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Dispersive Liquid-Liquid Microextraction Spectrophotometric Determination of Human Salivary Nitrite Content

Harez Sdiq Faraj*, Nabil Adil Fakhre

Department Of Chemistry, College of Education, Salahaddin University-Erbil, Erbil, 44002, Iraq.

Abstract

A novel and rapid dispersive liquid-liquid microextraction (DLLME) method was applied to intensify nitrite ions as a subsequent step to its spectrophotometric determination using the Griess reaction. Produced orange azo dye has (λ max.) of 460 nm., (ε) is 3.8432 x 10^3 L. $mol^{-1}.cm^{-1}$, and Beer's law was obeyed in the range 0.3-12.0 µg. mL^{-1}. It shows LOD, LOQ, and R^2 values of 0.2 µg. mL^{-1}, 0.3 µg. mL^{-1} and 0.9994, respectively. In the recommended DLLME technique, ethanol was used as the dispersant solvent and a minimal amount of chloroform was used as the extraction solvent. Then, the dye has been extracted into fine chloroform droplets. The solution was centrifuged for 3.0 min./5000 rpm, and the droplets sank to the bottom of a 10.0 mL conical test tube. The total amount of azo-dye enriched extracted phase was then measured using a spectrophotometer. The yellow chloroform extract exhibits ($\lambda_{max.}$) of 410 nm, (ε) is 8.6024 x 10^3 L. $mol^{-1}.cm^{-1}$and Beer's law was obeyed in the range 0.1-7.0 µg. mL^{-1}. The LOD, LOQ, and R^2 values of the proposed extraction method were 0.05 µg. mL^{-1}, 0.1 µg. mL^{-1} and 0.9997, respectively. Sensitivity enhancement was around 225%. The proposed technique was successfully applied to human saliva samples.

Keywords: Dispersive liquid-liquid microextraction, saliva samples, nitrite, spectrophotometry.

1. Introduction

For both plants and animals, nitrogen is an essential nutrient. Nitric oxide ($NO^{\cdot}$), nitrite (NO_2^-), and nitrate are its three environmental manifestations (NO_3^-). Routinely used samples including environmental, dietary, and biological samples always contain these ions [1]. Nitrogen is present in the form of nitrite ions in a highly unstable oxidation state. Nitrite can be further reduced by chemical and biological processes into a variety of compounds or it can be oxidised into nitrate [2].

As an intermediary step in the formation of nitrate, nitrifying bacteria in nature produce nitrite [3]. At least three conditions have been linked as risk factors to nitrate exposure: methemoglobinemia in infants due to receiving food and consuming water rich with nitrate [4], neural tube abnormalities in offspring whose mothers experience comparable pregnancy-related exposures [5], formation of carcinogenic N-nitroso compounds [6], hypothyroidism [7]. Through their interactions with secondary amines and amides, the production of N-nitrosamines under acidic conditions, such as the stomach, was linked to (NO_2^-). It is suspected that the lower molecular weight N-nitrosamines can cause cancer in both humans and animals. Nitrite is therefore regarded as a precursor to the formation of N-nitrosamines [8]. Determination of nitrite is also essential because it results in a widespread hazardous inorganic pollutant that exists in the environment, food additives, industrial, and physiological systems [9]. There are several methods that have been presented for determining (NO_2^-) cover Electrochemical [10, 11], Fluorescence [12, 13], Chemiluminescence [14, 15], spectrophotometry [16, 17], and Chromatographic method [18].

Due to its low cost and ease of use during the analysis stage, the UV-VIS spectrophotometer is still one of the most extensively used analytical systems for detecting analytes in environmental samples. Additionally, it may be found in all analytical chemical laboratories. However, the limited sensitivity

*Corresponding author e-mail: harez.faraj@su.edu.krd.; (Harez Sdiq Faraj).
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of the UV-Vis spectrophotometer severely restricts its use. Therefore, a separation/preconcentration process is needed to perform sensitive and accurate measurements. To meet the demands of green chemistry, analytical chemists have recently focused their efforts on the enhancement of separation and preconcentration procedures. For this reason, analytical chemists involved in green chemistry set the reduction or total elimination of the usage of harmful organic solvents which result in secondary laboratory waste as their top priority [19]. In general, ultra violet-visible spectrophotometry is the widest technique employed in quality control laboratories because of its inherent simplicity, sensitivity and cost effectiveness. Therefore, developing selective and sensitive methods using visible spectrophotometry is of paramount importance[20].

