# Griess Assay of Salivary Nitrite Content with Extraction Preconcentration

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**Abstract: Nitrite has been determined using a simple and sensitive spectrophotometric method. The suggested procedure is based on the diazotization reaction of p-bromoaniline in the presence of nitrite ion in a hydrochloric acid media, which produces diazonium salt. This salt is then combined with paracetamol in an ammonium hydroxide medium to produce an orange azo color with a maximum wavelength ($\lambda_{max.}$) of 460 nm, molar absorptivity (ε) is 3.8432 x $10^3$ L. $mol^{-1}.cm^{-1}$, and Beer's law was established between 0.3-12.0 µg. $mL^{-1}$. It displays LOD, LOQ, and $R^2$ values of 0.2 µg. $mL^{-1}$, 0.3 µg. $mL^{-1}$, and 0.9994 respectively. The dye can be extracted into chloroform. The yellow chloroform extract displays a maximum wavelength ($\lambda_{max.}$) of 410 nm, molar absorptivity (ε) is 9.93 x$10^3$ L. $mol^{-1}.cm^{-1}$, and Beer's law was observed between 0.1-5.0 µg. $mL^{-1}$. The LOD, LOQ, and $R^2$ values for the suggested extraction procedure were 0.05 µg. $mL^{-1}$, 0.1 µg. $mL^{-1}$, and 0.9996, respectively. The suggested method was successfully applied on samples of a human saliva.**

**Keywords: Nitrite, Griess Assay, Spectrophotometry, Liquid-Liquid Extraction, Paracetamol**

## 1. Introduction

For both plants and animals, nitrogen is an essential nutrient. Nitric oxide ($NO^{\cdot}$), nitrite ($NO_2^-$), and nitrate ($NO_3^-$) are its three environmental manifestations. Daily samples including environmental, dietary, and biological samples always contain them (Moshoeshoe & Obuseng, 2018). Nitrogen is present in the nitrite ion in an oxidation state that is highly unstable. Nitrite can be further reduced by chemical and biological processes into a variety of compounds or it can be oxidized into nitrate. (WHO, 2003). As an intermediary step in the creation of nitrate, nitrifying bacteria in nature produce nitrite (Schrenk et al., 2020). At least three conditions have been linked to nitrate exposure as risk factors: methemoglobinemia in infants receiving food and consuming water rich with nitrate (Adelana, 2005), the occurrence of neural tube abnormalities in offspring whose mothers experience comparable pregnancy-related exposures (Diaconu et al., 2001), formation of carcinogenic N-nitroso compounds (Parvizishad et al., 2017), hypothyroidism (Garcia Torres et al., 2022).

Through its interactions with secondary amines and amides, the production of N-nitrosamines in acid conditions, such as the stomach, was linked to $NO_2^-$. There is clear doubt that the lower molecular weight N-nitrosamines cause cancer in both humans and animals. Nitrite is therefore regarded a precursor to the formation of N-nitrosamine (Numan et al., 2021). There are several methods that have been presented for determining $NO_2^-$ such as, Electrochemical (Coviello et al., 2020; Zhang et al., 2018), fluorescence (Caroleo et al., 2022; Li et al., 2003), Chemiluminescence (Mikuška & Večeřa, 2003; Wang et al., 2020), spectrophotometry (Brizzolari et al., 2021; Hamoudi et al., 2020), and Chromatographic method (Zhao et al., 2015).

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Due to their simplicity and low cost, spectrophotometric methods are by far the most used techniques for determining nitrite and nitrate. These techniques mostly rely on the reaction of nitrite with some detecting reagents (such as diazotization or nitrosation reaction), After the reaction, a product's absorbance relates to the amount of nitrite present (Brizzolari et al., 2021) diazotization reaction (Hamoudi et al., 2020) and nitrosation reaction (Rished & Fakhre, 2008). The purpose of this study is to provide a sensitive and simple technique for the determination nitrite in human saliva utilizing the diazotization-coupling process. By extracting the unreacted color into the organic solvent, the method is rendered more sensitive.

## 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 pear-shaped separating funnel with a 100 ml capacity, and a shaking water bath, model Ycw-012S from Gemmy Co. 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-DeHaën AG) in (D.W). A pellet of sodium hydroxide and one milliliter of chloroform were added, and then distilled water was used to make the solution up to 100 milliliters. Working standard solutions of 100 g $mL^{-1}$ were freshly generated by diluting stock solution with distilled water (Rahim et al., 1983) A pellet of sodium hydroxide was added to prevent nitrite decomposition and 1.0 ml of chloroform to prevent bacterial growth (Veena & Narayana, 2009).

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.(Hamoudi et al., 2020).

