

**DEVELOPMENT AND VALIDATION OF FOUR NEW UVSPECTROMETRIC  
METHODS FOR THE DETERMINATION OF ISONIAZID (INH) IN PURE AND  
TABLET DOSAGE FORMS**

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**MARCH, 2019**

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DOSAGE FORMS**

**BY**

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**DEPARTMENT OF PHARMACEUTICAL AND MEDICINAL CHEMISTRY,  
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AHMADU BELLO UNIVERSITY,  
ZARIA, NIGERIA**

**MARCH, 2019**

### **Declaration**

I declare that the work in this Dissertation entitled “Development and Validation of Four New UV Spectrophotometric Methods for Determination of Isoniazid in Pure and Tablet DosageForm” was carried out by me in the department of pharmaceutical and medicinal chemistry ABU Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Maigari Hamza Kakudi

.....

.....

Name of student

Signature

Date

### **Certification**

This Dissertation entitled “Development and Validation of Four New UV Spectrophotometric Methods for Determination of Isoniazid in Pure and Tablet Dosage Form” by Maigari Hamza Kakudi meets the requirements for the award of the degree of Master of Science in pharmaceutical chemistry of the Ahmadu Bello University and was approved by the committee for its contribution to knowledge and literary presentation.

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## ABSTRACT

Tuberculosis (TB) is a serious infectious disease which is on increase since emergence of human immunodeficiency virus (HIV) leading to TB/HIV co infection, causing complications and treatment failure. Isoniazid is a first line drug for the treatment of TB therefore it is important to develop methods that will monitor its quality. In this study, four rapid, simple, relatively accurate, and precise spectrophotometric methods for the quantitative determination of isoniazid in pure and tablet dosage form were developed and validated. Methods 1 and 2 were based on dissolving standard isoniazid powder in distilled water and methanol respectively and scanning using UV-Vis spectrophotometer at a wavelength range of 200 - 400 nm to determine the wavelength of maximum absorption ( $\lambda_{max}$ ). Method 3 was based on condensation reaction between isoniazid and 2,4-di-nitrophenyl hydrazine (2,4 DNPH) and heating under acidic condition using 2 mL of 85 % hydrochloric acid (HCl) to form an orange-red hydrazone which was allowed to stand for few minutes for complete color development. Method 4 was based on the diazotization of isoniazid with sodium nitrite ( $\text{NaNO}_2$ ) and coupling with paraaminotoluidine under acidic condition (2 % HCl) and gently heating to give a coloured chromagen (diazo dye) which was allowed to stand for few minutes for complete color development. The coloured hydrazone and diazo dye formed were scanned at visible range of 400-750 nm to determine their  $\lambda_{max}$ s. Calibration curves were prepared using these methods. The developed methods were validated using ICH guidelines with respect to linearity, precision, accuracy, percentage recovery, limit of detection (LOD) and limit of quantification (LOQ). Standard isoniazid powder and a sample of isoniazid tablet were assayed using each method and compared with BP, 2009 method for the assay of isoniazid. Method 1 and 2 were observed to have a  $\lambda_{max}$  of 264 nm while an orange-red and a yellow chromagen colours were formed for methods 3 and 4

with time for complete development and  $\lambda_{\max}$  15 and 20 minutes, 464 and 420 nm respectively. Methods 1 and 2 obeyed Beer's law within a concentration range of 1-11  $\mu\text{g/mL}$  while the range for methods 3 and 4 was 1-18  $\mu\text{g/mL}$ . The correlation coefficient for the calibration plot was 0.999 in each case. The precision of the methods were  $\leq 1.57$  (percentage coefficient of variation, % CV) while the accuracy, percentage relative error (% Er) and percentage recoveries were  $\leq 4.130$  and  $\leq 102.01$  respectively. The methods have LOD and LOQ of  $\leq 0.145$  and  $\leq 0.559$   $\mu\text{g/mL}$  respectively. All the validation parameters were within the normal ranges. The percentage content of isoniazid in the standard powder and brand of the tablet assayed using all the developed methods were within the BP range of 99 - 101 % and 98.0 – 102 % respectively. There was no significant difference ( $p < 0.05$ ) between the percentages drug content assayed using the developed methods and that of the BP method, thus the developed methods can be interchanged with the BP method for quantitative estimation of isoniazid in pure and tablet dosage form.



