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# Two New Spectrophotometric Methods for the Determination of Isoniazid in Bulk form and Tablet Dosage Form

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## ABSTRACT

To develop two new spectrophotometric methods for the analysis of isoniazid in bulk form and tablets. The methods involved condensation of isoniazid with salicylaldehyde and diazo coupling with diazotized *p*-nitroaniline. Critical factors were optimised; evidence for new product formation, selection of analytical wavelengths, temperature and time and solvent for dilution. Validation was carried out according to ICH guidelines. The new methods were used for isoniazid tablets. Isoniazid formed an imine and azo adduct readily with the two reagents at 30 $^0$C after 5 and 20 mins, and determined at 405 and 420 nm, respectively. Low LODs were obtained for the two methods and recoveries were generally above 98%. The methods were successfully adopted for the assay of isoniazid in tablets and there were no significant differences in the contents when compared with the official titrimetric method of analysis. The methods could find application as in-process method in pharmaceutical industries.

**Keywords**: Isoniazid, spectrophotometric, hydrazone, *para*-nitroaniline, salicylaldehyde.

## INTRODUCTION

Tuberculosis is a communicable infectious disease characterised by weight loss, fatigue, productive cough and night sweats following alveolar implantation of

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the causative organism, *Mycobacterium tuberculosis* [1, 2]. With 1.6 million deaths in 2017 alone, tuberculosis (TB) is not only one of the top causes of death worldwide but the emergence of over half a million new cases of multidrug-resistant TB in the same year makes it a health-security threat [1]. Although there is only a 5-15% lifetime risk of developing the disease following infection, compromised immunity such as in people living with HIV increases the risk and fatality of TB. Nearly half of all HIV-negative and all of HIV-positive individuals with active TB will die without proper drug treatment which often consists of a combination of first line drugs including isoniazid [2]. Isoniazid, chemically pyridine-4-carbohydrazide, is converted to the active form which is bacteriostatic for latent bacteria and bactericidal for actively dividing microbes [3, 4].

Several methods have been reported for the quantitative estimation of isoniazid in bulk form, single- and multi-component dosage forms. These methods include HPLC analysis for both dosage and biological sample matrices using UV detection [5, 6] or less frequently mass spectrometry [7, 8], flow-injection chemiluminescence [9-11], fluorimetry [12], electrochemical methods [13] and titrimetry [14, 15]. Apart from the use of derivative spectroscopy and partial least squares [16, 17], the majority of spectrophotometric methods for the quantification of isoniazid takes advantage of the varied functionality and moderate reactivity of the drug which also serve to improve sensitivity and selectivity of the resulting methods. Thus, isoniazid because of its activated amino pyridine skeleton, has been used as coupling agents with diazotized reagents such as dapsone and 1-amino anthraquinone zinc chloride [18, 19]; in Schiff base formation with *cis*-cinnamaldehyde [20], 4-dimethylaminocinnamaldehyde [21] and as substrate for nucleophilic substitution reactions with epichlorohydrine and 4-hydroxyphenaylchloride [22]. The donor capability of the drug has also been employed in charge-transfer complexation with various acceptors including chloranil, bromanil [23] etc. and condensation reactions with 6,7-dichloroquinoline-5,8-dione, sodium 1,2-naphthoquinone-4-sulphonate [24, 25] etc. Colorimetric methods for the quantitative estimation of the analyte have also been reported based on the photodecomposition of Cu (II)-neocuproine complex and chlorpromazine free radical solutions following their reduction by isoniazid [14, 26].

These methods have their merits but suffer one or two drawbacks which include the use of expensive instrumentation and/or reagents that are not readily available in resource-limited countries, drastic reaction conditions such as elevated reaction times, long reaction times and strict control of pH.

The objective of this study was therefore to develop two simple, accurate and reliable colorimetric assay procedures for isoniazid following azo coupling with diazotized *p*-nitroaniline and Schiff base formation with salicylaldehyde.

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## METHODOLOGY

### *Chemicals and Reagents*

Salicylaldehyde, Isoniazid tablets, 1 M $H_2SO_4$, 96% ethanol, methanol, *n*-hexane, ethyl acetate, propan-1-ol, propan-2-ol, 1 M HCl, *para*-nitroaniline, sodium nitrite, potassium bromate powder, methyl red powder, potassium bromide powder, concentrated HCl (all analytical reagent grade from BDH, Poole, UK), isoniazid reference standard.

### *Equipment*

Analytical balance (Mettler H80), magnetic stirrer, pre-coated aluminium TLC plate, ultraviolet lamp 254/364 mm (PW Allen and Co., London), thermostated water bath, thermometer and Lambda 25 UV/VIS spectrophotometer (Perkin Elmer, UK).

### Methods

### *Preparation of isoniazid stock solution*

For the first method involving condensation with salicylaldehyde, a 0.0245 M solution of isoniazid (INH) was made by dissolving 0.0336 g of INH in sufficient quantity of ethanol and made up to 10 mL with ethanol. For method B involving diazo coupling reaction with *p*-nitroaniline, a $4.53\times10^{-2}$ M solution was prepared by weighing 0.0621 g quantity of INH and adding sufficient quantity of ethanol to make up to the 10 mL mark.

### *Preparation of reagents*

*Preparation of sulphuric acid*

Sulphuric acid (1 M) was prepared by diluting 13.5 mL of concentrated sulphuric acid with sufficient distilled water and making up to volume in a 250 mL volumetric flask.

*Preparation of 1 M HCl*

About 100 mL of distilled water was measured into a 250 mL volumetric flask; 44 mL of concentrated hydrochloric acid was also measured in the fume cupboard and added to the distilled water present in the volumetric flask. Distilled water was then added to make up to the 250 mL volume and the solution was shaken properly at intervals.

*Preparation of salicyaldehyde solution (Method A)*

A 0.0245 M solution of salicyaldehyde in 1M sulphuric acid was prepared by

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dissolving 0.13 mL of salicyaldehyde solution which is equivalent to 0.15 g of salicyaldehyde in 50 mL of sulphuric acid in a volumetric flask with vigorous shaking. The solution was then filtered to obtain a clear solution.

*Preparation of diazotized para-nitroaniline (DPNA) - Method B*

A 100 mg quantity of *para*-nitroaniline was weighed into a beaker and 15 mL 1 M HCl was added. This was placed on a hot water bath till it completely dissolved and then cooled to about 15 $^0$C and a 10%w/v solution of $NaNO_2$ (prepared by weighing 1 g of $NaNO_2$ into a 10 mL volumetric flask, distilled water was added to 10 mL) was added and stirred until one drop of the mixture turned starch iodide paper blue-black.