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

Saliva samples were obtained from adult volunteers; saliva sampling may be difficult because of insufficient saliva flow. so, they took 15-20 min. to collecting enough amount of saliva directly from mouth to 10 ml test tube.

Nitrite can be reduced to nitric oxide or ammonia by many species of bacteria (WHO). Nitrite and nitrate are interconvertible as the components of the nitrogen cycle in the natural environment as well as the human body via the nitrate–nitrite–nitric oxide pathway (Tatarczak-Michalewska et al., 2019).

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 samples was collected. NaOH is utilized as a stabilizer since nitrite is unstable in acid solution.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 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% underestimation of the nitrite levels(Diaconu et al., 2001).

### 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 appropriate volume. Using matching 1 cm quartz cells, the absorbance at 460 nm was assessed after 5.0 min. against a reagent blank.

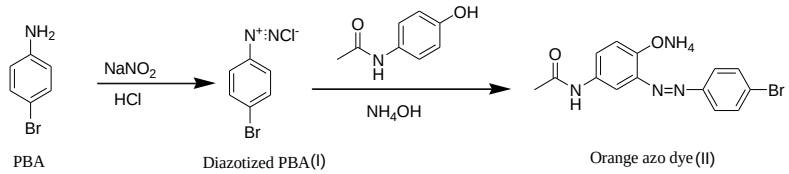
#### 2.4.2 Extraction Procedure

The produced azo dye was transferred from the 10 mL volumetric flask (as detailed in the 2.3.2 section above) to a 100 mL conical flask charged with 4.0 mL of chloroform, which was then put in a shaker water bath, the conical flask was aggressively shaken in a shaker at 25 $^0$C for 20 minutes before the solution was transferred to a separating funnel. The separation funnels were left in place for ten minutes. following equilibration to permit minute organic particles in the aqueous layer to cross over into the organic layer and finish phase separation. After 7.0 min. At 410 nm, the organic layer's absorbance was measured after 7.0 minutes in comparison to the reagent blank.

## 3. Results and Discussion

### 3.1 The Principal of the Method

The reaction that yields the colourful azo dye requires two steps. In the first stage, p-bromoaniline interacts with the nitrite ion to produce the p-bromophenyl 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 an alkaline solution and turns yellow upon extraction into chloroform. The following diagram illustrates the reaction:




Scheme 1: Schematic diagram of formation of azo dye.

### 3.2 Spectrophotometric Determination of Nitrite

It is detailed how spectrophotometry may be used to measure nitrite. It relies on the combination of the nitrite ion with an acidified p-bromoaniline solution to create a diazonium ion, which then combines with paracetamol in an alkaline medium to instantly form an orange colored, stable, and water-soluble azo dye

#### 3.2.1 Preliminary Investigation for Determination of Nitrite Ion

1.0 mL of 3.0 M hydrochloric acid 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 µg of $NO_2$- ion. The solution was then diluted to the appropriate volume with D.W in a 10 mL standard volumetric flask, and an orange dye resulted. The maximum absorbance at 442 nm can be seen in the colored dye's absorption spectra when compared to its comparable blank reagent, which exhibit little absorbance at this wavelength.

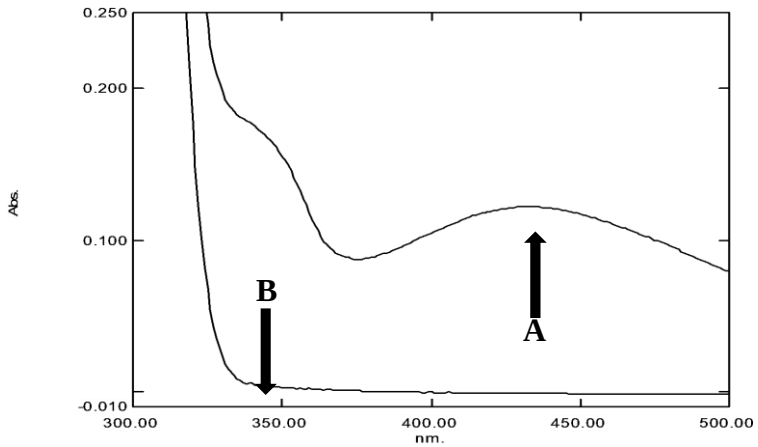



Figure 1: Absorption spectra of (A) azo dye against blank (B) blank against distilled water.

#### 3.2.2 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. (Numan et al., 2021).

##### 3.2.2.1 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, because $H_2SO_4$ and $HNO_3$ other than acidity have dehydration and oxidation property respectively then will disturb the reaction,

and $CH_3COOH$ and HCOOH are weak acids cannot dissociate well to provide high acidic solution. Table (1) shows the results.