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## ABBREVIATIONS

ABU	Ahmadu Bello University
BCG	Bromo cresol green
BPB	Bromo phenol blue
BP	British pharmacopoeia
CA	Chloranilic acid
DDQ	2, 3-dichloro-5, 6-dicyano- 1,4-benzoquinone
LOD	Limit of detection
LOQ	Limit of quantification
DNPH	Dinitrophenylhydrazine
EPI	Epichlorohydrine
FTIR	Fourier transform infrared
H <sub>2</sub> SO <sub>4</sub>	Sulphuric acid
H <sup>1</sup> NMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
HCl	Hydrochloric acid
HPC	4-hydroxyphenacylchloride
HNQ	2-hydroxy-1,4-napthoquinone

ICH	International conference on harmonization of requirements for registration of pharmaceuticals for human use
INH	Isoniazid
KIO <sub>4</sub>	Potassium meta per iodide
MPA	6-methyl-2-pyridine carboxaldehyde
NaIO <sub>4</sub>	Sodium meta per iodide
NaNO <sub>2</sub>	Sodium nitrite
NQS	1, 2-naphthoquinone-4-sulfonate
NAFDAC	National agency for food and drug administration and control
NBS	N-bromosuccinate
P-toluidine	paraaminotoluidine
%CV	Percentage coefficient of variation
%Er	Percentage relative error
p-DAB	paradimethylaminobenzaldehyde
PDT	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine
R <sup>2</sup>	Coefficient of determination
r	Coefficient of correlation
SD	Standard deviation

TB Tuberculosis

SPSS Statistical package for social sciences

TCNE Tetracyanoethelene

ThBThymol blue

UV-Vis Ultraviolet/Visible

$\lambda_{\max}$ Wavelength of maximum absorption

WHO World health organization

2, 4-DNPH

2,4-dinitrophenylhydrazine

## CHAPTER ONE

### 1.0 INTRODUCTION

Isoniazid (INH) also known as isonicotinyl hydrazide, is a synthetic derivative of isonicotinic acid hydrazide, chemically known as pyridine-4-carboxylic acid hydrazide (BP, 2009). It is first line drug in the treatment of pulmonary and extra pulmonary tuberculosis in combination with other anti-tubercular drugs such as rifampicin, ethambutol, pyrazinamide and streptomycin to avoid development of resistance (WHO, 2010, Niraimathi *et al.*, 2013). Isoniazid is a pro-drug and requires activation by the bacterial catalase-peroxidase enzyme (BP, 2009).

HPLC is the most sensitive and selective means of determining isoniazid in its pure and tablet dosage form and methods have been reported (Khuhawret *et al.*, 2005; Milan-segovia *et al.*, 2007; Dhal and Sharma, 2009; Abdallah *et al.*, 2016). However, the cost of procurement and the technical hands to operate the HPLC machine is a major challenge in most developing countries. On the other hand UV/Vis spectrophotometry is the most readily available instrument in research laboratories for quantitative determination of drugs in their pure and tablet dosage form. This could be due to its low cost, simplicity, relative accuracy and precision (Raza *et al.*, 2003, 2005a, 2005b).

Several UV/Vis spectrophotometric methods for the determination of isoniazid in pure and tablet dosage form have also been reported. A few of these reports include Babu *et al.*, 2002, Kamel, 2008, Oga, and 2010; Arifa *et al.*, 2013 among others. However, most of these methods suffer the disadvantage such as; the use of expensive chemicals which are not readily available [Rind *et al.*, 2005; (HNQ), Mana *et al.*, 2008; (BCG, ThB, BPB, DDQ). Niraimathi *et al.*, 2014; (p-DAB). , heating and cooling for long time (Devani *et al.*, 1985; Issopouloset *et al.*, 1991), indirect and tedious

procedure involving many steps and intermediates (Manalet *al.*,2008; Mohammed *et al.*,2015), low sensitivity (high values of LOD and LOQ) and requiring extraction(Niraimathiet *al.*, 2014)].