*Preparation of methyl red solution*

Methyl red (25 mg) was dissolved in a mixture of 0.93 mL of 0.1 M NaOH and 25 mL of ethanol (96%) in a 50 mL volumetric flask, sufficient distilled water was added to make up to 50 mL.

*Preparation of 0.0167 M Potassium bromate*

Potassium bromate (0.696 g) was weighed and transferred into a 250 mL volumetric flask, 100 mL of distilled water was added to dissolve the powder and distilled water was added to make 250 mL.

**Condensation of isoniazid with salicyaldehyde (Method A)**

***Evidence of condensation***

*Simple colour test (Spot test)*

A 0.5 mL aliquot of the stock solution of isoniazid was reacted with 0.5 mL of the stock solution of salicyaldehyde in a test tube. The change that occurred was observed immediately as well as after 20 minutes. The reaction was then repeated in another test tube. The test tube was thereafter immersed in a water bath at 60 $^0$C and the colour change was observed after 5 and 20 minutes.

*Thin layer chromatography*

Thin layer chromatographic analysis was carried out using pre-coated aluminium TLC plates. Samples of isoniazid, salicyaldehyde and adduct produced were spotted and the plates developed using three solvent systems; Ethyl acetate and Methanol (9:1); Ethyl acetate and Methanol (7:3) and Ethyl acetate and Hexane (5:5). The chromatographs were dried and visualized under visible light and UV lamp at wavelengths of 254 nm and 365 nm.

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### *Optimization studies*

*Selection of analytical wavelength*

An aliquot of isoniazid stock solution (0.5 mL) was reacted with 0.5 mL of the stock solution of salicyaldehyde. This was then made up to 5 mL with methanol in a sample bottle. 0.5 mL of the stock solution of isoniazid and 0.5 mL of the stock solution of salicyaldehyde were also made up to 5 mL with methanol in separate sample bottles. Using methanol as the blank for all three, the absorption spectra of isoniazid, salicyaldehyde and the reaction adduct were recorded using wavelength range of 190-800 nm. The spectra of the three solutions were then overlaid and the wavelength at which the reaction mixture showed absorbance without significant interference from the other two spectra was selected as the analytical wavelength.

*Optimization of solvent*

For this optimization step, the effect of completing the reaction with solvents such as methanol, ethanol, propan-1-ol and propan-2-ol was studied using reaction mixtures as previously described. The adduct in four separate test tubes were each made up to 5 mL with methanol, ethanol, propan-1-ol and propan-2-ol, respectively. Using each solvent as blank for the corresponding tubes, the absorbance of the condensation adduct in the different test tubes were determined at the selected analytical wavelength. The solvent which gave the highest absorbance reading was then selected as the best diluting solvent.

*Optimization of temperature*

A 0.1 mL quantity of the stock solution of INH was reacted with 0.1 mL of the stock solution of salicyaldehyde. The mixture was then incubated at 30 $^0$C for 5 minutes and 20 minutes. This was repeated at 50 $^0$C, 60 $^0$C and 70 $^0$C. In each case 4 mL of methanol was added to quench the reaction after cooling the tubes in an ice bath. This was done in duplicates. The absorbance of the sample mixture at each temperature was then measured to obtain optimal temperature.

*Optimization of time*

This reaction step was carried out by adding 0.1 mL of the drug solution to 0.1 mL of the salicyaldehyde solution in test tubes and incubated at the optimal temperature for 0, 2, 5, 10, 15, 20, 25 and 30 mins. The absorbance was measured at the selected analytical wavelength after terminating the reaction by cooling in an ice bath and thereafter adding 4 mL of methanol.

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*Optimization of acid concentration*

Different stock solutions of salicyaldehyde were prepared using different concentrations of sulphuric acid; 0.03125 M, 0.0625 M, 0.125 M, 0.25 M, 0.5 M, 1 M and 2 M solutions. 0.1 mL of the drug solution was then added to 0.1 mL of the different salicyaldehyde solution. The reaction was incubated at 30 $^0$C for 5 min and was terminated by cooling in ice-cold water and adding 4 mL of methanol. The absorbance of each mixture was then measured at 405 nm to determine the optimum acid concentration. This procedure was carried out twice.

*Stoichiometric ratio determination*

Equimolar (0.0245 M) solutions of INH and salicyaldehyde were prepared in their respective solvents. 0, 0.05, 0.066, 0.1, 0.134, 0.15, 0.2 mL of salicyaldehyde solution were measured into different test tubes and each was made up to 2 mL with the drug solution. The reaction mixtures were incubated at 30 $^0$C for 5 min. The reaction was terminated by cooling the reaction tubes in ice-cold water and making up to 5 mL with methanol. This was carried out in duplicate. The absorbance of each mixture was then measured at the selected wavelength.

***Calibration curve***

Calibration curve was prepared for the new condensation product within the concentration range 13.436 - 53.744 µg/mL of isoniazid (the linear part of the curve obtained from the determination of linearity of response) from the drug stock solution. To different test tubes containing 0.1 mL of salicyaldehyde solution each of 20, 30, 40, 50, 60, 70 and 80 µL of drug solution was added. The reaction was allowed to proceed at 30 $^0$C for 5 min. The reaction was quenched by cooling in ice-cold water and making the reaction mixture up to 5 mL with methanol. Triplicate preparations of each concentration were made and the absorbance determined at 405 nm against a reagent blank. The calibration curve was repeated on three consecutive days and the average values were used to generate a 3-day pooled calibration curve. The regression line equation and correlation coefficient were obtained from the calibration curve using linear regression analysis. The limits of detection and of quantification were obtained according to the ICH [27] guidelines using equations (1) and (2).

$$\mathbf{LOD} = \frac{3.3\sigma}{s} \quad \text{---------- (1)}$$

$$\mathbf{LOQ} = \frac{10\sigma}{s} \quad \text{---------- (2)}$$

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Where σ is the standard deviation of the blank signals and *s* is the slope of the calibration line.

### *Validation studies*

*Accuracy and Repeatability*

The new condensation reaction method was validated according to the International Conference on Harmonization (ICH) guidelines for validation of analytical procedures (ICH, 2005 [27]). Accuracy was evaluated at three concentrations (20.154, 33.59, 47.026 μg/mL) levels of the drug solution. To three test tubes containing 0.1 mL of salicylaldehyde solution, each of 30, 50, 70 μL of drug solution was added. The reaction mixtures were incubated at 30 $^0$C for 5 min and the reaction quenched by cooling in ice and making up to 5 mL with methanol. The absorbance of the reaction mixture was determined at 405 nm. The precision of the method was assessed with quadruple samples at each concentration and then estimated with percentage relative standard deviation (% coefficient of variation) while the accuracy was estimated with the recovery and percentage relative error.

