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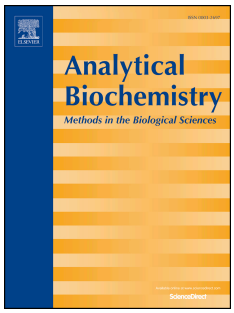

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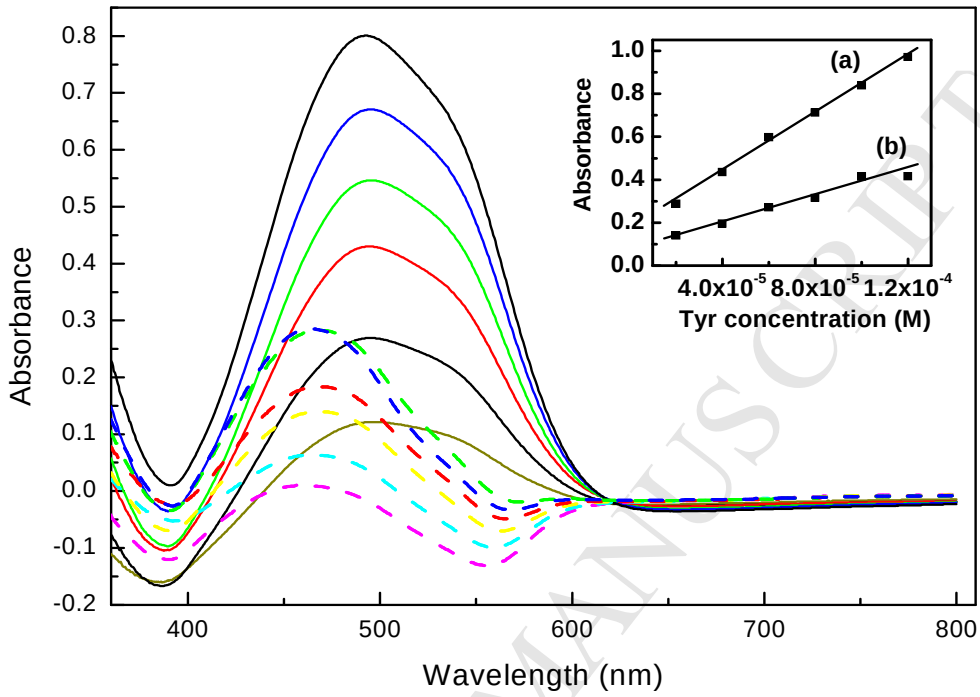

Electronic Spectra for the reaction of NN with different concentrations of Tyr in presence of GO (solid lines) and in absence of GO (dashed lines); Inset: Calibration curve (a) in presence of GO and (b) in absence of GO.

Effect of GO nanosheets on spectrophotometric determination of tyrosine in urine and serum using nitrosonaphthol

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Abstract

Here, we aimed to use graphene oxide to improve the selectivity and sensitivity of Tyr determination via the reaction with 1-nitroso-2-naphthol as a selective reagent of Tyr. The reaction between Tyr and 1-nitroso-2-naphthol in absence and presence of GO was studied spectrophotometrically. Different parameters such as concentrations, temperature, incubation time were optimized. The obtained data showed that the maximum absorbance was achieved by using 2 mL of 0.03 % 1-nitroso-2-naphthol at temperature 60 °C for 10 min. On the basis of calibration curve of various concentrations of Tyr in the presence of 20 μg mL^{-1} GO, the limit of detection was 6.4 x 10^{-6} M (1.15 μg mL^{-1}), where in absence of GO was 1.1 x 10^{-5} M (19.9 μg mL^{-1}). The selectivity of Tyr in presence of other amino acids and phenols was studied with and without GO. The data obtained revealed that the selectivity of Tyr in presence of GO with respect to some amino acids and phenols was improved. The proposed method has been applied for the determination of Tyr in urine and serum samples. Therefore, GO is a powerful catalytic surface for the sensitive and selective determination of Try in biological fluids.

Keywords: Graphene oxide; Spectrophotometric determination; Tyrosine; Amino acid; Selectivity; Sensitivity.

1. Introduction

Graphene oxide (GO) is an interesting nano-material, used in many applications. Recently, using of graphene and graphene oxide in biosensing and analytical application showed a great attention due to its unique properties of high surface area, biocompatibility, good water dispersibility and high reactivity of the oxygenated moieties present on its surface. This oxygenated lattice allows the interaction of non-covalent bonds with amines and phenyls functional groups through electrostatic interaction, $\pi - \pi$ stacking and hydrogen bonding [1-5].

Tyrosine (Tyr) is one of the most important amino acid, which assists the maintenance of a positive nitrogen in the body [6]. Tyr amino acid is involved in several diseases such as alkaptonuria, albinism, mental illness, lung disease, liver disease, and tyrosinemia. L-Tyrosine excretion in urine increased in patients suffering from diabetes mellitus. Also, it can be used for early detection and screening for a variety of cancer. So, the detection of Tyr in biological fluids as urine, serum and saliva may help in the early detection of human cancers, and in follow up of the treatment process of cancer diseases [7-10]. Various analytical methods have been reported and designed for determination of Tyr amino acid including spectrophotmetric [11-14], spectroflurometric [15-19], and electrochemical methods [20-25]. Due to the presence of Tyr with other amino acids in biological fluids and in pharmaceutical preparations, so the selective determination of Tyr is one of the interesting issues for analysts.