The process of identifying drugs and other materials by UV-Visible spectroscopy through their color was probably one of the earliest molecular absorption spectrophotometry for quantitative determination of drugs (Marczenko, 2000). The first measurements were made using eye as the detector and undispersed sunlight or artificial light as the light source, later it was found that the accuracy and the precision could be improved by isolating specific frequencies of light using optical filters. Further improvement of the measurement came with the use of prism and grating monochromators for wavelength isolation. Photoelectric detectors were developed, but quickly replaced with phototubes and photomultiplier tubes. The development of solid state microelectronics has now made available a wide range of detector type which if coupled with the computers; provide highly sophisticated readout electronic systems which subsequently leads to development of UV/Vis spectrophotometry (Marczenko,2000).

The presence of free  $\text{—NH}_2$  and  $\text{—C=O}$  groups in isoniazid can serve as suitable sites for coupling with suitable reagents to form Schiff bases or diazo dye. The colour intensity of these compound can serve as a means for determination of the drug concentration spectrophotometrically.

### **1.1Theoretical Frame Work**

The mechanism of UV-Vis spectrophotometry is based on absorption of light within UV and visible region and is considered as one of the important technique for quantitative analysis of drugs in their pure and tablet dosage form. Visible light represents a very small part of the electromagnetic spectrum and is generally considered to extend from 400-750 nm. When a solution or object transmits or absorbs only part of the radiation in the visible spectrum, it

appears colored. There is a close relation between the color of a substance and its electronic structure. A molecule exhibits absorption in the visible or ultraviolet range, when radiation causes an electronic transition, raising the molecule (ion) from the ground state to an excited state. The production or change of a color is connected with deformation of the normal electronic structure of the molecule. Irradiation causes variations in the electronic energy of the molecules containing one or more chromophoric groups like atomic groups with unsaturated linkages. Two or more chromophoric groups in a molecule often enhance one another's effect, to deepen the color by displacing the maximum absorption ( $\lambda_{max}$ ) towards longer wavelengths (from the ultraviolet towards the visible). This is called bathochromic shift. The displacement of the absorption maximum from the visible towards the ultraviolet is known as a hypsochromic shift (Blaedel and Meloche, 2001). The color of a molecule may be intensified by substituents called auxochromic groups. These groups may also cause bathochromic shifts. The color determining factor in a number of molecules is the introduction of conjugation of double bonds by means of electron donor and electron acceptor groups. The quantitative applicability of the absorption method is based on the fact that the number of photons absorbed is directly proportional to the number or concentration of atoms, ions or molecules (Harvey, 2000; Marczenko, 2000;Blaedel and Meloche, 2001).

## **1.2Statement of Research**

Isoniazid is a first line drug in the treatment regimen of tuberculosis, however several reports have indicated the proliferation of fake and substandard isoniazid brands in the market (WHO, 1999a, 2006a;Kelesidis and Falagas, 2015). Thus, there is need to ensure its quality through regular quality control test. However the sensitive HPLC machine for the analysis of isoniazidis not readily available in developing countries due to high cost of procurement and installation.

Although many UV/Vis methods have been developed for the analysis of isoniazid in its pure and tablet dosage form (Babu *et al.*, 2002; Kamel, 2008; Oga, 2010; Arifa *et al.*, 2013), they are tedious and require expensive reagents

### **1.3 Justification of the Research**

As a result of the installation and operating cost of the HPLC machine coupled with the limitations of the reported UV/Vis spectrophotometric methods for the analysis of isoniazid, there is need to develop simple, accurate and sensitive UV/Vis methods that will address the reported drawbacks and can be used for monitoring the quality of isoniazid which is very important in the clinical practice. In addition, the presence of NH<sub>2</sub> and C=O groups in isoniazid makes it a good candidate for the formation of coloured Schiff's bases and/or diazo dye that can be determined spectrometrically.