Numerous techniques have historically been used to preconcentrate different trace elements from their complicated matrix. The most well-known methods for purification and preconcentration include zeolite cation exchange, ion exchange purification, adsorption with chelating ion exchange resins, chromatography, liquid-liquid extraction, cloud point extraction, solid phase extraction, coprecipitation, solid phase microextraction, and liquid phase microextraction [21].

DLLME was firstly reported in 2006. The advantages of this approach are using the needle, a very tiny volume of a solvent and a dispersive combination is swiftly injected into an aqueous solution of a sample. As a result, the extractant is thoroughly disseminated into the aqueous sample as tiny drops with a large surface area, which encourages considerable increases in rate transfer and effective extraction of analyte in the droplets of extraction solvent [22].

In comparison to traditional procedures, solvent microextraction is quicker, easier, less costly, more sensitive, and more successful in removing interfering matrices. It also uses a lot less organic solvent. Additionally, since just one operational step is needed, issues with contamination and analyte loss disappear [23].

The combination of DLLME with UV-Visible Spectrophotometry for the measurement of nitrite was proposed in the current work. The purpose of this study is to supply a simple, exact, quick, and effective method for determining the presence of NO_2^- in human saliva utilising the diazotization-coupling reactions. The process is improved by extracting the unreacted dye into an organic solvent.

2. Experimental

2.1. Apparatus

A UV/Vis double beam spectrophotometer, the UV-1800 SHIMADZU, with 1-cm match quartz cells, was utilized for all spectrophotometric measurements in addition to recording the absorption spectra. To ascertain the quantity of hydrogen ions, a pH meter known as the HANNA pH-211 was employed. For extraction, a centrifuge model PLC-03, Gemmy industrial crop., was used.

2.2. Reagents

All of the chemicals were of the analytical reagent grade, and distilled water was used to produce the reagent preparations.

Stock nitrite solution (1000 µg. mL^{-1}): was prepared by dissolving 0.1499 g of $NaNO_2$ (Riedel-De Haën AG) in (D.W). A pellet of sodium hydroxide and one millilitre of chloroform were added, and then distilled water was used to make the solution up to 100 millilitres. Working standard solutions of 100 µg. mL^{-1}were freshly generated by diluting stock solution with distilled water [24], A pellet of sodium hydroxide was added to prevent nitrite decomposition and 1.0 ml of chloroform to prevent bacterial growth [25].

p-Bromoaniline solution (0.15 %): was prepared by dissolving 0.15 g (Riedel-De Haën) of p-bromoaniline in 1.0 mL of ethanol then 75.0 mL of distilling water was added, then it was shacked and warmed, In a volumetric flask filled with distilled water, the capacity was ultimately finished to 100 mL . The brown bottle used to store the solution in the refrigerator keeps it stable for at least three days. [16].

Hydrochloric acid solution (1.0 M): a volumetric flask containing 100 mL of distilled water and 8.280 mL of the concentrated hydrochloric acid (12.076 M) (Riedel-De Han) solution was used to make the solution.

Standard Paracetamol solution (0.25 %): was made by gently warming 0.25 g of pure paracetamol (Awamedica) in a known volume of distilled water before being diluted to a final concentration of 100 mL.

Ammonium hydroxide solution (2.0 M): was prepared by diluting 14.84 mL of concentrated ammonium hydroxide (13.48 N) (Riedel-De Han) with distilled water until it reached the desired proportion in a 100 mL volumetric flask.

2.3. Collection and Deproteinization of Samples

A saliva preservation method was employed to stop additional nitrate and nitrite reduction following saliva sampling. In glass tubes with 0.5 mL of 1.0 N NaOH, about 5.0 mL of whole saliva sample was collected. NaOH is utilized as a stabiliser since nitrite is unstable in an acid solution. A volume of 3.0 mL of saliva was used as an aliquot, and 0.2 mL of 0.5 M $ZnSO_4$ was introduced, followed by mixing. The mixture was then centrifuged for 10.0 minutes at 3000 rpm. $ZnSO_4$ treatment eliminates proteins and other components that can prevent nitrite from forming

chromogen. In the case of saliva, this step is especially crucial, and skipping it could result in an 80.0% underestimation of the nitrite levels [5].