In this study, we aimed to use GO nanosheets as one of the promising nano-materials for improvement of the sensitivity and selectivity of Tyr determination

during its reaction with NN reagent, which was firstly reported as a selective reagent for determination of Tyr amino acid [26]. GO nanosheets is an excellent adsorbent for amino acids and phenols on its surface. So, the idea of this work depends on adsorbing of Tyr and other interfering amino acids or phenols on GO surface, allowing the NN reagent to capture and react with Tyr amino acid as a selective reagent to form colored product 1,2-benzo-8-alanyl-3-phenoxazone (BAPZ). This product is not adsorbed on the GO surface and has the advantage of absorbing peak at 495 nm which is totally far from the absorption region of NN reagent at 379 nm. The data obtained showed a significant effect of GO nanosheet in terms of enhancing the sensitivity and selectivity, indicating that the GO may act as a catalyst for improvement of the reaction process. Also, GO act as a micro-adsorbent for Tyr amino acid and other interfering materials on its surface. By adjusting the reaction conditions between Tyr and NN reagent the selectivity could be enhanced by holding other interfering materials from the medium. This method was used for the determination of Tyr in urine and serum samples.

2. Experimental

2.1. Material and Instruments

Graphene oxide was obtained from Sigma Co. (Munich, Germany). The particle size of the GO was determined by Scanning Electron Microscopy (SEM-FEI, FEG Quants 250, Hillsboro, OR, USA, magnification 20–1,000,000× high resolution). All spectrophotometric measurements were carried out at 25 ± 1 °C using a UV-vis spectrophotometer model (UV-1800 SHIMADZU, Nakagyo-KU, Kyoto, Japan). Fourier Transform Infrared Spectroscopy (FTIR) was carried out using a Nicolet iS 10 Thermo scientific (Waltham, MA, USA). A bench-top sonicator of Model 1510 was used to disperse graphene oxide in water (Branson, Danbury, CT, USA).The Tyrosine, Catechol, Cysteine, Phenol, Pyrogallol, Resorcinol, Tryptophan, β-

Naphthol, Sodium Hydroxide, Sodium Nitrite, Nitric acid, and Sulfuric acid were delivered from Sigma Co. (Munich, Germany).

2.2. Methods

2.2.1. Prepaaration and characterization of Nitrosonaphthol (NN)

NN was prepared using the reported method [27, 28], (more details are presented in supplementary data).

2.2.2. Preparation of BAPZ

BAPZ was prepared using the reported method [29, 30], (more details are presented in supplementary data)

2.2.3. Using of GO for signal improvement of BAPZ

The reaction between NN and Tyrosine was fully characterized in presence and absence of GO, using spectrophotometer (UV-1800 SHIMADZU, Nakagyo-KU, Kyoto, Japan). UV-vis spectra were recorded in an aqueous solution of GO (0.2 μg mL^{-1}), Tyr ($5x10^{-5}$ M), NN (0.03 % W/V), BAPZ and BAPZ-GO. BAPZ in absence of GO showed a clear peak with maximum absorption wavelength at λ_{max} 468 nm.

2.2.4. Effect of Tyrosine Concentration in absence and presence of GO

The great enhancement in the peak absorption, has been utilized to prepare a calibration curve in absence and presence of GO, that can be used for Tyr determination. A stock solution of $1x10^{-3}$ M of Tyr was prepared in pure water with adding drops of 1 M NaOH till complete dissolution of Tyr. In absence of GO, 2 mL of an aqueous solution of (0.03 % W/V) NN reagent was added to the corresponding volumes (0.2, 0.4, 0.6, 0.8, 1.0 and 1.2 mL) of $1x10^{-3}$ M Tyr, followed by addition of 300 μL conc. HNO_3, then the final volume was completed to 10 mL with distilled water. The produced solution was heated at 60 ^oC for 10 min. The electronic spectra of the product, BAPZ, have been measured at different concentrations of Tyr ($2x10^{-5}$-$1.2x10^{-4}$ M), the blank solution was containing NN reagent and HNO_3. Warring: when

nitric acid is added to these reaction mixtures, the major reaction could be competed with other reactions such as nitration reaction [31].

In presence of GO, the same procedure has been used with addition of 200 μL of (1mg mL^{-1}) GO to the solution mixture of each concentration of Tyr. Then, the electronic spectra were recorded. The blank solution was containing NN, GO and HNO_3.

2.2.5. Reaction of different interferents with NN in absence and presence of GO

The reaction of NN with different interferents such as Catechol, Cystine, Phenol, Pyrogallol, Resorcinol, and Tryptophan has been studied in absence and presence of GO. 2 mL (0.03 % W/V) NN was added to 5 mL of each interferent (to final concentration $5x10^{-5}$ M), followed by addition of 300 μL conc. HNO_3, then the final volume was completed to 10 mL. The produced solution was heated for 10 min at 60 ^oC. Then, the electronic spectra were measured. The same method has been completely repeated in presence of GO.

2.2.6. Effect of interferents on the determination of Tyrosine in absence and presence of GO

The influence of the interferents on the BAPZ signal has been investigated in absence and presence of GO. 2 mL (0.03 % W/V) NN was added to 0.6 mL ($1x10^{-3}$ M) Tyr, then 5 mL ($1x10^{-4}$ M) of each interferent was added, followed by addition of 300 μL conc. HNO_3, then the final volume was completed to 10 mL. The produced solution was heated for 10 min at 60 ^oC. Then, the electronic spectra were recorded. The same procedure has been completely repeated in presence of GO.