### *Interference studies/ method selectivity*

The effect of common tablet excipients on the absorbance of reaction mixture was evaluated by weighing 5 mg of each of starch, talc, lactose, gelatin, magnesium stearate and a mixture of all 5 excipients into different test tubes. To each of the test tubes, 0.1 mL of salicylaldehyde solution was added. 0.05 mL of the isoniazid solution was added to each of the test tubes, the reaction mixtures were incubated at 30 $^0$C for 5 min and the reaction quenched by cooling in ice-cold water and making up to 5 mL with methanol. Quadruple sample preparations of each excipient mixture were made and the absorbance of each determined at 405 nm.

### *Analytical signal stability*

Volumes of salicylaldehyde and isoniazid solution corresponding to the mid-range concentration of the calibration curve (33.59 μg/mL) were measured into a test tube and the reaction mixture was incubated at 30 $^0$C for 5 min. The reaction was quenched by cooling the reaction mixture in ice cold water and making up to 5 mL with methanol. The absorbance of the reaction mixture was then determined at 0 min, 30 min, 1 hr, 1 hr 30 min, 2 hrs, 2 hrs 30 min, 3 hrs, 3hrs 30 min, 4 hrs and 24 hrs.

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### *Dosage form analysis*

Tablets of isoniazid were weighed and crushed and an amount of each brand equivalent to 0.03359 g of isoniazid was weighed and allowed to disperse in 10 mL of ethanol. The mixture was then filtered and 50 µL (33.59 µg/mL) of the solution was measured into a test tube containing 100 µL of the salicyaldehyde stock solution. The reaction mixture was incubated at 30 $^0$C for 5 min and the reaction was stopped by cooling in ice cold water and making up to 5 mL with methanol. The absorbance of the reaction solution was then determined at 405 nm. Five replicates were determined. This procedure was repeated for tablet brand B using an equivalent weight of 0.062 g.

## Diazo coupling reaction of isoniazid with DPNA (Method B)

### *Evidence of coupling reaction*

A 0.5 mL aliquot of diazotized *para*-nitroaniline (DPNA) was added to a test tube containing 0.5 mL of INH stock solution. The immediate colour change and the colour change after 20 minutes were observed. The colour formed was noted. The test tube was immersed in a water bath at 70 $^0$C for 5 minutes and 20 minutes and the colour change observed was noted. With significant colour change observed, TLC was carried out. On the TLC plate, the isoniazid (INH) stock, *para*-nitroaniline (PNA), DPNA and the azo adduct were spotted. The TLC plate was developed in mobile phase combinations as utilized for Method A.

### *Selection of analytical wavelength*

A 0.5 mL aliquot of DPNA was added to a test tube containing 0.5 mL of INH stock solution. The analytical wavelength was determined by recording the UV-VIS spectra from 190 to 800 nm using a scanning UV Spectrophotometer.

### *Optimization of coupling reaction temperature and time*

This reaction for the optimization of temperature was carried out as previously described for Method A using 0.1 mL aliquot of DPNA and 0.1 mL of INH stock solution. The reaction was terminated by placing the test tubes in an ice-bath. 10 mL of ethyl acetate was added to each reaction mixture and extracted. The absorbance value of the extract at 420 nm ($\lambda_{max}$) was recorded. All determinations were done in duplicates. For the optimization of reaction time, the procedure was repeated at 30 $^0$C for 0, 2, 5, 10, 15, 20, 25, 30, 35 and 40 minutes. The resulting reaction products were extracted into ethyl acetate and absorbance readings taken at 420 nm. All determinations were done in duplicates.

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### *Stoichiometric ratio of reagent-drug adduct formation*

Aliquots of INH stock solution: 0, 50, 66, 100, 134, 150 and 200 µL were transferred respectively into 7 test tubes. Into each test tube, 200, 150, 134, 100, 66, 50 and 0 µL of DPNA was added respectively. This was followed by incubation at 30 $^0$C for 20 minutes and 10 mL of ethyl acetate was added to each reaction mixture. The absorbance was measured at 420 nm and all determinations were done in duplicates.

### *Validation Studies*

*Calibration line*

Calibration lines using standard solutions of 6.212, 12.424, 18.636, 24.848, 31.06 and 37.272 µg/mL of INH and 0.1 mL of DPNA were generated on three successive days using the optimal analytical conditions as described above. Linear regression analysis was used to calculate the slope, intercept and the correlation coefficient (r) of the calibration line.

*Accuracy and Repeatability*

Three different concentrations; 12.424, 24.848 and 37.272 µg/mL of INH stock solution representing the lower, middle and upper range of the calibration line respectively were selected and each in turn was coupled with 0.1 mL of DPNA. This was done in quadruplicates and taken through the optimal analytical conditions already described. The absorbance values were determined at 420 nm. This determination was repeated for three days. Using the calibration line, the concentrations corresponding to these absorbance values obtained were determined and were used to calculate the errors, relative errors, standard deviations, relative standard deviations and recoveries of isoniazid from the quality control samples.

*Interference studies*

A 0.57156 µg/mL solution of INH was coupled with 0.1 mL of DPNA in 4 test-tubes each with 5 mg quantities of the excipients described for Method A. Five different excipients were used (talc, starch, gelatin, magnesium stearate and lactose. Also another set of 4 test-tubes contained a mixture of all the excipients. The coupled adduct was taken through the optimal analytical conditions and absorbance value was determined at 420 nm.

*Analytical Signal Stability Test*

A 0.57156 µg/mL solution of INH was coupled with 0.1 mL of DPNA, this was taken through optimal analytical conditions and the absorbance value was taken at 30 minutes interval for a period of 4 hours, as well as after 24 hours.

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### *Dosage form analysis*

For dosage form analysis using the newly developed method, a 0.57156 µg/mL solution was taken from the stock solution prepared and coupled with 0.1 mL of DPNA. This was taken through optimal analytical conditions and the absorbance value for six replicates was determined at 420 nm.

For the official method (British Pharmacopoeia [4]), the quantity of isoniazid powder (crushed tablets) equivalent to 0.250 g was dissolved in distilled water in a 100 mL volumetric flask and diluted to the 100 mL mark with distilled water. 100 mL of distilled water was added to 20 mL of the solution and 20 mL of concentrated HCl was added as well. 0.2 g of potassium bromide was weighed and added and 0.1 mL of methyl red solution was added as indicator. This was titrated drop-wise with 0.0167 M of potassium bromate until the red colour disappeared.

## RESULTS AND DISCUSSION

The two new procedures described in this research involved the spectrophotometric determination of isoniazid following condensation reaction with salicylaldehyde to form hydrazone as well as diazo coupling reaction of INH with diazotized *para*-nitroaniline. The two procedures provided accurate and simple approaches for the determination of this important anti-tubercular drug. Isoniazid still remains an important drug in the drug management of tuberculosis in the tropics. The need to provide readily adaptable methodologies for the determination of INH formed the primary motivation for this research design.