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2.4. Recommended Procedures

2.4.1. *Aqueous Procedure*

To a series of l0 mL volumetric flasks, 1.0 mL of 0.15 % p-bromoaniline and 1.0 mL of 1.0 M HCl have been introduced. Then aliquots of nitrite ion solution containing (3.0-120 µg) were added at room temperature. After that, 0.5 mL of 0.25%, paracetamol and 1.0 mL of 2.0 M ammonia solution were added simultaneously. Then, distilled water was used to dilute the mixture to the desired volume. Using matching 1.0 cm quartz cells, the absorbance at 460 nm was assessed after 5.0 min against a reagent blank.

2.4.2. *Extraction Procedure*

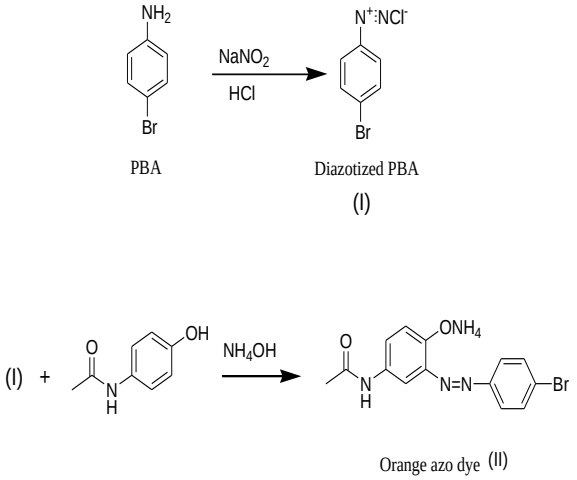
A test tube with a conical bottom was filled with the produced azo dye from the 10 mL volumetric flask (detailed in section 2.4.1). The aqueous dye solution was then quickly injected with a binary solution made up of 2.0 mL of ethanol (the solvent for dispersion) and 3.5 mL of chloroform (the solvent for extraction), 5.0 mL syringe has been used and the combination of water, ethanol, and chloroform produced a steady hazy solution. Then, chloroform was used to extract the nitrite ion into its tiny droplets. The tiny chloroform droplets were gathered together during the 3.0 min. at 5000 rpm centrifugation step, settled at the base of the conical test tube. A pipette was used to carefully remove the upper bulk aqueous phase. The final step was to transfer the resulting solution into a 1.0 mL quartz cell. At 410 nm, the absorbance value was measured versus the reagent blank.

3. Results and Discussion

The nitrite ion was handled following the suggested approach. The dye has a maximum absorption at 460 nm in water and 410 nm in chloroform.

3.1. The Principal of The Method

The reaction that yields the colourful dye requires two steps. In the first stage, p-bromoaniline interacts with the nitrite ion to produce the p-Bromo phenyl diazonium chloride ion in the presence of enough HCl (I). The diazonium ion and paracetamol are combined in the second stage to create an azo dye (II) that is orange in alkaline solution and turns yellow upon extraction into chloroform. The following diagram illustrates the reaction:



Scheme 1: Schematic diagram of azo-dye formation.

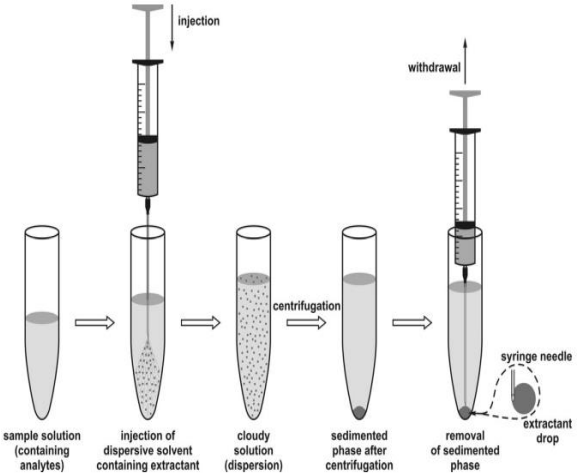


Figure 1. Mechanism of DLLME [26].

3.2. Preliminary Investigation for Determination of Nitrite Ion

A volume of 1.0 mL of 3.0 M HCl solution, 1.0 mL of 0.1% p-bromoaniline solution, 0.5 mL of 0.1% paracetamol solution, and 5.0 mL of 1.0 M ammonium hydroxide solution were added to a known volume of an aqueous sample containing 50.0 µg of (NO_2^-) ion. The solution was then diluted to the appropriate volume with D.W in a 10.0 mL standard volumetric flask, and an orange dye resulted. Maximum absorbance at 442 nm was observed in the coloured dye's absorption spectra when compared to its comparable blank reagents, which exhibit little absorbance at this wavelength.