2.3 Application in real samples

2.3.1 Determination of Tyr in urine

Urine sample were collected from 3 healthy adult volunteers. To each 10 mL urine sample, 1 mL of 4 M HCl was added, followed by addition of 1 mL acetonitrile. The solution mixture was left for overnight in the refrigerator at 4 ^oC. The mixture was filtered and centrifuged at 5000 rpm for 10 min. 1 ml of each urine sample solution spiked with Tyr was added to 2 mL (0.03 % W/V) NN followed by addition of 1 ml of GO solution (20 μg/ml) and 300 μL conc. HNO_3, then the final volume was

completed to 10 mL. The produced solution was heated for 10 min at 60 ^{o}C. Then, the electronic spectra were measured, and the Tyr quantity was calculated from the calibration curve.

2.3.2 Determination of Tyr in serum solution

The application of the proposed method for determination of Tyr in serum was performed by tacking 4.0 ml plasma of a real health three volunteers followed by addition of 3.0 ml of citric acid solution (5 %). The solution was centrifuged for 10.0 min at 5000 rpm to remove proteins, then the supernatant serum solution was placed in 10 mL volumetric flask. 2 mL (0.03 % W/V) NN was added to serum sample followed by addition of 1 ml of GO solution (20 μg/ml) and 300 μL conc. HNO_3, then the final volume was completed to 10 mL with distilled water. The produced solution was heated for 10 min at 60 ^{o}C. Then, the electronic spectra were measured, and the Tyr quantity was calculated from the calibration curve.

3. Results and discussion

The reaction of Tyr with 1-nitroso-2-naphthol (NN) had been used for determination of Tyr before [26]. This reported method was chosen to study the effect of GO nanosheets on the sensitivity and selectivity for Tyr amino acid. However, haze white color was formed during the reaction with time, this fine precipitate is not easy to be separated by centrifugation, making the method not accurate nor precise for the determination of Tyr [26]. In this work we aim to use GO nanosheets as a catalyst and micro-separating material for increasing the sensitivity and selectivity of Tyr determination.

3.1. SEM of GO

Morphological studies of the GO were performed by SEM. The SEM micrographs are shown in Figure 1. The graph showed that, the nanosheets of GO are clear and regular in shape.

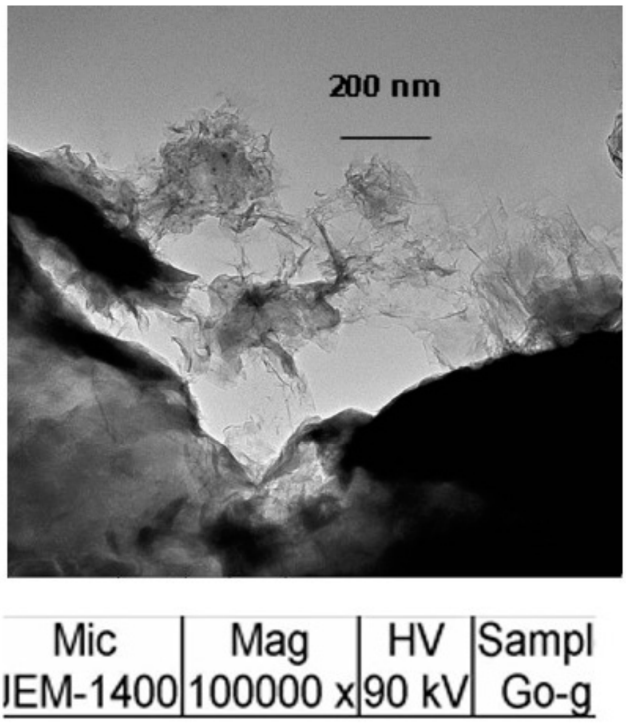


Figure 1. SEM analysis of GO.

3.2. Preparation and reactions of NN-Reagent with amino acids and phenols

Two different groups of biological interest compounds, L-amino acids and phenolic compounds, have been subjected to react with 1-nitroso-2-naphthol in the presence of nitric acid to produce colored compounds. Although, the reaction mechanism is not identical for all, the specificity of the reaction can best be described by treating each group separately.