### *Evidence of reaction between INH and reagents*

In both methods A and B, evidences for condensation and diazo coupling reactions were established by spot tests and analytical thin layer chromatographic analyses. In method A, both the INH and salicylaldehyde solutions hitherto colourless produced a yellow colour following contact with each other. The intense yellow colour became pronounced with time and at elevated temperatures. Likewise for method B, diazo-coupling reaction between INH and DPNA gave a yellow colour indicating the formation of an azo dye. Similarly, for the DPNA method, the colour produced became intense at elevated temperatures.

Thin layer chromatographic analyses revealed the formation of new products that are distinct from the starting materials. The results obtained for the thin layer chromatographic analyses are presented in Table 1. In method A, thin layer chromatography also showed that a new product was formed with $R_f$ value that is distinct from those of isoniazid and salicylaldehyde. From the struc-

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ture of salicylaldehyde (Fig. 1), the presence of the carbonyl group which is capable of hydrogen bond acceptance, coupled with the O-H bond, which allows for hydrogen bond donation, makes it polar but if compared to isoniazid, the latter has 2 sites for accepting hydrogen and a hydrazine moiety ($NHNH_2$) that can give out hydrogen which is less polar. Moreover, intramolecular hydrogen bond can be formed between the carbonyl group and the O-H bond which will make the system look like a two-ring system (Fig. 1). This also reduces the polarity of salicylaldehyde. It follows that its $R_f$ value should be the highest. For isoniazid, the presence of the pyridine ring in its structure contributes more to its polarity than benzene ring contributes to the polarity of salicylaldehyde. The hydrazine moiety ($NHNH_2$) is capable of hydrogen bond donation which is stronger compared to the O-H bond in salicylaldehyde (already involved in intramolecular hydrogen bonding). The resultant effect is that isoniazid behaves in a more polar fashion than salicylaldehyde. The condensation reaction between the amino functional group of isoniazid and the carbonyl of salicylaldehyde to give the imine group reduces the hydrogen bond donation capability of the hydrazine group in isoniazid, making it less polar that isoniazid. That loss is however compensated by the additional phenolic OH in its structure. Furthermore; the extra aromatic ring character in the condensation product makes the condensation product less polar than isoniazid but more polar than salicylaldehyde. Hence, the $R_f$ value obtained showed that the condensation product is of intermediate polarity between the slightly polar isoniazid and the non-polar salicylaldehyde. The non-polar nature of salicylaldehyde became clearly evident as it was the only component that migrated in the highly non-polar solvent combinations of ethyl acetate: hexane. Neither the drug nor the reagent moved from the origin in this particular mobile phase system. For method B, the $R_f$ values obtained also confirmed the presence of completely different compounds relative to the starting materials of INH and DPNA. Unlike the scenario observed for Method A, the azo adduct was the least polar of the three components. The non-polar nature became apparent while using the third mobile phase system (EtOAc: *n*-Hexane) where it was the only spot that migrated from the origin. The presence of two ring systems bridged by the azo linkage and the presence of the residual nitro group of DPNA must be responsible for this relatively non-polar behaviour.

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**Table 1.** Thin Layer Chromatographic analyses for the two methods

| | | Method A | | Method B | |
|---|---|---|---|---|---|
| **Solvent System** | **INH** | **Salicylaldehyde** | **Condensation product** | **DPNA** | **Azo adduct** |
| EtOAc: MeOH (7:3) | 0.47 | 0.73 | 0.59 | 0.51 | 0.70 |
| EtOAc: MeOH (9:1) | 0.22 | 0.6 | 0.43 | 0.51 | 0.77 |
| EtOAc: n-Hex (5:5) | 0 | 0.61 | 0 | 0 | 0.49 |

EtOAc = Ethyl acetate; MeOH = Methanol; *n*- Hex = *n*- Hexane; DPNA = Diazotized *para*-nitroaniline

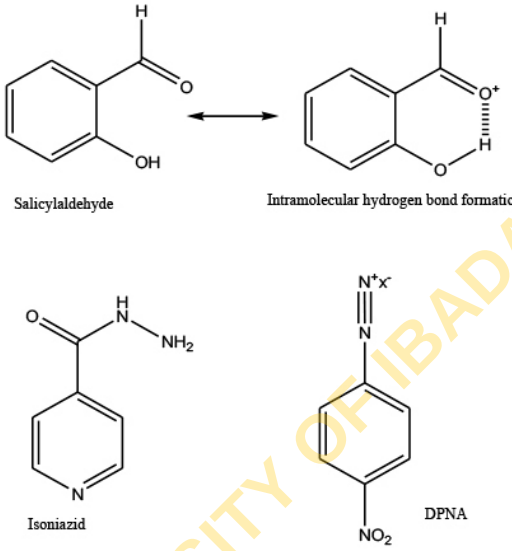


**Figure 1.** Structures of salicylaldehyde, pseudo ring structure of salicylaldehyde, Isoniazid and Diazotized *para* nitroaniline (DPNA).

### *Selection of Analytical Wavelengths*

The results obtained for the selection of analytical wavelengths for methods A and B are presented in Figures 2 and 3 respectively. The UV-Vis spectra for the condensation reaction between INH and salicylaldehyde (method A) as presented in Figure 2 shows that absorbance for INH and salicylaldehyde are almost non-existent beyond 380 nm. The condensation reaction produced a new spectral pattern for the reaction with INH which an evidence of condensation reaction is. The spectrum of the condensation adduct however resulted in a hyperchromic effect in the spectra; that is, an increase in the intensity of absorption. This new absorption maximum is also accompanied by slight bathochromic shift ($+\Delta\lambda$ = 2-5 nm). Optimal difference in absorptivity between

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salicylaldehyde and the condensation product was found at 405 nm which was selected as the analytical wavelength for subsequent analytical work. In figure 3, it can be observed that absorbances for INH and DPNA were almost non-existent beyond 350 nm wavelength. However, both adduct and PNA had significant absorbance beyond 400 nm, that is, there was a bathochromic shift. Also, for the new adduct, a hyperchromic effect was observed. At around 420 nm, there was an observed difference in absorbance of PNA and adduct hence 420 nm was selected as the analytical wavelength. The observed difference between absorbance of PNA and adduct was further confirmed by taking the absorbance of both solutions at selected wavelengths around visible region and this was used to determine the final choice of analytical wavelength at 420 nm.