Table 1: Effect of different acids on diazotization of (0.1 %) PBA.

| Acid solution (1.0 M) | HCl | $H_2SO4$ | $HNO_3$ | $CH_3COOH$ | HCOOH |
|---|---|---|---|---|---|
| Absorbance | 0.225 | 0.070 | 0.100 | 0.077 | 0.060 |

### 3.2.2.2 Effect of Hydrochloric Acid Amount

The influence of hydrochloric acid volume on the diazotization reaction has been examined over the range of (0.25- 2.0) ml. Best absorption intensities were achieved at the addition of 1.0 mL of 1.0 M HCl, because there is no significant difference from 1.0 mL to 2.0 mL, Table (2) shows this effect. Then 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 dramatically decrease. In higher concentrations the excess of acid converts the diazonium ion into diazonium salt (Kokhasmile, 2016). The results shown in Table (3). According to the observations of both Tables, the best absorption was given at 1.0 ml of 1.0 M with (pH=1.55), using less than 1.0 ml, and 1.0 M causing higher pH which is insufficient for diazotization.

Table 2: Effect of (1.0 M) HCl volume.

| HCl volume (mL) | 0.25 | 0.50 | 1.00 | 1.50 | 2.00 |
|---|---|---|---|---|---|
| Absorbance | 0.119 | 0.288 | 0.279 | 0.270 | 0.274 |

Table 3: Effect of HCl concentration.

| HCl concentration (M) | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 | 7.0 |
|---|---|---|---|---|---|---|---|
| Absorbance | 0.285 | 0.277 | 0.256 | 0.073 | 0.054 | 0.040 | 0.004 |

### 3.2.2.3 Effect of Diazotized P-Bromoaniline Concentration

The effect of various concentrations of 1.0 ml p-bromoaniline was studied on maximum absorption of the coloured azo-dye over the range of (0.05 - 0.25) %. Results show in Table (4) that the solution's optimal concentration for achieving the highest absorption was 0.15 %.

Table 4: Effect of p-bromoaniline concentration.

| Concentration of p-bromoaniline (%) | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 |
|---|---|---|---|---|---|
| Absorbance | 0.293 | 0.289 | 0.306 | 0.297 | 0.305 |

### 3.2.2.4 Effect of Paracetamol Concentration

The effect of 0.5 mL paracetamol concentration ranged from (0.05-0.25) % has been studied. From the results, as shown in Table (5) 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 and more than this amount of paracetamol is not soluble in water have to dissolve it by adding another chemical which may later interfere the reaction some way.

Table 5: Effect of paracetamol solution concentration.

| Paracetamol concentration (%) | 0.05 | 0.10 | 0.15 | 0.20 | 0.25 |
|---|---|---|---|---|---|
| Absorbance | 0.264 | 0.304 | 0.344 | 0.369 | 0.379 |

### 3.2.2.5 Effect of Base Types on Azo Dye

The effect of (5.0 mL, 1.0 M) of different type of strong and weak bases such as ($Na_2CO_3$, NaOH, KOH, and $NH_4OH$) on the coupling reaction of diazonium ion with paracetamol was investigated. The results shown in Table (6) indicates that the colored azo dye is formed in an alkaline medium and ammonium hydroxide solution gave maximum absorption among other bases.

Table 6: Effect of different base solutions on absorbance.

| Different base solutions | $NH_4OH$ | KOH | NaOH | $Na_2CO_3$ |
|---|---|---|---|---|
| Absorbance | 0.375 | 0.005 | 0.041 | 0.300 |

### 3.2.2.6 Effect of Ammonium Hydroxide Amount

The effect of different concentrations of ammonium hydroxide solution 5.0 mL was also investigated ranging from (0.5-3.0) M. The results shown in Table (7) indicate that 2.0 M (pH = 10) of the solution has the highest absorbance. Because the excess of hydroxide ion reacts with the reagents (the diazonium ion $ArN_2^+$) and tends to convert them to unionized compounds ($ArN_2OH$) which do not couple with the reagent (Kokhasmile, 2016). Then The influence of 2.0 M of base volume from (1.0-6.0) mL has been studied to achieve the maximum color density of the azo dye on absorbance were investigated. The result shown in Table (8) suggests that the absorbance decreased when the reagent volume was increased and reached maximum when 1.0 ml of 2.0 M was used. Therefore, it was recommended that 1.0 mL of $NH_4OH$ solution with a (pH = 9.88) be added for the studies that followed. It was not possible to use less than 1.0 ml because 0.5 ml of HCl cause (pH=7.9) which is too low for coupling reaction to occur properly.

