Martin, C. Smith and J. S. Beckman, Peroxynitrite-mediated tyrosine nitration catalyzed by superoxide dismutase, *Arch. Biochem. Biophys.*, 1992, **298**, 431–437.
- 7 M. Romero-Goomez, L. Grande, I. Camacho, S. Benitez and J. Irlles, Altered response to oral glutamine challenge as prognostic factor for overt episodes in patients with minimal hepatic encephalopathy, *J. Hepatol.*, 2002, **37**, 781–787.
- 8 M. Attia and N. Al-Radadi, Nano photo probe binuclear Pt-2 pyrazinecarboxylic acid–bipyridine for enhancement of the efficiency of 3-nitrotyrosine biomarker for early diagnosis of liver cirrhosis with minimal hepatic encephalopathy, *Biosens. Bioelectron.*, 2016, **86**, 406–412.
- 9 J. P. Gaut, J. Byun, H. D. Tran and J. W. Heinecke, Artifact-free quantification of free 3-chlorotyrosine, 3-bromotyrosine, and 3-nitrotyrosine in human plasma by electron capture-negative chemical ionization gas chromatography mass spectrometry and liquid chromatography-electrospray ionization tandem mass, *Anal. Biochem.*, 2002, **300**, 252–259.
- 10 L. F. Gamon, C. Guo, J. He, P. Häggglund, C. L. Hawkins and M. J. Davies, Absolute quantitative analysis of intact and oxidized amino acids by LC-MS without prior derivatization, *Redox Biol.*, 2020, **36**, 101586.
- 11 Y. Kato, N. Dozaki, T. Nakamura, N. Kitamoto, A. Yoshida, M. Naito, M. Kitamura and T. Osawa, Quantification of modified tyrosines in healthy and diabetic human urine using liquid chromatography/tandem mass spectrometry, *J. Clin. Biochem. Nutr.*, 2009, **44**, 67–78.
- 12 H. J. C. Chen and W. L. Chiu, Simultaneous detection and quantification of 3-nitrotyrosine and 3-bromotyrosine in human urine by stable isotope dilution liquid chromatography tandem mass spectrometry, *Toxicol. Lett.*, 2008, **181**, 31–39.
- 13 A. R. Mani, A. S. Pannala, N. N. Orie, R. Olsson, D. Harry, C. A. Rice-Evans and K. P. Moore, Nitration of endogenous para-hydroxyphenylacetic acid and the metabolism of nitrotyrosine, *Biochem. J.*, 2003, **374**, 521–527.
- 14 D. Tsikas, E. Schwedhelm, F. K. Stutzer, F. M. Gutzki, I. Rode, C. Mehls and J. C. Frolich, Accurate quantification of basal plasma levels of 3-nitrotyrosine and 3-nitrotyrosinoalbumin by gas chromatography–Tandem mass spectrometry, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2003, **784**, 77–90.
- 15 H. Ohshima, M. Friesen, I. Brouet and H. Bartsch, Nitrotyrosine As A New Marker For Endogenous Nitrosation and Nitration Of Proteins, *Food Chem. Toxicol.*, 1990, **28**, 647–652.
- 16 Y. Hui, M. Wong, S. S. Zhao, J. A. Love, D. M. Ansley and D. D. Y. Chen, A simple and robust LC-MS/MS method for quantification of free 3-nitrotyrosine in human plasma from patients receiving on-pump CABG surgery, *Electrophoresis*, 2012, **33**, 697–704.
- 17 D. Yi, B. A. Ingelse, M. W. Duncan and G. A. Smythe, Quantification of 3-nitrotyrosine in biological tissues and fluids: Generating valid results by eliminating artifactual formation, *J. Am. Soc. Mass Spectrom.*, 2000, **11**, 578–586.
- 18 I. Verrastro, S. Pasha, K. T. Jensen, A. R. Pitt and C. M. Spickett, Mass spectrometry-based methods for identifying oxidized proteins in disease: advances and challenges, *Biomolecules*, 2015, **5**, 378–411.
- 19 Y. C. Sun, P. Y. Chang, K. C. Tsao, T. L. Wu, C. F. Sun, L. L. Wu and J. T. Wu, Establishment of a sandwich ELISA using commercial antibody for plasma or serum 3-nitrotyrosine (3NT). Elevation in inflammatory diseases and complementary between 3NT and myeloperoxidase, *Clin. Chim. Acta*, 2007, **378**, 175–180.