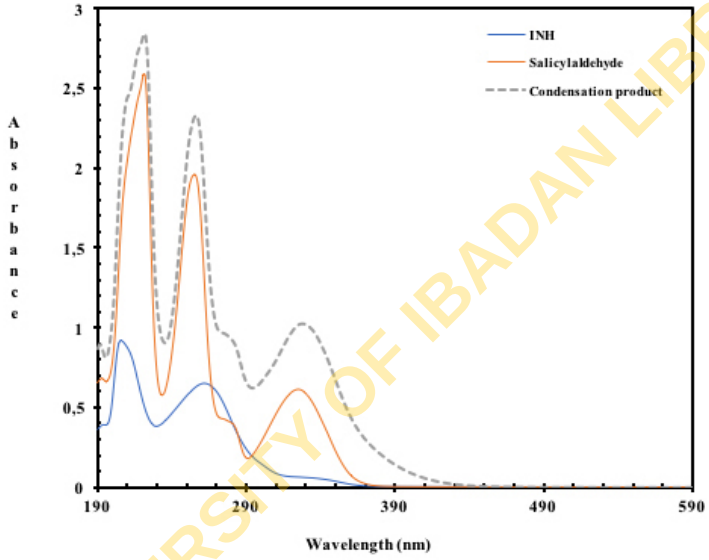


**Figure 2.** Overlaid absorption spectra for the condensation reaction between INH and Salicylaldehyde.

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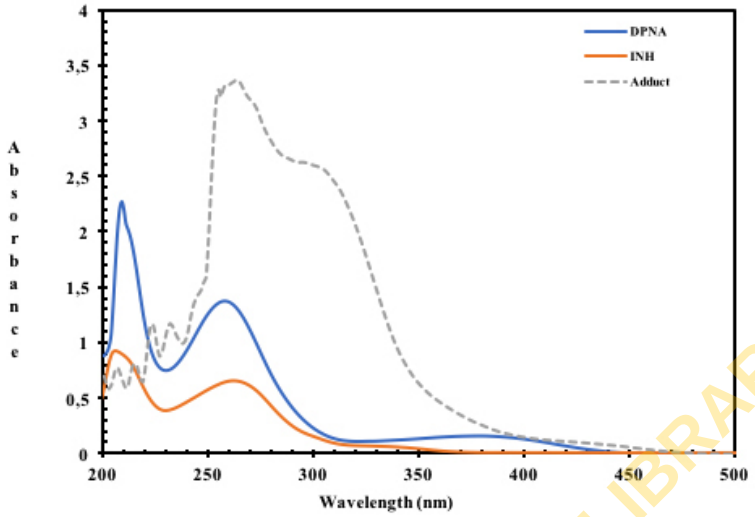


**Figure 3.** Absorption spectra for the azo dye formation between INH and DPNA.

### *Optimisation studies*

Several factors that can affect the formation of coloured products between INH and the two derivatisation reagents were identified and optimised in a series of steps in order to improve the accuracy of the new methods.

*Optimisation of solvent for dilution*

For method A, the condensation reaction will involve expulsion of water as INH reacts with salicylaldehyde. Thus, the formation of the Schiff base will be promoted by a solvent that will remove the water molecule so that it will not catalyze the decomposition of the product formed. In this assessment the alcohols, methanol, ethanol, propan-1-ol and propan-2-ol were utilized. Methanol gave the optimum absorbance value among the alcohols and was therefore selected as the optimal solvent. Methanol is more effective than the other solvents listed above in extracting water thereby stabilizing the product as the equilibrium is moved more towards the right, favoring formation of the Schiff base (adduct) rather than its hydrolysis back to the aldehyde. The suitability of methanol may be explained by its 50:50 ratio of hydrophobic to hydrophilic component. For method B, extraction of the azo adduct formed into ethyl acetate was found to give the optimal result.

*Optimisation of temperature and time of reaction*

The results obtained for the optimisation of temperature are presented in Figure 4 for both methods A and B. For method A using salicylaldehyde and meth-

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od B using diazotised *p*-nitroaniline, the optimisation of temperature which was done at 5 different temperatures showed that 30 $^0$C was the optimum temperature. The absorbance was the highest both at 5 and 20 minutes, the absorbance values thereafter declined at higher temperatures. This decrease in absorbance at increased temperature could be due to thermal decomposition of the reaction products formed. However, for method B, the temperature pattern was not readily deciphered for the 5 min profile. This might be due to the insufficient energy available to drive the reaction at such low duration of exposure. In the assessment for method B, 50 $^0$C was required before optimal absorptivity could be attained and thereafter attained a plateau at 60 $^0$C. Since a higher advantage will be derived from determining INH at a lower temperature of 30 $^0$C (a gain of over 45% in absorptivity between the two temperature levels) at 20 mins, the effect of varying reaction condition at this temperature was further investigated.

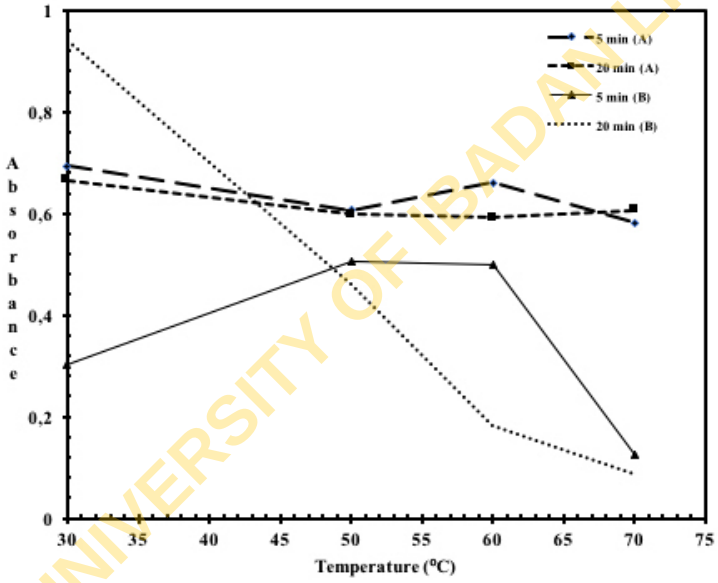


**Figure 4.** Optimization of temperature for the two new methods.

At the optimum temperature of 30 $^0$C, the time required for maximum absorbance was investigated for the two methods. The results are presented in figure 5. Salicylaldehyde was established to react with isoniazid at 30 $^0$C for 5 minutes. The optimum time was determined at the following reaction times; 0, 2, 5, 10, 15, 20, 25 and 30 min. The absorbance was highest at 5 minutes. Hence, 5 minutes was selected as the optimum time for reaction. For the DPNA method, optimum time was found to be 20 minutes. This is in consonance with the result obtained for the temperature optimisation.