Table 7: Effect of ammonium hydroxide concentration.

| $NH_4OH$ concentration (M) | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
|---|---|---|---|---|---|---|
| Absorbance | 0.004 | 0.375 | 0.431 | 0.441 | 0.438 | 0.413 |

Table 8: Effect of the ammonium hydroxide volume.

| $NH_4OH$ Volume (mL) | 1.0 | 2.0 | 3.0 | 4.0 | 5.0 | 6.0 |
|---|---|---|---|---|---|---|
| Absorbance | 0.428 | 0.386 | 0.353 | 0.329 | 0.305 | 0.305 |

### 3.2.2.7 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 (III) (PBA + HCl + $NO_2^-$ + APAP + $NH_4OH$) was used in following tests because it was found from the results that it gives the highest absorption of the produced azo dye. Table (9) indicates the results.

Table 9: Order of addition.

| Component of Reaction | Order Number | Absorbance |
|---|---|---|
| $NO_2^-$ + HCl + PBA + APAP + $NH_4OH$ | I | 0.453 |
| PBA + $NO_2^-$ + HCl + APAP + $NH_4OH$ | II | 0.447 |
| PBA + HCl + $NO_2^-$ + APAP + $NH_4OH$ | III | 0.463 |
| PBA + HCl + $NO_2^-$ + $NH_4OH$ + APAP | IV | 0.182 |

### 3.2.2.8 Development Time and Stability Period

The constancy and development of the colored dye depend on time, as a result, it is investigated under the ideal experimental circumstances mentioned above. According to the testing results, the chromophore forms to its maximum after 5.0 min from dilution to the mark and is stable for around 160 min before gradually fading, according to that its error % was 4.3 % and dos not out of accepted value. As indicated in Table (10).

Table 10: The stability of formed azo dye.

| Time (min) | Abs. |
|---|---|
| 0.0 | 0.399 |
| 5.0 | 0.440 |
| 10 | 0.439 |
| 15 | 0.439 |
| 20 | 0.435 |
| 30 | 0.437 |
| 40 | 0.433 |
| 50 | 0.429 |
| 80 | 0.429 |
| 110 | 0.428 |
| 140 | 0.427 |
| 160 | 0.421 |

### 3.2.2.9 Final Absorption Spectra

After obtaining the optimum conditions for the formation of the azo dye, the final absorption spectra checked, and it was found that the spectrum was the same as in Figure (2) except the maximum absorption was shifted to 460 nm and the intensity was increased.

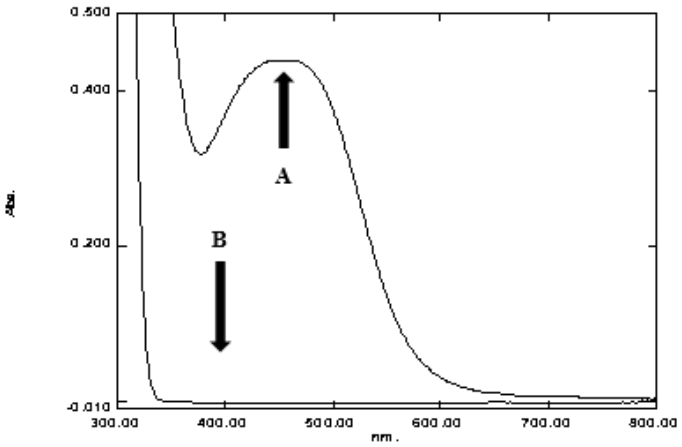



Figure 2: Absorption spectrum of (A) azo dye against reagent blank (B) blank against distilled water.

### 3.2.2.10 Recommended Procedure and Calibration Graph

Under the optimum conditions, to 10 mL standard flasks containing 1.0 mL of 0.15 % p-bromoaniline solution and 1.0 mL of 1.0 M HCl solution, a series of aqueous solutions containing (0.3-12.0) µg. ml-1 of nitrite was prepared and 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. calibration curve was obtained as shown in Figure (3) Beer's law was obeyed within the range of (3.0-120) µg / 10 ml and Table (11) shows statistical data of the method.

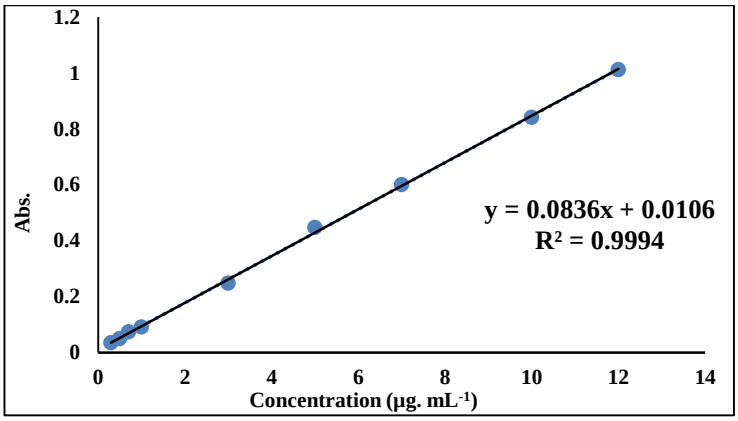



Figure **Error! No text of specified style in document.**.3: Calibration curve of nitrite ion determination.