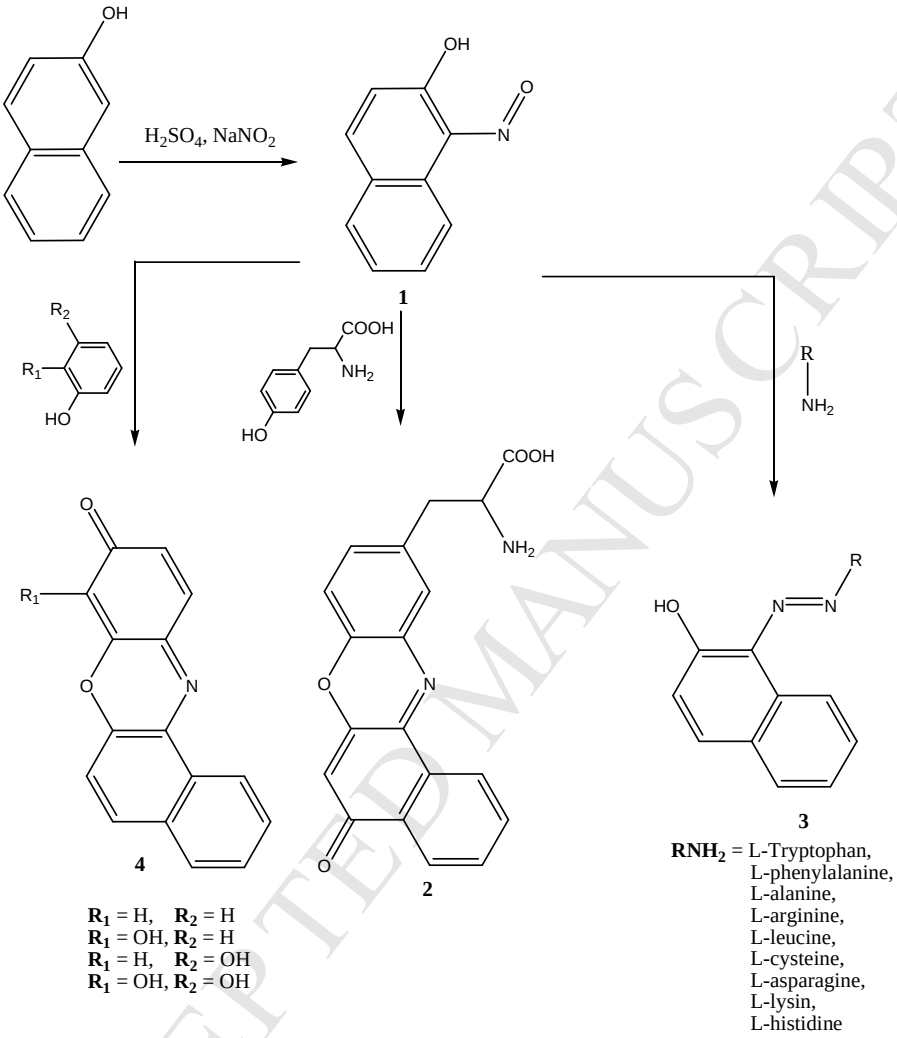
3.2.1. Synthesis of 1-nitroso-2-naphthol

Basic solution of 2-naphthol reacted effectively with nitrous acid solution (sodium nitrite and Sulfuric acid) to afford 1-nitroso-2-naphthol. The compound was confirmed by reported melting point and spectra data (IR and UV-visible) [27].

3.2.2. Reaction of 1-nitroso-2-naphthol with L-amino acids and phenols

In the presence of nitric acid, Tyr (phenolic amino acid) reacts with 1-nitroso-2-naphthol to produce 1,2-benzo-8-alanyl-3-phenoxazone (BAPZ) (**2**), pink-purple water-soluble compound. The reaction mixture was concentrated to one third then left to cool. The precipitated product was filtered and recrystallized from ethanol-water. BAPZ was confirmed by its IR spectra [29, 32, 33]. IR spectra showed abroad band at

3550-2600 cm^{-1} corresponding to NH_2 and OH groups. C=O showed a peak at 1632 cm^{-1} (scheme 1).