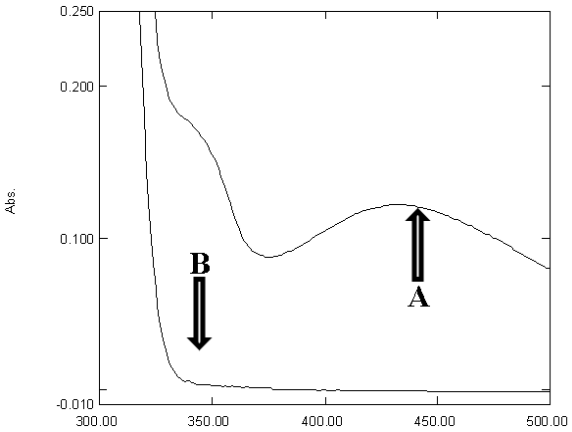


Figure 2. Absorption spectra of (A) azo dye of nitrite ion against blank (B) blank against D.W.

3.2.1. *Optimization of Experimental Conditions*

Linearity, accuracy, precision (repeatability), LOD, LOQ, and stability (robustness) were among the performance criteria of the proposed method that were validated. [8].

3.2.1.1. *Effect of Different Acid*

Various acidic solutions (HCl, H_2SO_4, HNO_3, CH_3COOH and HCOOH) with 1.0 M of concentration were tested for diazotization reaction. Due to its greatest absorbance values and stability concerns for diazotization, HCl was discovered to be the most effective among them.

3.2.1.2. *Effect of Amount of Hydrochloric Acid*

The influence of the volume of HCl on the diazotization reaction has been examined over the range (of 0.25- 1.5) mL. Best absorption intensities were achieved in addition to 1.0 mL of 1.0 M HCl because there is no significant difference from 1.0 mL to 2.0 mL and in this range, results show stability for diazotization. The role of the various concentrations of 1.0 mL HCl solution has been studied. The absorbance gradually declined by increasing concentration from 1.0- 7.0 M, after which absorbance experienced a dramatic decrease. In higher concentrations, the excess acid converts the diazonium ion into diazonium salt [21]. According to the observations, the solution's maximum absorption is at 1.0 M.

3.2.1.3. *Effect of Diazotized P-bromoaniline Concentration*

The effect of various concentrations of 1.0 ml p-bromoaniline was studied on the maximum absorption of the coloured azo-dye over the range of (0.05 - 0.25) %. Results show that the solution's optimal concentration for achieving the highest absorption was 0.15 %.

3.2.1.4. *Effect of Paracetamol Concentration*

The effect of 0.5 mL paracetamol concentration ranged from (0.05-0.25) % has been studied. From the results, it can be observed that 0.25 % paracetamol is the more suitable amount which gives the highest value of intensity for the azo dye formed.

3.2.1.5. *Effect of Base Types on Azo Dye*

The effect of (5.0 mL, 1.0 M) of different types of strong and weak bases such as (Na_2CO_3, NaOH, KOH, NH_4OH) on coupling reaction of diazonium ion with paracetamol was investigated. The results indicate that the coloured azo dye is formed in alkaline medium and ammonium hydroxide solution gives maximum absorption among other bases.

3.2.1.6. *Effect of Amount of Ammonium Hydroxide*

The role of different concentrations of NH_4OH solution 5.0 mL was also investigated, ranging from 0.5- to 3.0 M. The results indicate that 2.0 M of the solution has maximum absorption. Because the excess of hydroxide ions reacts with these reagents (the diazonium ion ArN_2^+) and tends to convert them to unionized compounds (ArN_2OH) which are not coupled with the reagent [27]. Then the influence of 2.0 M of base volume from 1.0- to 6.0 mL has been studied to obtain the maximum colour density of the azo dye on absorbance investigated. The obtained results indicate that the absorbance declined with raising the reagent volume and reached the maximum on using a volume of 1.0 ml of 2.0 M. Therefore, the addition of 1.0 mL NH_4OH solution with (pH = 9.7) was recommended for the subsequent experiments.

3.2.1.7. *The Addition Order of Reaction Components*

To investigate the impact of altering the order of additions on the absorption of the azo colour produced. Four different sequences were chosen, each with a different solution addition. The sequencing (PBA + HCl + NO_2^- + APAP + NH_4OH) was used in following tests because it was found from the results that it gave the highest absorption of the produced azo dye.