Table 11: The calibration curve's statistical information, as determined by spectrophotometric analysis of the nitrite ion.

| Parameter | Characteristic |
|---|---|
| λ max (nm) | 460 |
| Color | Orange |
| Beer's law (µg. $ml^{-1}$) | 0.3-12.0 |

| | |
|---|---|
| Detection limit (µg. $ml^{-1}$) | 0.2 |
| Quantitation limit (µg. $ml^{-1}$) | 0.3 |
| Coefficient of determination, $R^2$ | 0.9994 |
| Molar absorptivity (L. $mol^{-1}.cm^{-}$ | 3.8432 x $10^3$ |
| Sandell's index (µg. $cm^2$) | 0.0119 |

### 3.2.2.11 Accuracy and Precision

According to the virtues of the relative standard error (Error %) and relative standard deviation (RSD %) 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 (12) shows that the method's accuracy and precision are adequate.

Table: 12 Accuracy and Precision of the proposed spectrophotometric method.

| Concentration of nitrite (µg.$ml^{-}$ | RSD % | Error % |
|---|---|---|
| 0.3 | 0.1 | -4.0 |
| 5.0 | 0.8 | 4.5 |
| 12.0 | 2.5 | -0.2 |

### 3.3 Liqui-Liquid Extraction Spectrophotometric Determination of Nitrite

Analytical chemistry includes the solvent extraction technique, which has been acknowledged as a superior separation approach because to its flexibility, speed, and simplicity. The analytical chemist can benefit greatly from extraction processes, which can be carried out with equipment as simple as a separatory funnel, in just a few minutes at most, and are applicable to both trace and macrolevels of metals. The diazo-coupling reaction is the foundation of the majority of the commonly used spectrophotometric techniques for nitrite measurement. Many diazo coupling techniques have limited sensitivity and need long sample times. Lower detection limits, increased sensitivity, selectivity, and faster sample times are all provided by techniques based on the extraction of the azo dye into an appropriate organic solvent. (Rasheed, 2003).

### 3.3.1 Preliminary Experiment

The content of the 10.0 mL volumetric flask of the prepared azo dye (described in 3.2.2.10) after 5.0 min. was transferred to a 100 mL conical flask and charged with 5.0 mL of chloroform, afterthought placed in shaker water bath, the conical flask was shaken vigorously for 20 min. at 15 0C and transferred to a separating funnel. The separating funnels were kept standing for 10.0 min. after equilibration to allow tiny organic particles in the aqueous layer to enter the organic layer and complete phase separation. Absorption spectrum of the organic phase against reagent blank shows that maximum absorption at 410 nm as well as color of azo dye turned to yellow with higher intensity.

### 3.3.2 Absorption Spectra

The absorption spectra seen in Figure (4) was obtained after the nitrite ion experienced the aforesaid extraction technique. In order to find the optimal conditions for the extraction of azo dye to the organic layer, the maximum absorption of the extracted azo dye was measured at 410 nm. At this point, the blank almost completely lacks absorbance.

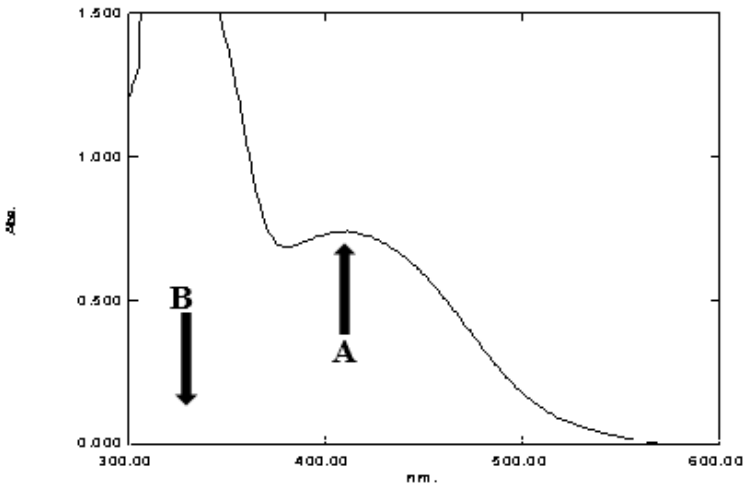



Figure 4: Absorption spectrum of (A) azo dye in organic phase against reagent blank (B) blank against solvent.