Scheme 1: Reaction of 1-nitroso-2-naphthol with L-amino acids and phenols

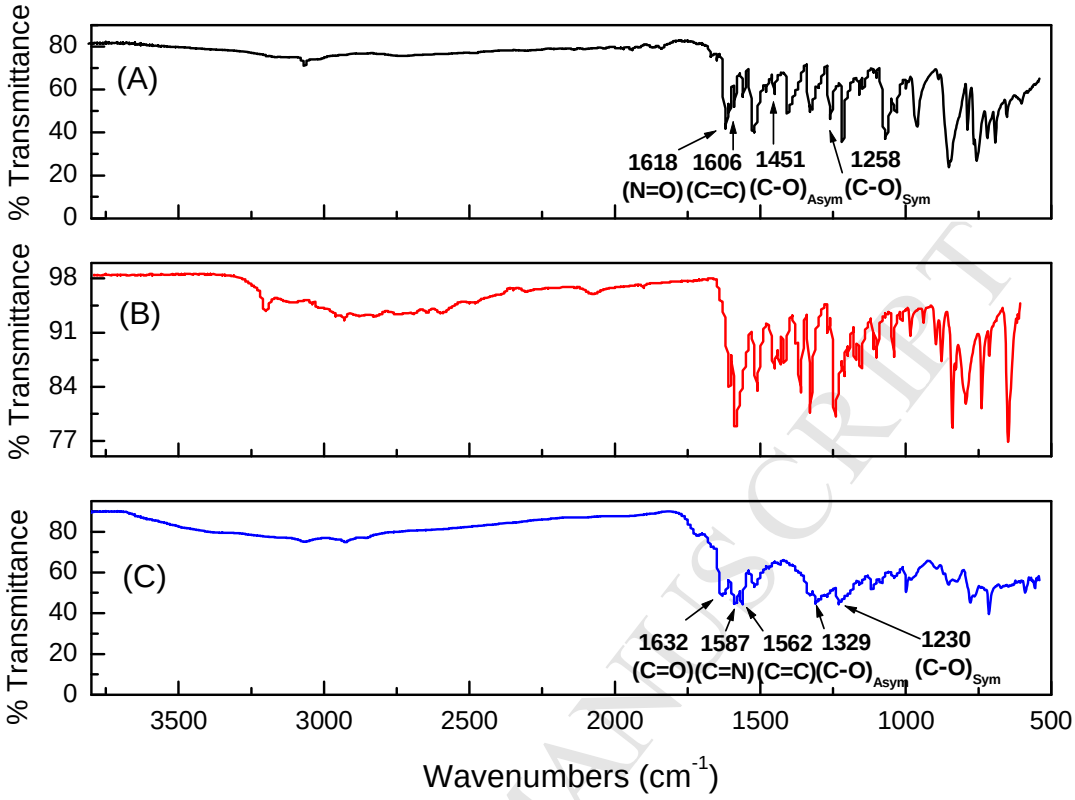
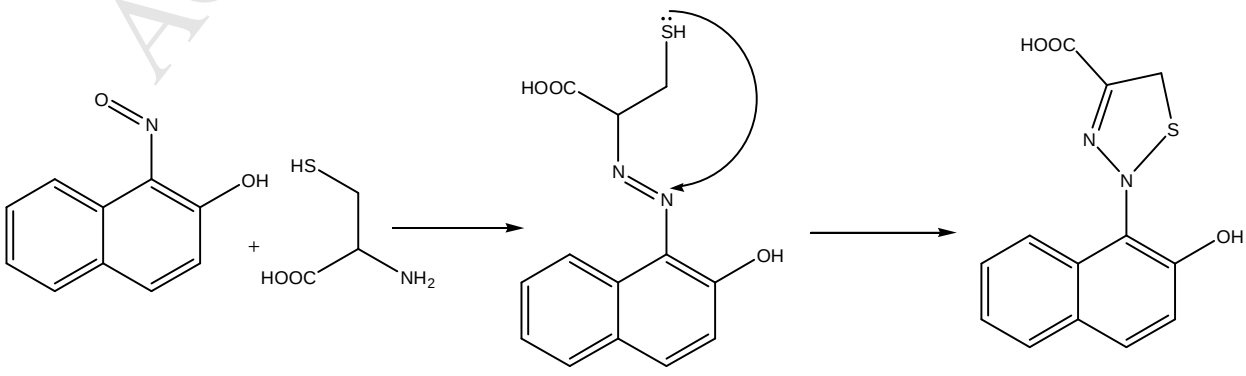


Fig. 2 FT-IR specta of (A) 1-Nitroso-2-Naphthole (NN), (B) Tyr, and (C) BAPZ.

On the other hand, amino acids under investigation, do not have a phenolic part, proceed in the same reaction condition to afford aliphatic azo-compounds (**3**) [34]. These azo compounds showed maximum absorbance at the UV range 435-465 nm, while cysteine showed two absorbance peaks at 400 and 550 nm, this could be attributed to its ability for the cyclization of its α-mercapto-group to afford thiadiazole derivative (scheme 2).



Scheme 2: Proposed mechanism for the reaction of 1-nitroso-2-naphthol with L-cysteine

Similarly, the reaction of phenolic derivatives, except pyrogallol, with 1-nitroso-2-naphthol in presence of nitric acid, produced relatively stable pink-purple water-soluble compounds that began to fade within a few hours [35] . The hydroxyl group, and its position of the phenolic compounds, both appear to be critical. The reaction proceeds smoothly for all phenolic compounds to afford 9*H*-Benzo[*a*] phenoxazine (**4**) derivatives except for pyrogallol where no color has been appeared. On the other hand, reaction of resorcinol and 1-nitroso-2-naphthol in the presence of nitric acid proceeded effectively to produce the highest intense reddish violet color which has absorption maximum at 538 nm.

3.3. Using of GO for signal improvement of BAPZ

The reaction of Tyr amino acid with NN in absence of GO showed a clear peak with maximum absorption wavelength at λ_{max} 468 nm, which is totally far away from the absorption band of NN and/or Tyr. NN and Tyr spectra showed maximum wavelengths at 379 and 273 nm, respectively. Where GO spectral absorption band was found at 223 nm. In presence of GO, The recorded spectrum of BAPZ-GO showed a fantastic increase in absorbance (~220 %) at 495 nm with a 27 nm red shift, compared to BAPZ in absence of GO (Fig. 3). This red shift in λ_{max} of BAPZ in presence of GO is an evidence of its adsorption on GO surface [36].

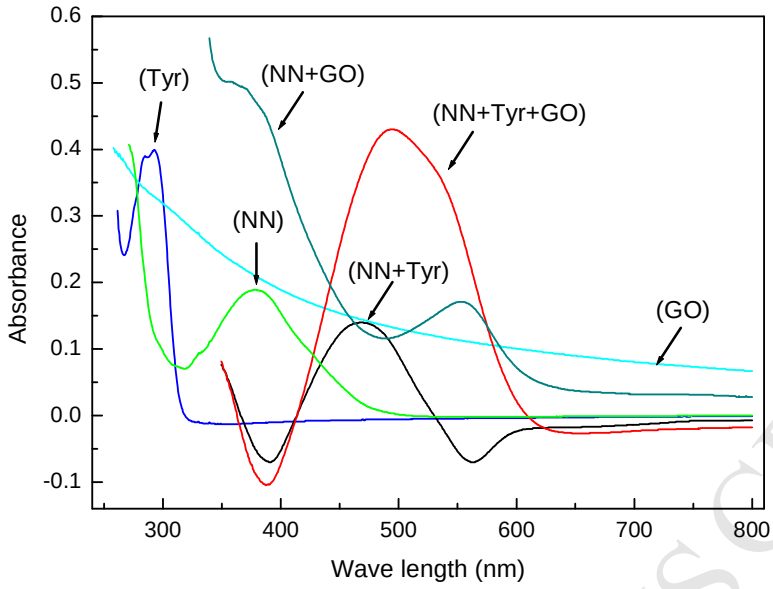


Fig. 3 Electronic Spectra for Tyr, NN, GO, NN+GO, BAPZ, and BAPZ-GO.