3.2.1.8. *Development Time and Stability Period*

The constancy and development of the coloured dye depend on time; as a result, it is investigated under the ideal experimental circumstances mentioned above. According to the testing results, the chromophore reaches its maximum

after 5.0 min from dilution to the mark and is stable for around 160 min before gradually fading.

3.2.1.9. *Final Absorption Spectra*

The final absorption spectrum was evaluated after obtaining the ideal circumstances for the spectrophotometric determination of nitrite, and it was shown in Figure (3).

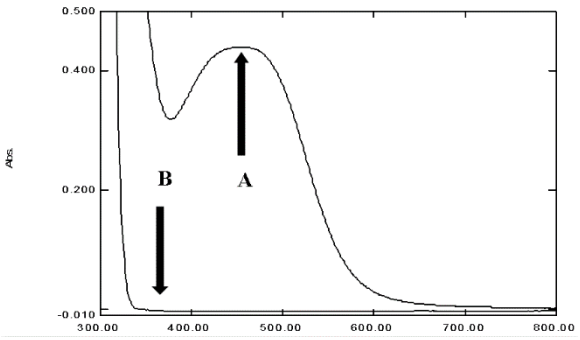


Figure 3. Absorption spectrum of (A) azo dye against reagent blank (B) blank against distilled water.

3.2.1.10. *Recommended Procedure*

To 10 mL standard flasks containing 1.0 mL of 0.15% p-bromoaniline solution and 1.0 mL of 1.0M HCl solution, a known amount of aqueous sample containing 50 g of nitrite has been added. then 0.5 mL of a 0.25% solution of paracetamol. The combination was turned into a caustic solution by addition of 1.0 mL of 2.0 M ammonium hydroxide solution. It was appropriately diluted with distilled water. After 5.0 minutes, the absorbance at 460 nm was measured in comparison to a reagent blank.

3.2.1.11. *Calibration Graph*

The Beer's law was seen to be followed across the range of (0.3-12 µg. mL^{-1}), as shown in figure (4), according to the calibration curve acquired by the DLLME technique. The nitrite ions detection limit was 0.05 µg. mL^{-1}. Table (1) displays the calibration curve's statistical data.

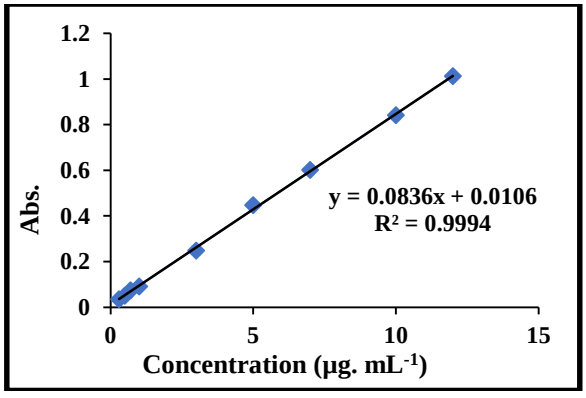


Figure 4. Calibration curve of nitrite ion determination.

Table 1. The calibration curve's statistical information, as determined by spectrophotometric analysis of the nitrite ion.

Parameter	Characteristic
λ max (nm)	460
Colour	Orange
Beer's law (µg. ml^{-1})	0.3-12.0
Detection limit (µg. ml^{-1})	0.20
Quantitation limit (µg. ml^{-1})	0.30
Coefficient of determination, R^2	0.9994
Molar absorptivity (L. $mol^{-1}.cm^{-1}$)	3.8432×10^3
Sandall's index (µg. cm^2)	0.0119

3.2.1.12. *Accuracy and Precision*

According to the virtues of the relevant (RSD %) and (Error %) for three replicate samples at 3 different concentration levels (within Beer's law range), the accuracy and precision of the estimation of NO_2^- ion were evaluated. The outcome shown in Table (2) shows that the method's accuracy and precision are adequate.

Table: 2 Accuracy and precision of the suggested spectrophotometric method.

Concentration of nitrite (µg.ml^{-1})	RSD %	Error %
0.3	0.1	-4.0
5.0	0.8	4.5
12	2.5	-0.2

3.3. DLLME Spectrophotometric Determination of Nitrite

The experimental conditions to achieve extraction of the formed azo dye utilising dispersive liquid-liquid microextraction were established. The investigation involves the dispersive to extractant volume ratio, speed of centrifuge and centrifugation time. Regarding all optimization conditions above, a preliminary dispersive solvent extraction investigated increasing intensity of absorption.