### **1.4 Aim of the Research**

To develop and validate four new UV/Vis Spectrometric methods for the determination of isoniazid in pure and tablet dosage forms.

#### **1.4.1 Specific objectives**

The specific objectives are to;

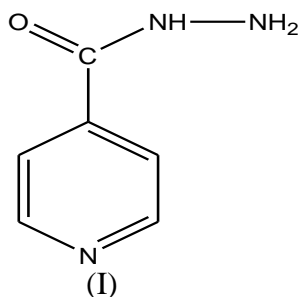
- (1) condense isoniazid solution with 2, 4-dinitrophenylhydrazine to form coloured hydrazone,
- (2) couple isoniazid solution with p-aminotoluidine to form a diazo dye,
- (3) determine the  $\lambda_{max}$  of isoniazid in distilled water and methanol
- (4) apply the developed methods in the determination of isoniazid in pure and tablet dosage form.

## **CHAPTER TWO**

### **2.0 LITERATURE REVIEW**

#### **2.1 Chemistry of Isoniazid**

Isoniazid (I) is a derivative of isonicotinic acid, chemically called pyridine-4-carboxylic acid hydrazide. It has a molecular formula and molecular weight of  $C_6H_7N_3O$  and 137.14 respectively. It is white crystalline powder with melting point range of 170-173 °C. It is freely soluble in polar solvents (eg water, methanol and ethanol) and insoluble in non-polar solvents (eg chloroform, ether and benzene). It is a weak base due to presence of a hydrazine group. It is stable in dimethyl sulfoxide and water (BP, 2009) and optically inactive because it has no chiral center (Laurence *et al.*, 1989).



### 2.1.1 Mechanism of action of isoniazid

Isoniazid is bactericidal against metabolically active (rapidly-dividing) bacilli and bacteriostatic against resting (slow-growing) bacilli (BP, 2009). It inhibits biosynthesis of mycolic acids which are essential components of the mycobacterial cell wall and also interferes with mycobacterial metabolism of vitamin B<sub>6</sub> (Kamel, 2008).

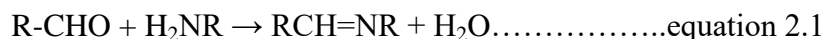
### 2.1.2 Uses of isoniazid



Isoniazid is the drug of choice in the treatment of pulmonary and extra pulmonary tuberculosis in combination with other anti-tuberculosis drugs like rifampicin, ethambutol, streptomycin and pyrazinamide to avoid development of resistance. It is also used in combination treatment of leprosy with Dapson (Watson, 2005).

## 2.2 Chemistry of Schiff's Bases

Hugo (Ugo) Schiff, a German chemist was the first to discover Schiff bases and other imines. Schiff bases are compounds containing an azomethazine group (-CH=N-) formed by condensation of a primary amine with a carbonyl compound (equation 2.1).



Where R = an aliphatic or an aromatic group.

Schiff bases of aliphatic aldehydes are relatively unstable and readily polymerize while those of aromatic aldehydes, having an effective conjugation system, are more stable (Kumar, 2010). Condensation of amines with aldehydes and ketones has numerous applications which include preparative use, identification, detection and determination of aldehydes or ketones, purification of carbonyl or amino compounds and protection of these groups during complex action or sensitive reactions (Kumar, 2010). Schiff bases are important intermediates in a number of enzymatic reactions involving interaction of an enzyme with an amino or carbonyl group of the substrate (Kumar, 2010). One of the most prevalent types of catalytic mechanisms in biochemical processes involves condensation of primary amine in an enzyme, usually that of lysine residue, with a carbonyl group of the substrate to form imine or Schiff bases (Kumar, 2010).