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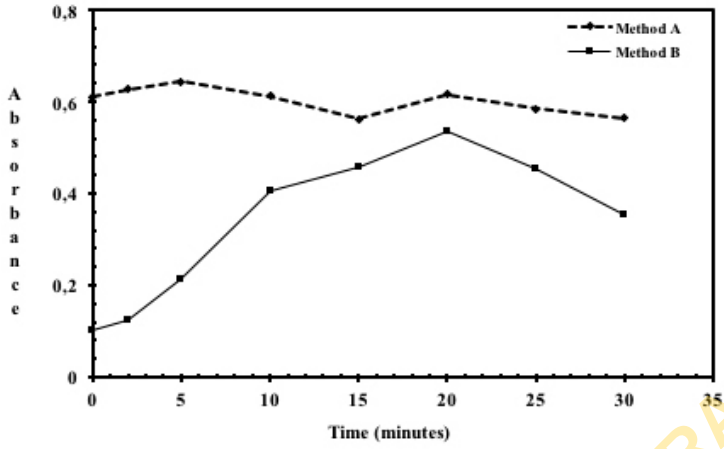


**Figure 5.** Effect of time allowed for reaction at optimal temperatures for methods A and B.

*Effect of acid concentration (method A)*

The effect of varying the concentration of $H_2SO_4$ used to prepare salicylaldehyde solution was studied at different concentrations from 0.03125 to 2 mol/L. The absorbance peaked at 1mol/L acid concentration, after which it declined. If the reaction medium is too acidic, the amine in the isoniazid structure becomes protonated such that it lacks the unshared electrons and is no longer nucleophilic. This inhibits the first step in the nucleophilic addition of the amine to the carbonyl function in salicylaldehyde. However, the reaction medium must be sufficiently acidic to promote the carbonyl oxygen of salicylaldehyde as this makes coupling easier since the carbonyl carbon then becomes more electrophilic (as seen in fig. 6). The optimum acid concentration selected was 1 mol/L.

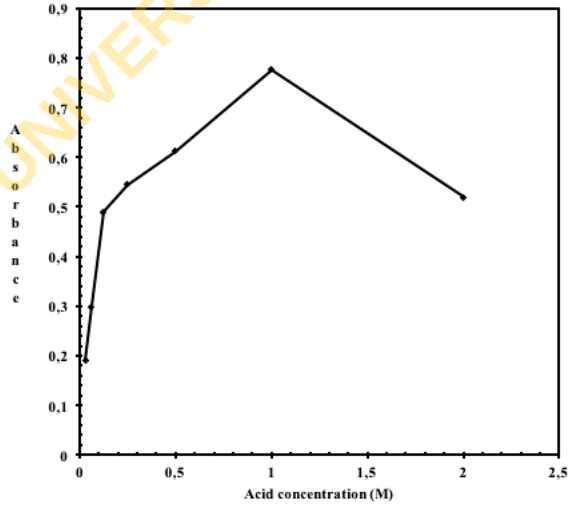


**Figure 6.** Effect of acid concentration on the condensation reaction (Method A).

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### *Stoichiometric ratio determination*

Optimal absorbance was observed at the drug-adduct ratio 1:1 for both methods A and B as presented in fig. 7. Hence, subsequent analytical work was carried out with this drug-adduct ratio. In the reaction of INH with salicylaldehyde, the result obtained implies that the nucleophilic addition involved the primary amine in isoniazid rather than its secondary amine. For the nucleophilic addition to occur with the secondary amine, a dehydrating agent will be required to expunge water resulting in the corresponding enamine product. However, in this case, the primary amine can be said to undergo nucleophilic addition in the absence of a dehydrating agent to give the imine containing compound, a hydrazine. The reaction between INH and DPNA (method B) giving a 1:1 mole ratio also implies that only one site is available on the INH molecule for the electrophilic attack as evidenced also by a single spot on TLC analysis.

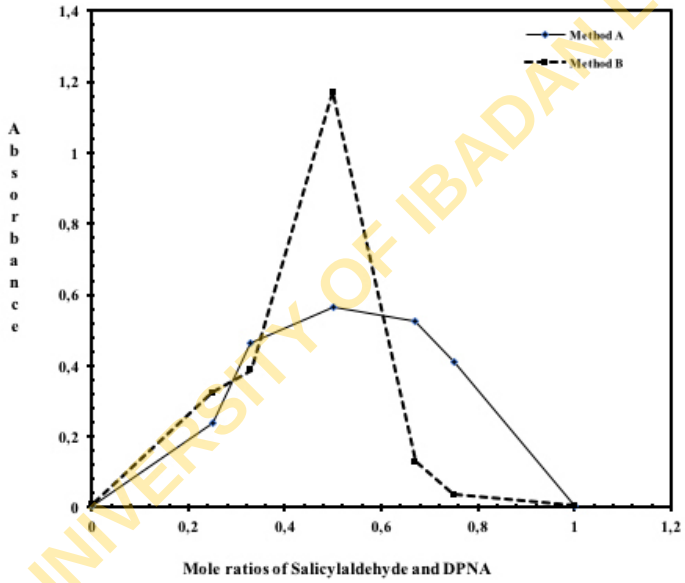


**Figure 7.** Stoichiometric ratio determination for INH using methods A and B.

### *Validation studies*

The results obtained for the analytical and validation parameters for the assessment of INH by the two new spectrophotometric methods are presented in Table 2. For the utilisation of salicylaldehyde (method A), calibration curves were prepared under the established conditions, by plotting absorbance as a function of the corresponding concentrations for the average of the results obtained on each of 3 consecutive days. A linear relationship was observed

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between absorbance at 405 nm and concentrations of isoniazid in the range of 13.436 - 53.744 µg/mL. Linear regression was obtained for the reaction between salicyaldehyde and isoniazid with a correlation coefficient of 0.9967 and a coefficient of determination of 0.9935. Molar absorptivity was calculated from the calibration curve. For method B, a pooled calibration data for three days over the concentrations range of 6.212 – 37.272 µg/mL gave an excellent curve with a correlation coefficient (r) of 0.9965 and coefficient of determination ($r^2$) of 0.993. In both methodologies adopted for the analysis of INH, low values were obtained for the LOD and LOQ. These results point to the sensitivity and suitability of the methods for the determination of INH.

**Table 2.** Analytical and Validation parameters for the two spectrophotometric methods

| **Parameter** | **Method A** | **Method B** |
|---|---|---|
| Analytical wavelength (nm) | 405 | 420 |
| Beer's law limit (µg/mL) | 13.436 - 53.744 | 6.212 – 37.272 |
| Correlation coefficient (r) | 0.9967 | 0.9965 |
| Coefficient of determination ($r^2$) | 0.9935 | 0.9930 |
| Slope ±95% Confidence interval | 0.0096 ± 0.0008 | 0.0358 ± 0.000058 |
| Intercept ± 95% Confidence interval | 0.0552 ± 0.0176 | 0.2068 ± 0.0007 |
| Molar absorptivity (L $mol^{-1}$ $cm^{-1}$) | $1.597 \times 10^3$ | $4.04 \times 10^3$ |
| LOD (µg/mL) | 0.230 | 0.101 |
| LOQ (µg/mL) | 0.695 | 0.306 |

*Assessments of Accuracy and Repeatability*

Using the optimised conditions for both methods A and B, the accuracy and repeatability assessments were carried out using a 3-day period. The results for the intra-day and inter-day accuracy and precision are presented in Tables 3 and 4 respectively.