3.3.1. *Preliminary Experiment*

During the extraction 2.0 mL of ethanol (dispersive solvent) was mixed with 3.5 mL of chloroform (extractant solvent) and then dispersed using a 5.0 mL syringe through 10.0 mL of aqueous azo dye solution (described in section 3.2.1.10) containing (5.0 µg. mL^{-1}) in a 10.0 mL conical bottom

test tube the solution became blurred rapidly, The upper bulk aqueous phase was carefully extracted using a pipette following a 3.0 min./5000 rpm centrifugation stage. Finally, the resultant solution was transferred into a 1.0 mL quartz cell for spectrophotometric nitrite detection. The absorption spectrum of the organic phase against reagent blank shows that maximum absorption changed from 460 nm to 410 nm as well as the colour of azo dye turned to yellow with higher absorption intensity.

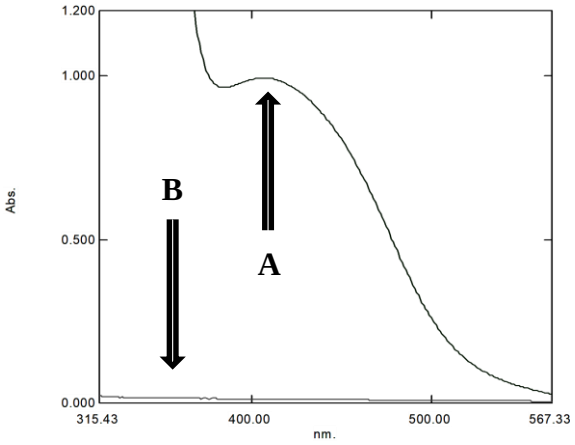


Figure 5. Absorption spectrum of (A) Extracted azo dye against reagent blank, and (B) blank against solvent.

3.3.2. *Optimization of the Experimental Conditions*

3.3.2.1. *Selection of Type and Volume of The Disperser Solvent*

To improve the contact area between the extractant and sample solution, a dispersive solvent's primary function is to disperse an extraction solvent as tiny droplets in the sample solution. The extraction solvent and sample solution should both be miscible with the dispersive that is being utilised. DLLME procedures essentially rely on the volume of a dispersive solvent. The extraction solvent's solubility and ability to disperse in the sample solution depend on the amount of disperser solvent used. Additionally, the extractant is satisfactorily disseminated as fine droplets while the analyte solubility in the aqueous phase increases at large volumes of the disperser, decreasing the extraction efficiency [28].

Different quantities of ethanol, including 0.5, 1.0, 1.5, and 2.0 mL, were mixed with 3.5 mL of chloroform and submitted to the same DLLME technique to obtain the best volume of disperser solvent. According to the findings, the extraction solvent (ER) was almost at its greatest in volumes of 2.0 mL. For the next trials, the ethanol volume was set at 2.0 mL.

Table 3. Volume of ethanol dispersive solvent.

Volume ethanol (mL)	Absorbance
0.0	0.772
0.5	0.913
1.0	0.930
1.5	0.921
2.0	0.968

3.3.2.2. *Effect of Centrifuging Rate*

The DLLME definition of extraction time is the interval between injecting the binary solvent and starting the centrifugation. With consistent experimental conditions, the impact of extraction time was investigated between 1 and 10 minutes. There is an indefinitely wide surface area between the extraction solvent and the aqueous phase. As a result, the complex is quickly transferred from the aqueous phase to the extraction solvent. Because the equilibrium state is quickly reached as a result, the DLLME technique has the advantage of having a very quick extraction time. [29].

Different centrifugation rates were used for a series of identical solutions. For 5.0 minutes, the centrifugation speed was changed from 1000 to 7000 rpm. When the rate is increased to 4000 rpm, the absorbance gradually increases and then roughly remains constant. It was determined that 5000 rpm was the ideal rate for centrifuging. According to the findings, the extraction rate (ER) was almost constant throughout the board and only slightly varied at 5000 rpm, which is why this rate was chosen for the next studies.

Table 4. Centrifuging rate.

centrifuging rate (rpm)	Absorbance
1000	0.932
2000	0.918
3000	0.944
4000	0.917
5000	0.973
6000	0.909
7000	0.905

3.3.2.3. *Centrifuging Time*

Effects of different centrifuging times were mixed to the same DLLME procedure. The results indicate that the ER was nearly highest at 3 min and other results are quite close to it. Therefore, the time was fixed at 3 min for the next experiments.