The obtained data indicated that, GO could act as a catalyst for the reaction between Tyr and NN, with an excellent increase in absorption with a red shift of 27 nm. Moreover, the colored product BAPZ was not adsorbed on GO surface. The desorption of BAPZ from the GO surface could be attributed to the involvement of the free phenolic hydroxyl group, responsible for H-bonding with active groups present on GO surface, in BAPZ formation. This advantage lead to the possible use of GO as an optical sensor for determination of Tyr amino acid.

3.4 Effect of reagent (NN) concentration, temperature, and reaction time

The effect of NN concentration on the absorbance of BAPZ was achieved by taking different volumes 1 to 5 mL of 0.03 % W/V NN. The obtained data showed that, the maximum absorbance of BAPZ was obtained at 2 mL of the reagent. The change in absorbance was studied at different temperatures from 25 – 80 ℃. The maximum absorbance was achieved at 58 – 62 ℃, therefore, 60 ℃ was used as the optimum temperature during all the experiments. The effect of reaction time between Tyr amino acid and NN was also investigated at 5, 10, 15, and 20 min. The obtained results, showed that after 10 min., the absorbance was reached to its steady state, indicating that the reaction reached its equilibrium point. Different volumes of conc.

nitric acid (100-500 µL) were added to accelerate the reaction [26]. The maximum absorbance of the product was obtained with 300 µL of conc. HNO_3, and was used as an optimum volume during all experiments.

3. 5. Effect of Tyrosine Concentration in absence and presence of GO

The great enhancement in the absorption peak, has been utilized to prepare a calibration curve in absence and presence of GO for Tyr determination. The test solutions were prepared by adding different concentartions ranged from $2x10^{-5}$ to 1.2 $x10^{-4}$ M of Tyr, and 2 mL of NN reagent solution (0.03 %). The mixture was heated in a water bath at 60 °C for five min., then 300 µl of conc. HNO_3 was added, and the mixture was heated for more five min. The developed red colour was measured at 468 nm. The same calibration curve was measured at 495 nm in presence of 20 µg mL^{-1} of GO. The limit of detection (LOD) was calculated as (3.3σ/S), where σ is the standard deviation of the intercepts and S is the slope of the calibration curve. The data obtained from Fig 4, showed that the limit of detection was improved from 1.1 x 10^{-5} M to 6.4 x 10^{-6} M in absence and presence of GO nanosheets, respectively.

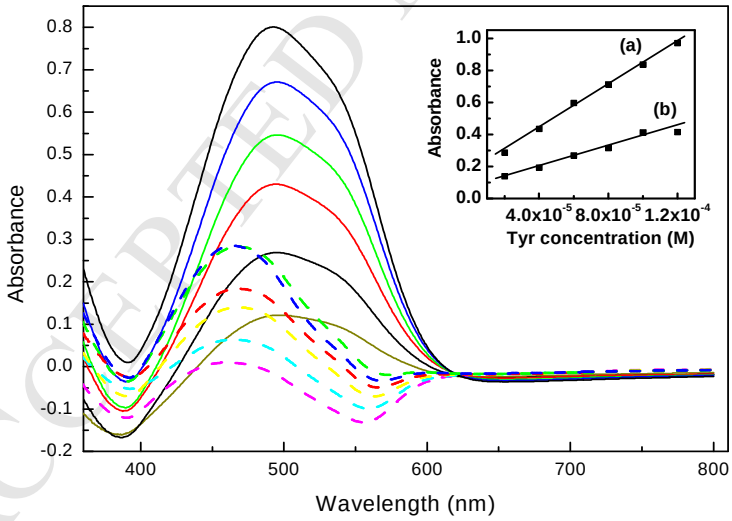


Fig. 4 Electronic Spectra for the reaction of NN with different concentrations of Tyr ($2x10^{-5}$ – 1.2 x 10^{-4} M) in presence of GO (solid lines) and in absence of GO (dashed lines); Inset: Calibration curve (a) in presence of GO and (b) in absence of GO.

3. 6. Effect of interferents on the determination of Tyr in absence and presence of GO

The influence of interfering compounds (Tryptophan, L-phenylalanine, L-alanine, L-arginine, L-leucine, L-cysteine, L-asparagine, L-lysin, L-histidine amino acids, and phenol, pyrogalol, catechol and resorcinol) on the BAPZ signal has been investigated in absence and presence of 20 μg mL^{-1} GO. A 2 mL (0.03 % W/V) of NN reagent was added to 0.5 mL ($1x10^{-3}$ M) Tyr, then 5 mL ($1x10^{-4}$ M) of each interferent was added, followed by addition of 300 μL conc. HNO_3, then the final volume was completed to 10 mL.