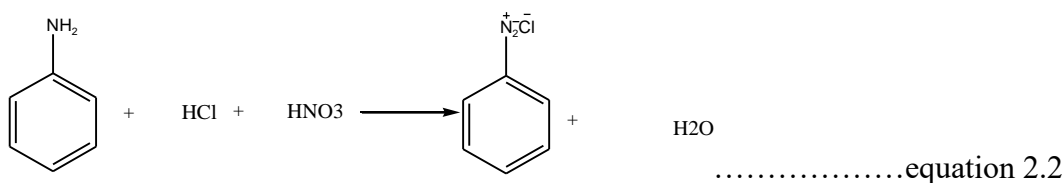
Stereochemical investigations carried out with the aid of molecular models showed that Schiff bases formed between methylglyoxal and the amine groups of the lysine side chains of proteins

can bend back in such a way towards the nitrogen atoms of the peptide groups that a charge transfer can occur between these groups and the oxygen atoms of the Schiff bases. (Kumar, 2010).

Certain polymeric Schiff bases have been reported to possess antitumor activity (Mukamel, 2000). These Schiff bases have the highest degree of hydrolysis at pH 5 and the solubility in water is also highest at this pH. Their antitumor activity considerably increases with a slight increase in water solubility (Paula and Julio, 2009). Another important role of Schiff base structure is in transamination, the transaminases appear to have the prosthetic group i.e. pyridoxal phosphate which is non covalently linked to the enzyme protein (Kumar, 2010).

### 2.3 Chemistry of Diazo dye

Aromatic amines when treated with nitrous acid in cold mineral acid solution yield very important class of compounds known as aryl diazonium salts example, aniline reacts with nitrous acid in hydrochloric acid solution at 0-5°C to form a solution of benzenediazonium chloride (John Peter *et al.*, 1858).



Diazonium salts were discovered by John Peter Griesis in 1858 and the reaction producing them is referred to as diazotization (John Peter *et al.*, 1858). These diazonium salts are valuable in the synthesis of organic compounds and are comparable with Grignard reagent in versatility (Kumar, 2010).

## **2.4 Analytical Methods for the Determination of isoniazid**

### **2.4.1 UV spectrophotometric method using distilled water and methanol.**

### **2.4.2 Colourometric method using 2,4-dinitrophenylhydrazine and diazotization.**

Several analytical methods have been reported for quantitative determination of isoniazid in its pure and tablet dosage form. Both official (compendial) and unofficial methods have been reported

#### **2.4.2 Compendial method of analysis**

British Pharmacopoeia 2009 (BP, 2009) describes titration of isoniazid with potassium bromate in presence of potassium bromide using methyl red as indicator while United State pharmacopoeia (USP) describes HPLC method using L1 column (4.6 mm× 25 cm) and a mobile phase consisting of methanol: water (40:60) (pH adjusted to 2.5 with H<sub>2</sub>SO<sub>4</sub>) with a flow rate of 1.5 mL/ min and UV detection at 254 nm (USP)

#### **2.4.3 Non compendial methods of analysis**

##### *2.4.2.1 Titrimetric methods*

Four titrimetric procedures for the assay of isoniazid in dosage form were reported. Three of the methods were redox titrations developed using N-bromosuccinimide, bromamine-B and bromamine-T as titrants (Mohan *et al.*, 1984; Rajuet *et al.*, 2001; Nagendra *et al.*, 2002).

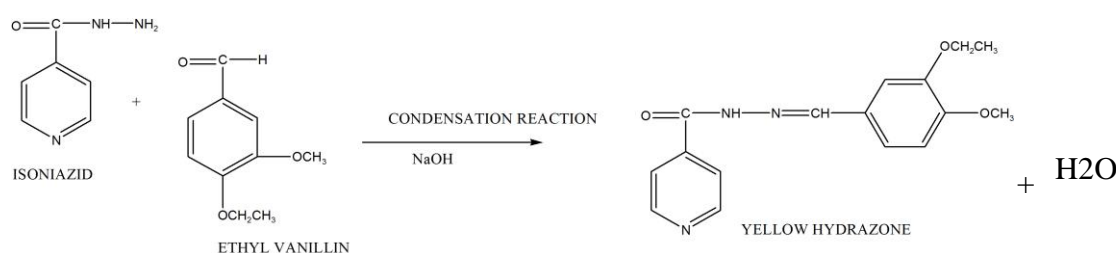
Another method involves the titrimetric determination of isoniazid using N-bromophthalimide as a titrant. It is determined either directly using methyl red or amaranth as indicator, or by a back titration method in which a known excess of N-bromophthalimide solution is added to isoniazid solution and then the residual unreacted reagent is determined (El-Brashy and El-Ashry, 1992).