- 20 F. Khan, A. A. Siddiqui and R. Ali, Measurement and significance of 3-nitrotyrosine in systemic lupus erythematosus, *Scand. J. Immunol.*, 2006, **64**, 507–514.
- 21 A. J. Gow, C. R. Farkouh, D. A. Munson, M. A. Posencheg and H. Ischiropoulos, Biological significance of nitric oxide-mediated protein modifications, *Am. J. Physiol.: Lung Cell. Mol. Physiol.*, 2004, **287**, 262–268.
- 22 D. Tsikas and M. W. Duncan, Mass spectrometry and 3-nitrotyrosine: Strategies, controversies, and our current perspective, *Mass Spectrom. Rev.*, 2014, **33**, 237–276.
- 23 D. Teixeira, R. Fernandes, C. Prudêncio and M. Vieira, 3-Nitrotyrosine quantification methods: Current concepts and future challenges, *Biochimie*, 2016, **125**, 1–11.
- 24 M. S. Attia, M. H. Khalil, M. S. A. Abdel-Mottaleb, M. B. Lukyanova, Yu. A. Alekseenko and B. Lukyanov, Effect of Complexation with Lanthanide Metal Ions on the Photochromism of (1, 3, 3-Trimethyl-5-Hydroxy-6-Formyl-Indoline-Spiro2, 2-[2H] chromene) in Different Media, *Int. J. Photoenergy*, 2006, 1–9.
- 25 M. S. Attia, A. A. Essawy, A. O. Youssef and M. S. Mostafa, Determination of Ofloxacin using a Highly Selective Photo Probe Based on the Enhancement of the Luminescence Intensity of Eu<sup>3+</sup> - Ofloxacin Complex in Pharmaceutical and Serum Samples, *J. Fluoresc.*, 2012, **2**, 557–564.
- 26 M. S. Attia, A. M. Othman, A. O. Youssef and E. El-Raghi, Excited state interaction between Hydrochlorothiazide and Europium ion in PMMA polyme, and its application as photo probe for Hydrochlorothiazide in tablet and serum samples, *J. Lumin.*, 2012, **132**, 2049–2053.
- 27 M. S. Attia, A. O. Youssef and A. A. Essawy, A novel method for tyrosine assessment in vitro by using fluorescence enhancement of the ion-pair tyrosine-neutral red dye photo probe, *Anal. Methods*, 2012, **4**, 2323–2328.
- 28 M. S. Attia, A. O. Youssef and R. H. El-Sherif, Durable diagnosis of seminal vesicle and sexual gland diseases using the nano photo probe thin film sm-doxycycline complex, *Anal. Chim. Acta*, 2014, **835**, 56–64.



- 29 M. S. Attia, M. Diab and M. F. El-Shahat, Diagnosis of some diseases related to the histidine level in human serum by using the nano photo probe Eu–Norfloxacin complex, *Sens. Actuators, B*, 2015, **207**, 756–763.
- 30 M. S. Attia, A. O. Youssef, Z. A. Khan and M. N. Abou-Omar, Alpha fetoprotein assessment by using a nano photo probe thin film binuclear Pt-2-aminobenzimidazole-Bipyridine for early diagnosis of liver cancer, *Talanta*, 2018, **186**, 36–43.
- 31 M. S. Attia and N. S. Al-Radadi, Progress of pancreatitis disease biomarker alpha amylase enzyme by new nano photo probe, *Biosens. Bioelectron.*, 2016, **86**, 413–419.
- 32 M. S. Attia and N. S. Al-Radadi, Nano photo probe binuclear Pt-2-pyrazinecarboxylic acid-bipyridine for enhancement of the efficiency of 3-nitrotyrosine biomarker for early diagnosis of liver cirrhosis with minimal hepatic encephalopathy, *Biosens. Bioelectron.*, 2016, **86**, 406–412.
- 33 M. S. Attia, Nano optical probe samarium tetracycline complex for early diagnosis of histidinemia in new born children, *Biosens. Bioelectron.*, 2017, **94**, 81–86.