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**Table 3.** Intra-day accuracy and repeatability determination

| Method A | | | | | | |
|---|---|---|---|---|---|---|
| **Concentration taken (µg/mL)** | **Concentration found (µg/mL)** | **Recovery (%)*** | **S.D.** | **RSD (%)** | **Error** | **Relative error (%)** |
| 20.154 | 20.55 | 101.98 | 0.37 | 1.78 | 0.39 | 1.94 |
| 33.59 | 33.03 | 98.32 | 0.47 | 1.42 | 0.56 | 1.71 |
| 47.026 | 49.02 | 104.23 | 2.01 | 4.09 | 1.99 | 4.06 |
| **Method B** | | | | | | |
| 12.424 | 12.180 | 98.04 | 0.05 | 0.39 | 0.24 | 1.96 |
| 24.848 | 25.162 | 101.26 | 0.09 | 0.36 | 0.31 | 1.26 |
| 37.272 | 37.257 | 99.96 | 0.02 | 0.06 | 0.02 | 0.04 |

**n = 4 for each concentration level; SD = standard deviation; RSD = Relative standard deviation*

**Table 4.** Assessments of Inter-day accuracy and repeatability

| Method A | | | | | | |
|---|---|---|---|---|---|---|
| **Concentration taken (µg/mL)** | **Concentration found (µg/mL)** | **Recovery (%)*** | **S.D.** | **RSD (%)** | **Error** | **Relative error (%)** |
| 20.154 | 20.33 | 100.86 | 0.45 | 2.20 | 0.17 | 0.85 |
| 33.59 | 33.75 | 100.47 | 0.78 | 2.31 | 0.16 | 0.46 |
| 47.026 | 48.45 | 103.03 | 2.22 | 4.58 | 1.43 | 2.94 |
| **Method B** | | | | | | |
| 12.424 | 12.199 | 98.19 | 0.06 | 0.45 | 0.23 | 1.81 |
| 24.848 | 25.169 | 101.29 | 0.07 | 0.27 | 0.32 | 1.29 |
| 37.272 | 37.259 | 99.97 | 0.03 | 0.08 | 0.01 | 0.03 |

**n = 12 for each concentration level; SD = standard deviation; RSD = Relative standard deviation*

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For method A, the percentage relative error for intra-day accuracy was less than 5%, with recovery of 98.32–105.06%, indicating good accuracy. The percentage relative standard deviation for the intra-day precision did not exceed 5%, indicating good repeatability. For inter-day accuracy, the percentage relative error was less than 5%, with recovery of 100.47–103.03%. The percentage relative standard deviation was 2.20 – 4.58%, indicating good reproducibility. Recovery studies also measure the effectiveness of sample preparation. The results showed that 33.59 µg/mL was the optimal analyte size recommended for routine use of this assay procedure. For method B, the recoveries for the intra-day assessment ranged between 98.04 and 99.96% while the RSD and relative errors were generally less than 0.5% and 2.0% respectively. For the inter-day analysis of the recovery studies, 98.19 – 101.29 range was obtained with RSD and % relative error less than 0.5% and 2.0% respectively. The high recoveries obtained coupled with low errors and low relative standard deviations attest to the suitability of the two methods for the analysis of INH in bulk drug.

***Interference liabilities***

A study of possible interference by common excipients used in formulation (starch, lactose, talc, magnesium stearate and gelatin) of pharmaceuticals showed no interference from any of the excipients. The results are presented in Table 5. The percentage recoveries obtained for method A were between 95.37-103.44% which shows good accuracy with relative errors generally less than 4%. Similarly, for method B, there was no interference from the matrix of the tablet excipients as recoveries of 99.22 – 101.41% were obtained. In addition, the relative error and relative standard deviation were less than 0.5 and 1.5% respectively. These results once again establish the ability of the two methods to selectively determine INH in the presence of tablet excipients and thus conferring great measure of accuracy.

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**Table 5.** Interference liabilities with common tablet excipients

| Excipients | Method A | | | Method B | | |
|---|---|---|---|---|---|---|
| | Concentration taken (µg/mL) | Concentration found (µg/mL) | Recovery ±S.D. (%) | Concentration taken (µg/mL) | Concentration found (µg/mL) | Recovery ±S.D. (%) |
| Talc | 33.59 | 32.40 | 96.46 ± 0.23 | 21.742 | 21.663 | 99.63 ± 0.08 |
| Starch | 33.59 | 32.04 | 95.37 ± 0.53 | 21.742 | 21.718 | 99.89 ± 0.03 |
| Lactose | 33.59 | 33.86 | 100.80 ± 0.89 | 21.742 | 21.607 | 99.38 ± 0.09 |
| Gelatin | 33.59 | 34.74 | 103.44 ± 0.71 | 21.742 | 21.573 | 99.22 ± 0.05 |
| Magnesium stearate | 33.59 | 34.59 | 102.97 ± 0.77 | 21.742 | 21.814 | 100.33 ± 0.08 |
| Mixture of excipients | 33.59 | 34.69 | 103.28 ± 1.12 | 21.742 | 22.048 | 101.41 ± 0.05 |

**n = 4 for each concentration level; SD = standard deviation*

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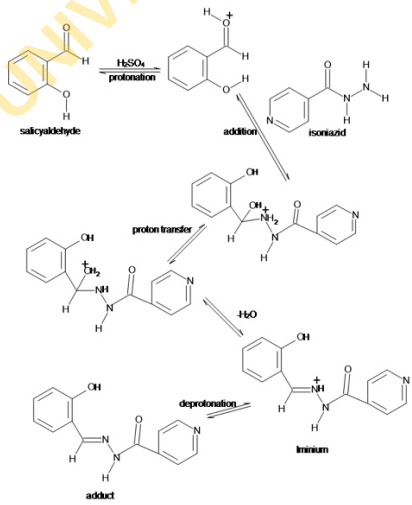
### *Analytical signal stability*

For method A, good analytical signal stability was obtained for the entire period. However, for method B, it was observed that the wrapped adduct was more stable than the unwrapped. Over a period of 30 minutes, absorbance values of both wrapped and unwrapped samples attained constant stability but the variation in stability for the wrapped was relatively higher when compared to the unwrapped for the entire study period.