The data obtained (Fig. 5) showed that, in absence of Tyr and GO, most of interferents interact with NN forming the corresponding NN-amino acid with no significant peaks in the absorption region of Tyr. Where, their absorbance strengths at 495 nm were completely negligible with respect to BAPZ signal for the same concentration. This behavior may be attributed to the absence of phenolic hydroxyl group in all of the amino acids under interest, except Tyr. However, in case of Cys, there were an absorption peaks at λ_{max} 400 and 550 nm, this could be explained in the term of high conjugated pattern of the proposed product (scheme 2). In absence of Tyr and in presence of GO, the signal of the corresponding NN-interfered amino acids was increased by around 3-7 %, indicating that GO has no significant effect on the reaction between most of the interferents and NN.

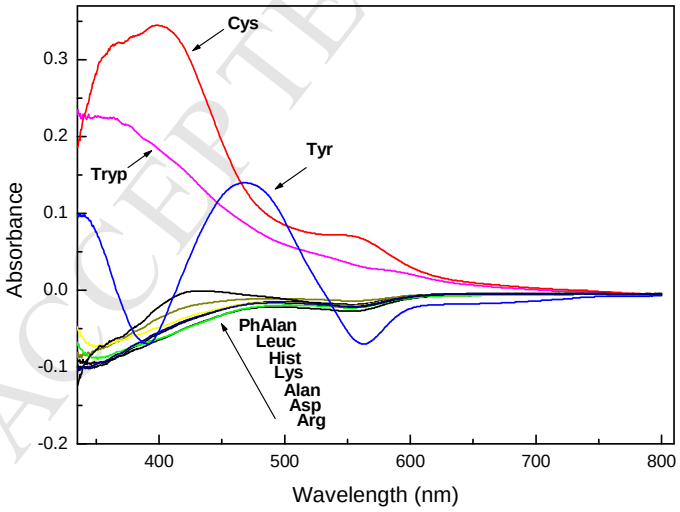
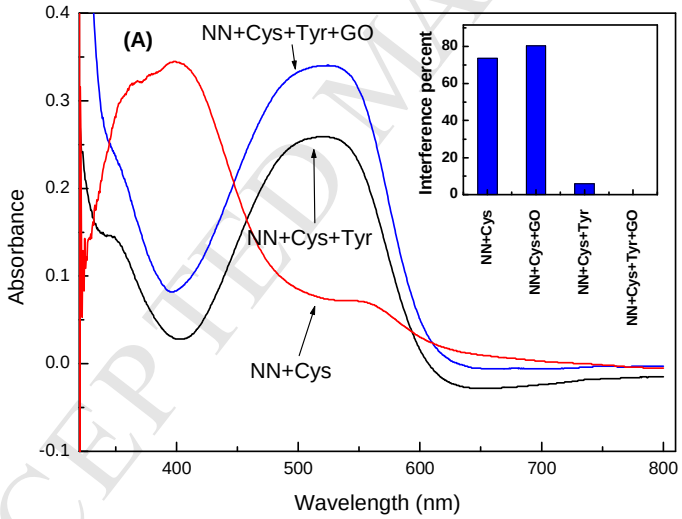


Fig. 5 Electronic Spectra for the reaction of NN with ($5x10^{-5}$ M) amino acids.

On the other hand, in presence of Tyr and in absence of GO, the reaction of NN with Tyr to form BAPZ is more preferable than the reaction of NN with other

interfering amino acids, due to the selectivity of NN toward Tyr, showing a tremendous decrease in percent of interference, especially with Cys (Fig. 6 A). While, in presence of Tyr and GO, the interference was decreased to about 0-1 %, due to the action of GO as a powerful catalyst for the reaction between NN and Tyr. Moreover, GO may help in holding the other interfering materials on its surface [2, 5], encouraging the NN to capture Tyr from GO surface forming BAPZ, which is not adsorbed on GO. Phenolic compounds showed a moderate interfering effect in absence of Tyr and GO, due to the presence of hydroxyl groups, readily to react with NN. Similarly, as previously described in amino acids, GO was used to reduce the interference effect (Fig. 6 B). Therefore, It could be concluded that, graphene oxide nanosheets had increased the selectivity of the proposed method for the determination of Tyr using NN in presence of different interfering amino acids and phenols.



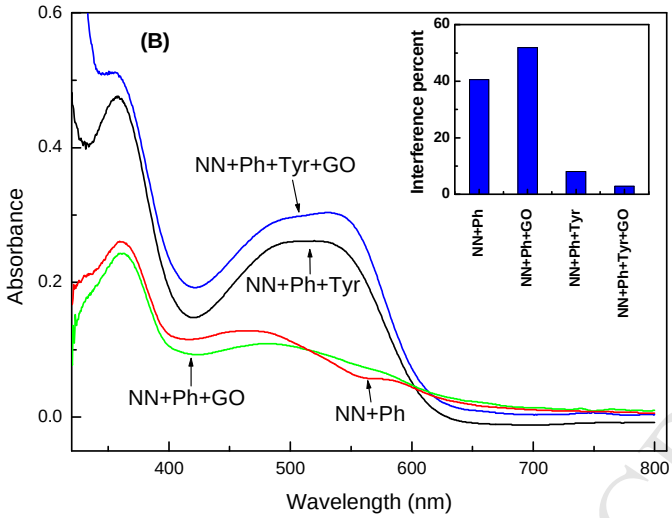


Fig. 6 Electronic Spectra for the reaction of NN with ($5x10^{-5}$ M) of interferents (A) Cys and (B) Ph, in absence and presence of ($6x10^{-5}$ M) Tyr and 20 µg $mL^{-1}$of GO; Inset: Corresponding interference percent.