### 3.3.3 Optimum Conditions

To obtain the optimum conditions for the extraction of the azo dye, utilizing liquid-liquid extraction the following investigation were examined involves the type of organic solvent, the shaking time, volume of extraction, effect of time on absorbance after extraction, and the extraction temperature.

#### 3.3.3.1 Effect of Organic Solvent

In the LLE technique, the selection of extraction solvent is an important step. Generally, the suitable extraction should have a high affinity to analyte, low solubility in water, higher density and lower miscibility than the aqueous phase (Mzban et al., 2020). Efficiency of different organic solvents have been examined against their blank at 25 $^{0}$C as shown in Table (13) bellow results indicate that chloroform shows the greatest extraction ability among the studied solvents.

Table 13: Effect the Different Extraction Solvent.

| Extraction solvent | Chloroform | Tetrachloromethane | Hexane | Benzene |
|---|---|---|---|---|
| Absorbance | 0.935 | 0.521 | 0.148 | 0.599 |

#### 3.3.3.2 Effect Variation of Equilibration Time

A study to investigate the impact of shaking time on the extraction and final absorption intensity was examined by allowing the extraction to proceed by varying periods. The period of equilibration was varied from 5.0 to 30 min., while the other variables kept constant. The results in Table (14) reveal that the shaking time had a slight effect on both extraction and absorbance, and it was enough to shake it for more than 20 min. Then 20 min. was chosen as optimal because 25 min. is causing the process to be time consuming while there is not such a dramatic difference between their absorbance.

Table 14: Effect of extraction time.

| Extraction time (min.) | 5.0 | 10 | 15 | 20 | 25 |
|---|---|---|---|---|---|
| Absorbance | 0.712 | 0.692 | 0.749 | 0.910 | 0.926 |

### 3.3.3.3 Effect of Time on Stability of Azo Dye in Organic Phase

Impact of elapsing time interval after separating phases and before taking absorbance of organic phase has been studied in the range of 5.0 - 50 min. with constant experimental conditions. The results as shown in Table (15) indicate that the absorbance increases over time and reached maximum in 20.0 min. then gradually decrease until reaches constant value from 35-50 min, its recommended to read the absorbance of extracted azo dye after 7.0 min. to saving time, because there is no significant difference between absorbance of 20.0 min. and 7.0 min.

Table 15: Effect of time on stability of azo dye.

| Time (min.) | 5.0 | 10.0 | 15.0 | 20.0 | 25.0 | 30.0 | 35.0 | 40.0 | 50.0 |
|---|---|---|---|---|---|---|---|---|---|
| Absorbance | 1.062 | 1.062 | 1.302 | 1.353 | 1.309 | 1.350 | 1.258 | 1.266 | 1.250 |

### 3.3.3.4 Temperature Effect on Extraction of Azo Dye

The most significant parameter in solvent extraction is temperature. The viscosity of the solvent decreases as the temperature goes up, enhancing its capacity to moisten the matrix and solubilize the target analytes. The extract's equilibrium capacity, which varies depending on whether the process is exothermic or endothermic, is another influence (Mohammad & Fakhre, 2021).The extraction of species into an organic phase involves large changes in enthalpy (solvation processes) and in entropy (solvent orientation and restructuring), leading to considerable temperature effects (Orabi, 2013).

To study the effect of temperature on extraction of azo dye, extractions were carried out by mechanical shakier in controlled temperature condition for 20 min. Temperature was varied in the range 10 to 40 $^0$C. The results are presented in Table (16) showing that the absorbance of extracted organic phase was directly proportional with temperature and 25 $^0$C was chosen as best, because its room temperature and chloroform is a volatile solvent its volume will decrease if we use higher temperature the resulting extracted volume will not be sufficient to fill the cell.

Table 16: Temperature Effect on Extraction of Azo Dye.

| Temperature ($^0$C) | 10 | 15 | 20 | 25 | 30 | 35 | 40 |
|---|---|---|---|---|---|---|---|
| Absorbance | 0.964 | 1.010 | 1.090 | 1.002 | 1.119 | 1.398 | 1.539 |

### 3.3.3.5 Effect of Organic to Aqueous Volume Ratio

The preconcentration factor and sensitivity of the liquid-liquid extraction are highly dependent on the volume of organic phase (Mzban et al., 2020) therefore, Various volume of organic solvent used for extraction were studied. Various volumes of extraction solvent (chloroform) was studied. The results presented in Table (17) clearly showed that when greater volumes of extraction solvent (chloroform) were used in the ratio yields decreasing the absorbance value of the extracted dye. This is due to the dilution effect that helps to decrease the concentration of an analyte in the sediment phase. The ratio

of 4:10 organic to aqueous ratio was fixed due to that 3.0 mL of organic solvent is not enough to fill used cell.