Table 5. Centrifuging time.

Centrifuging time (min.)	Absorbance
3.0	0.940
5.0	0.923
7.0	0.844
10	0.950
15	0.913

3.3.2.4. *Final Absorption Spectra*

The final absorption spectrum was evaluated after obtaining the ideal circumstances for the DLLME determination of nitrite, and it was shown in Figure (6).

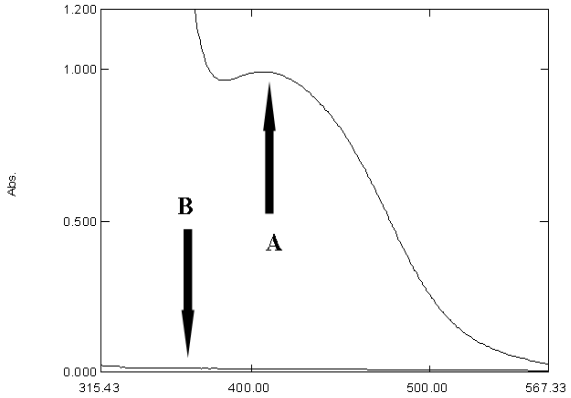


Figure 6. Absorption spectrum of (A) azo dye in organic phase against reagent blank (B) blank against solvent.

3.3.2.5. *Recommended Extraction Procedure*

The produced azo dye (5.0 µg. mL^{-1}) from the 10.0 mL volumetric flask (discussed in section 3.2.1.9) was put into a test tube with a conical bottom. Then, a binary solution containing 2.0 mL of ethanol (the disperser solvent) and 3.5 mL of chloroform (the extraction solvent) was swiftly injected into the sample solution to produce a stable hazy solution (water, ethanol, and chloroform). Then, chloroform was used to extract the nitrite ion into tiny droplets. The tiny chloroform droplets were brought together during the 3.0 min. at 5000 rpm centrifugation step, sediment at the base of the conical test tube. A pipette was used to carefully remove the upper bulk aqueous phase. The final step was to transfer the resulting solution into a 1.0 mL quartz cell. At 410 nm, the absorbance value was measured against a reagent blank.

3.3.2.6. *Calibration Curve*

Beer's law was seen to be followed across the range of 1-70 µg of nitrite ion in a final volume of 10 mL (i.e., 0.1-7 µg. mL^{-1}), as shown in figure (5), according to the calibration curve acquired by the DLLME technique. The nitrite ion detection limit was 0.0107 µg. mL^{-1}. Table (6) displays the calibration curve's statistical data.

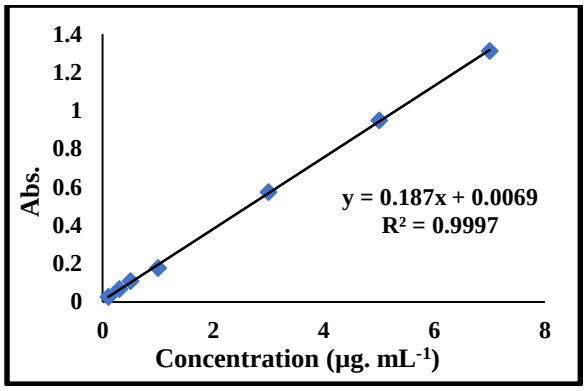


Figure 7. Calibration graph of Nitrite ion determination by DLLME method.

Table 6. The statistical data of the calibration curve obtained using DLLME-spectrophotometric determination of nitrite ion.

Parameter	Characteristic
λ max (nm)	410
Colour	Yellow
Beer's law (µg. ml^{-1})	0.1-7.0
Detection limit (µg. ml^{-1})	0.05
Quantitation limit (µg. ml^{-1})	0.10
Coefficient of determination, R^2	0.9997
Molar absorptivity (L. $mol^{-1}.cm^{-1}$)	$8.6024x10^3$
Sandall's index (µg. cm^2)	0.0053

3.3.2.7. *Accuracy and Precision*

According to the virtues of the relevant (RSD %) and (Error %) for three replicate samples at 3 different concentration levels (within Beer's law range), the accuracy and precision of the estimation of (NO_2^-) ions were evaluated. The outcome shown in Table (7) shows that the method's accuracy and precision are adequate.

Table 7. Accuracy and Precision of the proposed spectrophotometric method.