### *Reaction Mechanisms of the two new spectrophotometric methods*

The proposed reaction pathways for the two methods for the determination of INH are presented in Schemes 1 and 2 for the salicylaldehyde and DPNA respectively. For the condensation reaction (Scheme 1), a nucleophilic reaction between INH and salicylaldehyde occurs *via* the formation of an iminium and eventually a mole of water was expunged to create a stable molecule, (*E*)-N`-(2-hydroxybenzylidene) isonicotinohydrazide. For the diazo coupling reaction of INH with diazotized *para*-nitroaniline (method B, Scheme 2), an aromatic ring undergoing attack by a diazonium must generally contain a powerful nucleophile (an electron donating group) such as –OH, -$NH_2$ etc. Possibly due to the size of the attacking species, coupling takes place almost always at the *para* position to the activating group except if the *para* position is occupied, in which case, coupling occurs at the *ortho* position to the activating group (Adegoke, 2012) [28]. Since the *para* position to the activating group in INH is occupied by a hydrazide moiety, which is actually a *meta*-directing group, this leaves the *ortho* position to the activating group as the available coupling site. This *ortho* position is also at the *meta* position relative to the hydrazide hence there is further reinforcement of the position for coupling to occur to give the molecule (*Z*)-2(2-(4-nitrophenyl)diazenyl)pyridine-4-carbohydrazide as the azo adduct.




**Scheme 1.** Proposed condensation reaction between INH and salicylaldehyde in methanol.

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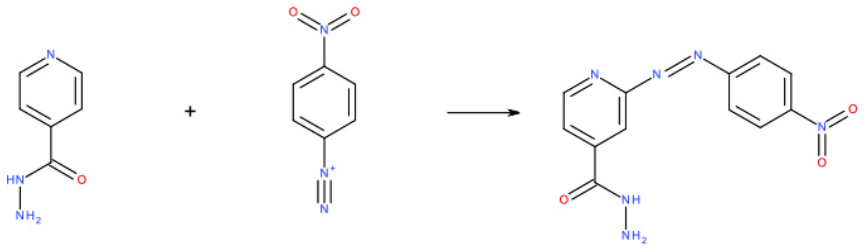

**Scheme 2.** Coupling pattern between INH and DPNA in ethyl acetate medium.

### *Dosage forms Analysis*

The proposed methods were adopted for the determination of INH in two commercial brands of isoniazid tablets containing 300 mg isoniazid active ingredient. The samples passed the weight uniformity test and the quantitative determination using the new methods. The recoveries obtained were 98.75%±0.69 and 99.67%±1.58 for brands A and B respectively using the condensation method. For the second method, recoveries obtained for tablet brands A and B are 99.13%±1.73 and 99.13%±1.47 respectively. On calculating the recoveries of isoniazid from the tablet brands relative to the official titrimetric assay, values close to 100 % were obtained by both methods. This implies that the two new methods can accurately determine the contents of isoniazid in tablets. The *F* test was used to estimate the difference in variance between the new method and the official method, while Student's *t* test was used to compare the mean recovery, with 95% confidence interval. The results obtained are presented in Table 6. The two tests showed lack of statistical significant differences in the precision and accuracy of the two new methods compared to the official method and thus implies that the methods are equivalent.

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**Table 6.** Comparative tablet analysis using methods A, B and official titrimetric method

| Tablet Formulation | Method A | | | | Statistics[b] | | Method B | | | | Statistics[b] | | Official method | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Found (%) | RSD (%) | Recovery (%)[a] | Error (%) | t-test | F-test | Found (%) | RSD (%) | Recovery (%)[a] | Error (%) | t-test | F-test | Found (%) | RSD (%) |
| A | 98.75 | 0.69 | 99.86 | 0.142 | 0.78 | 0.67 | 99.13 | 1.75 | 100.24 | 0.242 | 0.78 | 0.05 | 98.89 | 0.58 |
| | | | | | | | | | | | | | | |
| B | 99.67 | 1.58 | 99.81 | 0.191 | 0.86 | 0.32 | 99.13 | 0.14 | 99.27 | 0.736 | 0.18 | 0.06 | 99.86 | 0.84 |

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[a]*Mean value calculated from the recoveries of new methods and official assay (n=6)* [b]*p-values*

Many spectrophotometric methods reported in the literature either lack sensitivity or specificity. However, the specificity of this method has been proven by the determination of the drug in the presence of commonly used excipients and no interference was recorded. The azo adduct and the condensation products obtained also showed good stability over 4hrs and 24hrs.

The 3D optimized structures of the resultant products from the two reaction mechanisms are presented in Figure 8. The predicted Log P values for the respective adducts using condensation and azo adduct formation are 1.64 and 1.08. This explains the results obtained from the TLC analyses where significant migrations were observed for the new products under the normal phase mode. A cursory look at the 3D structures for the products revealed the staggered natures of the products and hence accounting for the stabilities observed on the bench. The staggered natures of the bonds allowed for optimal stability with minimal steric hindrance. The heat of formation of the condensation product was predicted as -214.17kJ/mol justifying the spontaneity of the formation of the condensation product in the presence of a mineral acid.

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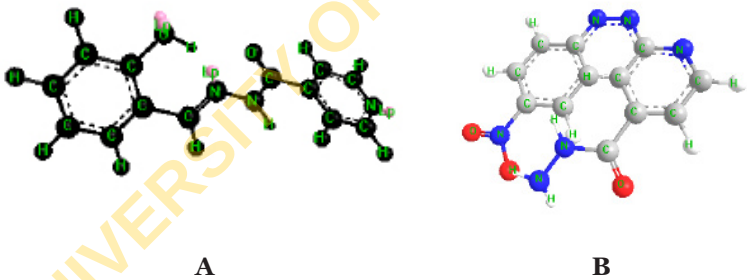

A B

**Figure 8.** 3D optimized structures for the reaction products of isoniazid with salicylaldehyde and diazotized p-nitroaniline.

### *Advantages of the methods over previously reported methods*

The new methods as proposed in this report are simple and easy to carry out compared to the HPLC method described in the United States Pharmacopoeia [30]. The methods also have lower limits of detection when compared with previously reported methods. The methods are also simpler than the method developed by Jennings *et al.* (1974) [30] which involves the conversion of the analyte

from one oxidation state to another before its determination.

The proposed methods do not require the use of sophisticated equipment when compared with the method developed by Lapa *et al*. (2000) [12] which involves fluorimetric analysis. The reagents used for the proposed methods are readily available and environmentally friendly when compared with the reagents required for the method developed by El-Brashy *et al*. (1992) [14] which requires the use of reagents such as neocuproine and epichlorohydrine which may not be readily available to the analyst.

Two new spectrophotometric methods involving relatively available reagents were successfully developed for the assay of isoniazid in bulk and tablet dosage forms. The methods are simple and could find applications in the in-process quality control for the manufacture of isoniazid tablet and as rapid analytical methods for the assay of the drug.

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