Table 17: Effect of Organic Solvent Volume.

| Volume (mL) | 3.0 | 4.0 | 5.0 | 7.0 | 10 |
|---|---|---|---|---|---|
| Absorbance | 1.679 | 1.062 | 0.957 | 0.643 | 0.462 |

### 3.3.3.6 Final Absorption Spectra

After obtaining the optimum conditions for the extraction preconcentration process, the final absorption spectra checked, and it was found that the spectrum was the same as in (Figure 5) except the maximum absorption was shifted to 410 nm and the intensity was increased.

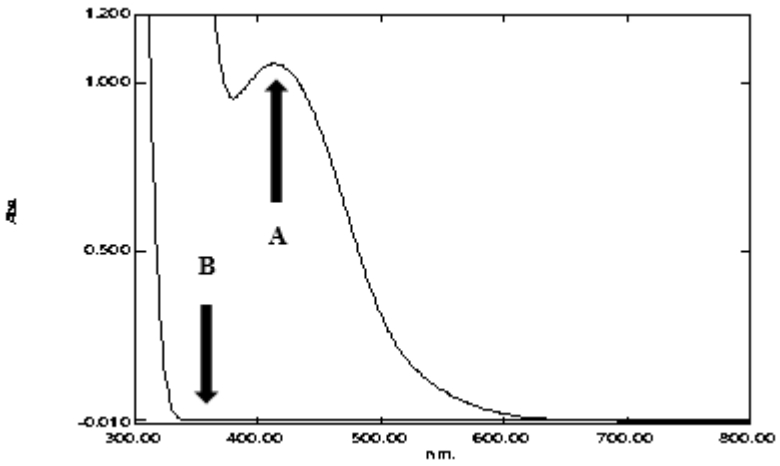



Figure 5: Absorption spectrum of (A) azo dye in organic phase against reagent blank (B) blank against solvent.

### 3.3.3.7 Recommended Extraction Procedure

The contents of the 10 mL volumetric flask of the prepared azo dye (described in section 3.2.2.10) was transferred to a 100 mL conical flask charged with 4.0 mL of chloroform, afterthought placed in shaker water bath, the conical flask was shaken vigorously in a shaker for 20 min. in 25 $^{0}$C and transferred to a separating funnel. The separating funnels were kept standing for 10 min. after equilibration to allow tiny organic particles in the aqueous layer to enter the organic layer and complete phase separation. After 7.0 min. (from optimization section 3.3.3.3) absorbance of organic layer was read at 410 nm against chloroform.

### 3.3.3.8 Calibration Curve

The calibration curve obtained by the extraction procedure showed that Beer's law obeyed over the range of 1.0-50 µg of nitrite ion in a final volume of 10 mL (i.e. 0.1-5.0 µg. mL-1) as shown in (Figure 6) The detection limit was (0.05 µg. mL-1) of nitrite ion. The statistical data of the calibration curve is shown in Table (18).

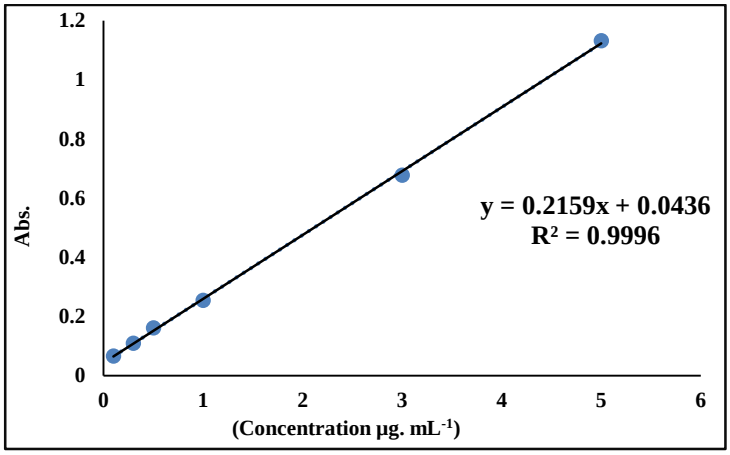



Figure 6: Calibration curve of the nitrite ion using liquid-liquid extraction spectrophotometric method.