Concentration of nitrite (µg mL^{-1})	RSD %	Error %
0.3	0.355	4.1
3.0	2.540	0.9
7.0	4.384	-0.3

3.3.3. *Application of the Method*

The recommended study was applied successfully to the determination of nitrite in human saliva samples. To prove that the results are accurate a known amount of nitrite ion solution was spiked into half of samples. The results of spiked and un-spiked samples were compared and the results shown in Table (8).

Table 8. Analytical Results of Nitrite in Real Human saliva.

Parameters	sample	Saliva
Spiked (µg. ml^{-1})	0	5
Found (µg. ml^{-1})	0.5502	5.7869
RSD (n = 4)	0.0057	0.679
Recovery (%)	-	104.7
Error (%)	-	4.7336

3.4. Comparison of The Methods

In the comparison of the proposed spectrophotometric and DLLME methods with the reference methods, the novelty of the proposed methods is that they can determine for a wide range of analytes, New reagents were used in the Griess reaction, small LOD and LOQ (clear linearity in the range of physiological and pathological concentrations), high sensitivity, the less organic solvent used for extraction, and have a high coefficient of determination with good accuracy and precision, simplicity, inexpensive, free from toxic and difficultly soluble compounds, free from complex sample treatment, and extreme heating. as shown in Tables (9 and 10).

Table 9. Comparison of proposed spectrophotometric method for determination of nitrite with other spectrophotometric methods.

Reagents	Beer's law (µg. ml^{-1})	Sensitivity (L. $mol^{-1}.cm^{-1}$)	LOD (µg. ml^{-1})	LOQ (µg. ml^{-1})	R^2	Ref.
Nuclear fast red + potassium Bromate	2.0–45	0.6643 $x10^3$	0.7	2.5	0.997	[30]
Sulfanilic acid + α-Naphthylamine	0.01-0.1	0.1472 x 10^3	0.0015	0.0051	0.9992	[31]
Sulphanylamide + N-(1-naphthyl) ethylenediamie	0.23–9.2	4.1000 x 10^3	0.0621	0.23	0.9998	[16]
p-Bromoaniline +	0.3-12	3.8432 x 10^3	0.2	0.3	0.9994	P.W

Table 10: Comparison of proposed DLLME method for determination of nitrite with other extraction methods.

Method		Cloud-point extraction	In-syringe liquid microextraction	DLLME
Beer's law	(µg. ml^{-1})	0.01-1.0	$5x10^{-5}$-0.01	0.1-7
Sensitivity	(L. $mol^{-1}.cm^{-1}$)	747.64	4291.8	8.6024 $x10^3$
LOD	(µg. ml^{-1})	0.001	$1.3x10^{-5}$	0.05
Analyte		NO^{2-}	NO^{2-}	NO_2^-
Solvent		Triton X-114 (10%)	$[C_4MIM]PF6$	Chloroform-ethanol
Extractant volume (ml)		0.2	1000	3.5
Reference		[18]	[32]	Present work

3.5. Statistical Analysis

Statistical analysis was performed on the results obtained by the proposed methods and the reported method [30] for determination of human saliva using the Student's t- and F- tests at P=0.05, regarding both accuracy and precision; no significant difference was found as shown in Table. The data analysis was carried out by IBM SPSS statistic 23.

Table 11: Statistical analysis

Method	Human Saliva	
	DLLME-UV-Vis (proposed method)	HPLC-DAD (reference method)

Mean Recovery	104.7	104.23
%RSD	0.679	3.34
n	4	6
Student t-test* 2.353	0.6103	
F-value *	0.1673	

*Theoretical values of t and F at (P= 0.05).

4. Conclusion

The present study demonstrates the use of the DLLME-UV-Vis spectrophotometric method for the estimation of salivary nitrite content easily and a difficult sample treatment doesn't require. Saliva sample collection is non-invasive and reasonably simple. This procedure relies on the diazotization of p-bromoaniline in the presence of nitrite ions in an acidic medium to produce the appropriate diazonium ion, which is then combined with paracetamol in the presence of an ammonia solution to produce a stable and water-soluble azo-dye. We have proposed the use of DLLME for extracting nitrite ions by assembling azo dye from real human saliva samples. Since less extraction fluid is utilised than with LLE or SPE, this method is quick, sensitive, affordable, and has a high enrichment factor and low toxicity. In comparison to other detection methods like FAAS and ICP/OES, utilising spectrophotometry as a detection system also demonstrates a cheap initial and operational cost (no requirement for the combustion of gases).

5. Conflicts of Interest

There are no conflicts to declare.

6. Formatting of Funding Sources

By authors only.

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