##### *2.4.2.2 Spectrophotometric methods*

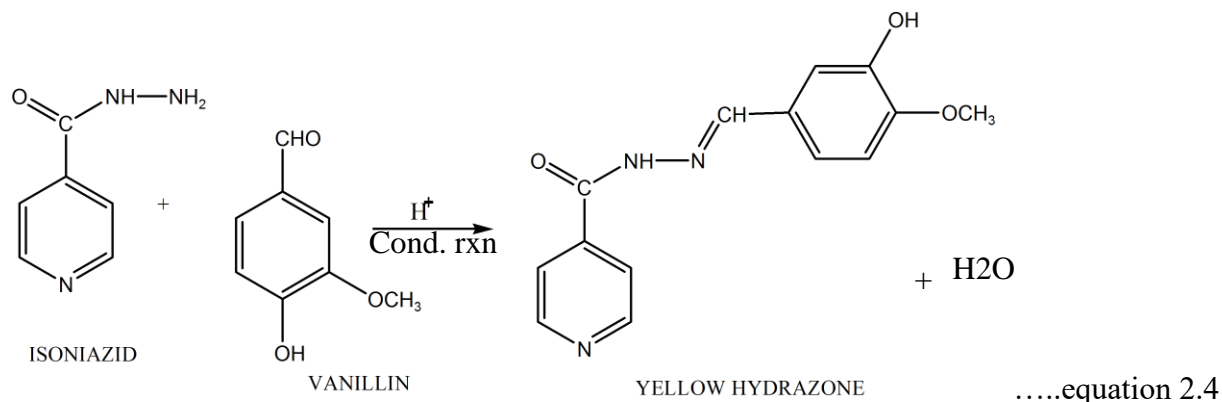
Several UV (200 -400 nm) and visible spectrophotometric (400 -750 nm) methods have been

reported for the determination of isoniazid in pure form and in its tablet dosage form. Some of these methods include:

A method based on condensation reaction between isoniazid and ethyl vanillin in basic medium (NaOH) to form a colored hydrazone (equation 2.3) with  $\lambda_{\max}$  of 410 nm. Beer's law was obeyed in the range 2-16  $\mu\text{g/mL}$  (Ravalkashyap *et al.*, 2012). The method is direct, simple and rapid however, other parameters such as limit of detection and limit of quantification were not reported.

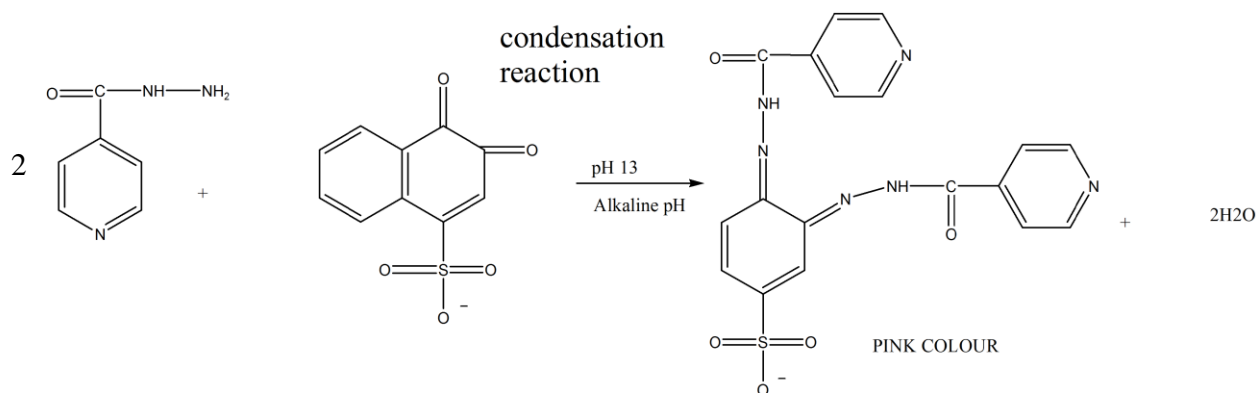


A similar method was reported in which the reagent was vanillin in acidic medium (HCl) resulting in a product with a  $\lambda_{\max}$  of 405 nm. Beer's law was obeyed in the range of 1-12  $\mu\text{g/mL}$  (Oga, 2010). This method has advantage of being rapid, simple, sensitive and low cost however there is poor linearity ( $r = 0.96$ ) and limit of detection and limit of quantification, were not indicated.