Table 18: The statistical data of the calibration curve obtained using LLE-spectrophotometric determination of nitrite ion.

| Parameter | Characteristic |
|---|---|
| λ max (nm) | 410 |
| Color | Yellow |
| Beer's law (µg. $ml^{-1}$) | 0.1-5.0 |
| Detection limit (µg. $ml^{-1}$) | 0.05 |
| Quantitation limit (µg. $ml^{-1}$) | 0.1 |
| Coefficient of determination, $R^2$ | 0.9996 |
| Molar absorptivity (L. $mol^{-1}.cm^{-}$ | 9.93 $x10^3$ |
| Sandell's index (µg. $cm^2$) | 0.0046 |

### 3.3.3.9 Accuracy and Precision

The accuracy and precision of the determination of nitrite ion were studied depending on the value of the (Error %) and (RSD %) for three replicate samples respectively in three different concentration levels (within Beers law range). The result listed in Table (19) indicates an acceptable accuracy and precision of the method.

Table 19: Accuracy and Precision of the proposed spectrophotometric method.

| Concentration of nitrite (µg. $ml^{-}$ | RSD % | Error % |
|---|---|---|
| 0.1 | 0.59 | 2.36 |
| 1.0 | 2.36 | -2.41 |
| 5.0 | 2.02 | 0.76 |

### 3.3.3.10 Application of The Method

The recommended procedure was applied successfully to the determination of nitrite in human saliva samples. To proving that the results are accurate a known amount of nitrite ion solution was spiked into half of the samples. The results of spiked and un-spiked samples were compared and the results as shown in Table (20).

Table 20: Analytical Results of Nitrite in Real Human saliva.

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

### 3.4 Comparison of The Methods

In comparison of the proposed methods with the reference methods, it seems that the present methods have a wide range of determination, New reagents were used, smaller LOD and LOQ (clear linearity in the range of physiological and pathological concentrations), higher sensitivity, less organic solvent used for extraction, and have higher coefficient of determination as shown in Table (21 and 22).

Table 21: Comparison of the Spectrophotometric Methods

| Reagents | Beer's law (µg. $ml^{-1}$) | Sensitivity (L. $mol^{-1}.cm^{-1}$) | LOD (µg. $ml^{-1}$) | LOQ (µg. $ml^{-1}$) | $R^2$ | Reference |
|---|---|---|---|---|---|---|
| Nuclear fast red + potassium Bromate | 2.0–45 | 0.6643 $x10^3$ | 0.7 | 2.5 | 0.9970 | (Mousavi & Shirkhanloo, 2009) |
| Sulfanilic acid + α-Naphthylamine | 0.01-0.1 | 0.1472 x $10^3$ | 0.0015 | 0.0051 | 0.9992 | (Senra-Ferreiro et al., 2010) |
| Sulphanylamide + N-(1-naphthyl) ethylenediamie | 0.23–9.2 | 4.1000 x $10^3$ | 0.0621 | 0.23 | 0.9998 | (Brizzolari et al., 2021) |
| p-Bromoaniline + Paracetamol | 0.3-12 | 3.8432 x $10^3$ | 0.2 | 0.3 | 0.9994 | Present work |

Table 22: Comparison of the Extraction Methods

| Method | Beer's law (µg. $ml^{-1}$) | Sensitivity (L. $mol^{-1}.cm^{-1}$) | LOD (µg. $ml^{-1}$) | LOQ (µg. $ml^{-1}$) | Extractant (ml) | Reference |
|---|---|---|---|---|---|---|
| Cloud-Point Extraction | 0.01-1.0 | 0.7476 $x10^3$ | 0.001 | N/A | 0.2 | (Zhao et al., 2015) |
| In-syringe liquid microextraction | 0.05$x10^{-3}$ -0.01 | 4.2918 $x10^3$ | 0.000013 | N/A | 1000 | (Roohparvar et al., 2018) |
| Liquid-Liquid Extraction | 0.1-5.0 | 25.000$x10^3$ | N/A | N/A | 10 | (Tarafder & Roychowdhaury, 2018) |

| | | | | | | |
|---|---|---|---|---|---|---|
| Liquid-Liquid Extraction | 0.1-5.0 | 9.9296 x$10^3$ | 0.05 | 0.1 | 4.0 | Present work |

## 4. Conclusion

By using this spectrophotometric method, salivary nitrite can be estimated easily and a particular endowment doesn't require. Saliva sample collection is not interfering and reasonably simple. This procedure relies on the diazotization of p-bromoaniline in the presence of nitrite ion to producing the diazonium ion, which is then combined with paracetamol in the presence of an ammonia solution to produce a stable and water soluble azo-dye. Liquid–liquid extraction has been proposed for extracting nitrite ions assembling azo-dye from real human saliva samples. this approach is quick, easy, sensitive, affordable, and has a high enrichment factor and low toxicity Since very little extraction solvent is used, it has minimal toxicity. In comparison to other 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).

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