- 34 M. S. Attia, K. Ali, M. El-Kemary and W. M. Darwish, Phthalocyanine-doped polystyrene fluorescent nanocomposite as a highly selective biosensor for quantitative determination of cancer antigen 125, *Talanta*, 2019, **201**, 185–193.
- 35 M. S. Attia, W. H. Mahmoud, A. O. Youssef and M. S. Mostafa, Cilostazol Determination by the Enhancement of the Green Emission of Tb<sup>3+</sup> Photo probe, *J. Fluoresc.*, 2011, **21**, 2229–2235.
- 36 M. S. Attia, M. N. Ramsis, L. H. Khalil and S. G. Hashem, Spectrofluorimetric assessment of chlorzoxazone and Ibuprofen in pharmaceutical formulations by using Eu-tetracycline HCl photo probe doped in sol-gel matrix, *J. Fluoresc.*, 2012, **22**, 779–788.
- 37 O. Stern and M. Völmer, Decay time of the fluorescence, *Z. Phys.*, 1919, **20**, 183–188.
- 38 A. A. Elabd and M. S. Attia, A new thin film photo probe for assessment of UO<sub>2</sub><sup>2+</sup> based on the fluorescence quenching of Trimetazidine doped in sol gel matrix, *J. Lumin.*, 2015, **165**, 179–184.
- 39 A. A. Elabd and M. S. Attia, Spectrofluorimetric assessment of UO<sub>2</sub><sup>2+</sup> by the quenching of the fluorescence intensity of Clopidogrel embedded in PMMA matrix, *J. Lumin.*, 2016, **87**, 165–172.
- 40 A. A. Essawy and M. S. Attia, Novel application of pyronin Y fluorophore as high sensitive photo probe of glucose in human serum, *Talanta*, 2013, **107**, 18–24.
- 41 E. Hamed, M. S. Attia and K. Bassiony, Synthesis, spectroscopic and thermal characterization of Copper (II) complexes of folic acid and their absorption efficiency in the blood, *Bioinorg. Chem. Appl.*, 2009, **1–7**.
- 42 *International conference on harmonisation of technical requirements for registration of pharmaceuticals for human use, (ICH) Q2 (R1) validation of analytical procedures: text and methodology* 2005, vol. 1, pp. 1–15.
- 43 C. Leeuwenburgh, M. Hardy, S. Hazen, P. Wagner, S. Oh-ishi, U. Steinbrecher and J. Heinecke, Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima, *J. Biol. Chem.*, 1997, **272**, 1433–1436.
- 44 G. Romer, *et al.*, Value of the critical flicker frequency in patients with minimal hepatic encephalopathy, *J. Hepatol.*, 2007, **45**, 879–885.
- 45 E. Schwedhelm, D. Tsikas, F. Gutzki and J. Frölich, Gas chromatographic-tandem mass spectrometric quantification of free 3-nitrotyrosine in human plasma at the basal state, *Anal. Biochem.*, 1999, **276**, 195–203.
- 46 D. Tsikas, E. Schwedhelm, F. K. Stutzer, F. M. Gutzki, I. Rode, C. Mehls and J. C. Frölich, Accurate quantification of basal plasma levels of 3-nitrotyrosine and 3-nitrotyrosinoalbumin by gas chromatography-tandem mass spectrometry, *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2003, **784**, 77e90.
- 47 W.-Z. Zhang, C. Lang and D. M. Kaye, Determination of plasma free 3-nitrotyrosine and tyrosine by reversed-phase liquid chromatography with 4-fluoro-7-nitrobenzofurazan derivatization, *Biomed. Chromatogr.*, 2007, **21**, 273e278.
- 48 M. Pourfarzam, A. Movahedian, N. Sarrafzadegan, G. Basati and S. Z. Samsamshariat, Association between plasma myeloperoxidase and free 3-nitrotyrosine levels in patients with coronary artery disease, *Int. J. Clin. Med.*, 2013, **158e164**.
- 49 A. Seven, M. Aslan, S. Incir and A. Altintas, Evaluation of oxidative and nitrosative stress in relapsing remitting multiple sclerosis: effect of corticosteroid therapy, *Folia neuropathologica/Association of Polish Neuropathologists and Medical Research Centre, Pol. Acad. Sci.*, 2013, **51**, 58e64.