3.7 Application in real samples

The proposed method has been applied for the determination of Tyr in urine and serum samples of 3 healthy adult volunteers, using NN reagent in presence of (20 µg/mL) GO as shown in table 1. 1 mL urine and serum samples after treatment, were spiked with Tyr to final concentrations of 20, 60, 100 x 10^{-6} M in measured solution. The actual Tyr amount found in urine samples were 21.2 ± 0.42, 18.7 ± 0.17 and 22.1 ± 0.10, respectively. In serum samples, the actual Tyr found were 26.5 ± 0.51, 28.3 ± 0.27 and 27.9 ± 0.15, respectively. The data obtained by the Liquid Chromatography standard method of British Pharmacopoeia [37] showed a good agreement with the results of the proposed method. The obtained data reveals that the proposed method can be used as a sensitive and selective method for the determination of Tyr in biological fluids.

Table 1 Determination of Tyr in urine and serum samples

Sample no.	Added ($x10^{-6}$ M)	Total Found ($x10^{-6}$ M)	Actual Found ($x10^{-6}$ M)	Reference method [37] B.P. (LC) (Actual found $x10^{-6}$ M)
Urine	20	41.2 ± 0.82	21.2 ± 0.42	20.8 ± 0.36
	60	78.7 ± 0.70	18.7 ± 0.17	19.2 ± 0.25
	100	122.1 ± 0.56	22.1 ± 0.10	21.3 ±0.15
Serum	20	46.5 ± 0.90	26.5 ± 0.51	25.8 ± 0.48
	60	88.3 ± 0.83	28.3 ± 0.27	27.3 ± 0.35
	100	127.9 ± 0.65	27.9 ± 0.15	26.8 ± 0.18

3.8 Comparison with other methods

The present work was compared with other reported methods [24, 38-40] for the determination of Tyr as shown in Table 2. Although the present method has a moderate sensitivity in comparison with other methods [38-40], it has the advantages of application in real samples and intensive interference study compared with the reported methods [38, 39]. On the other hand, the reported method [40] needs more than 24 hours for sensor preparation, making this method more tedious and time consuming than the present study. The selectivity and LOD of the present work are better than the reported one based on filtered multi-walled carbon nanotubes on Teflon net [24].

However, using of GO showed an improvement in the selectivity and sensitivity by deacreasing LOD, which encourage the use of GO in other spectrophotometric techniques.

Table 2 A comparison of Tyr determination using NN in presence of GO with other reported methods

Used strategy	Technique	Linear range	LOD	application	Ref
MSNP- CPE	DPV	5×10^{-7} to 6×10^{-4} M	1.5 x 10^{-7} M	Artificial urine.	[38]
MWCNT - PBP- CPE	Amp	2×10^{-6} to 100×10^{-6} M	1.9 x 10^{-7} M	Milk & serum	[39]
AuNPs - GO - GCE	DPV	1×10^{-9} to 2×10^{-8} M	1.5 x10^{-10} M	Milk	[40]
MWCNT – Teflon - SPE	DPV	25×10^{-6} to 750×10^{-6} M	8×10^{-6} M	plasma & blood	[24]
NN	Spectro-photometry	2 x 10^{-5} to 1.2 x 10^{-4} M	1.1 x 10^{-5} M	----	present work
NN - GO			6.4 ×10^{-6} M	Urine & Serum	

* MWCNT = Multi-walled carbon nanotubes
* CPE = Carbon paste electrode
* SPE = Screen printed electrode
* GCE = Glassy carbon electrode
* PBP = Poly bromocresol purple
* MSNP = Mesoporous silica nanoparticles
* DPV = Differential pulse voltammetry
* Amp = Amperometry

4. Conclusions

GO nanosheets have been used for enhancement of the selectivity and sensitivity of spectrophotometric determination of Tyr in aqueous media. In presence of GO, the signal was enhanced, indicating that GO may act as a powerful catalyst for the reaction between NN and Tyr. The limit of detection was significantly decreased from 1.1x10^{-5} to 6.4x10^{-6} M in absence and presence of GO, respectively. The selectivity was improved with respect to interfering amino acid and phenols, due to holding of interfering materials on GO surface, giving the chance of NN to capture Tyr from GO surface to form BAPZ. The proposed method has been applied for the determination of Tyr in urine and serum samples. The actual Tyr amount found in urine samples were 21.2 ± 0.42, 18.7 ± 0.17 and 22.1 ± 0.10, respectively, while in serum samples, the actual Tyr found were 26.5 ± 0.51, 28.3 ± 0.27 and 27.9 ± 0.15, respectively. These data are in good correlation with that obtained by standard method of British

Pharmacopoeia . Therefore, the proposed method could be used as a selective and sensitive optical sensor for Tyr and protein with free Tyr amino acids determination in biological fluids in presence of different interfering amino acids and phenols.

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5. References

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- Determination of tyrosine amino acid (Tyr) in presence of graphene oxide
- GO improves the selectivity and sensitivity of Tyr determination.
- The lower limit of detection was significantly increased.
- The selectivity was improved with respect to interfering amino acid and phenols.
- The method has been applied for the determination of Tyr in urine and serum samples
- The method could be used as optical sensor for Tyr determination.