Isoniazid was reported to react with 1, 2-naphthoquinone-4-sulfonate (NQS) at pH 13 to form a pink-colored product which was measured at 495 nm. Beer's law was obeyed in the range 0.5-30

$\mu\text{g/mL}$ . (Ravalkashyap *et al.*, 2012). This method requires an expensive reagents that are not readily available.

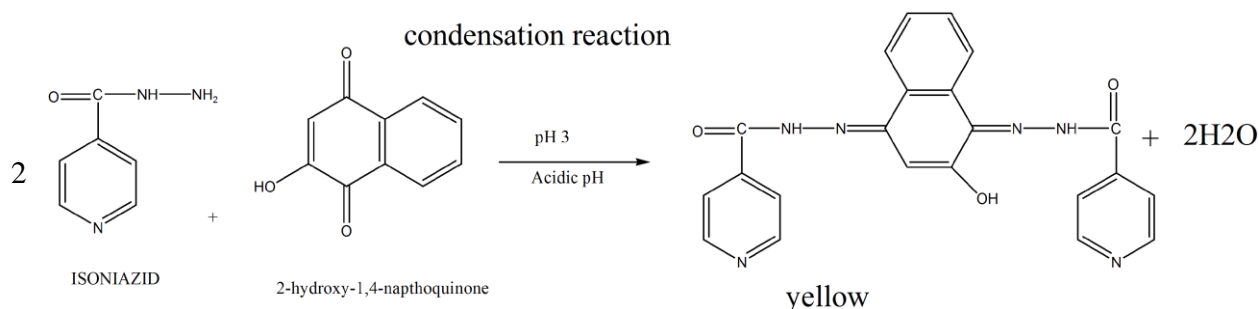


.....equation 2.5

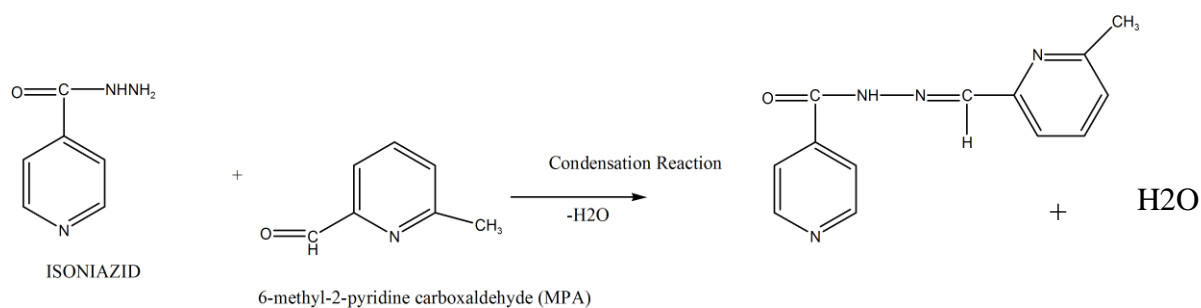
Another method with similar problem is the reaction of isoniazid with isatin in acidic (pH 1.4) to form isatin-isonicotinyl hydrazone (yellow colored product) with  $\lambda_{\text{max}}$  of 340 nm. Beer's law was obeyed in the range of 0-32  $\mu\text{g/mL}$  (AbdelHamid *et al.*, 1992).

Isoniazid was also reported to react with 2-hydroxy-1,4-naphthoquinone (HNQ) at pH 3 in aqueous-methanolic solution to give a yellow colored product having absorption maximum at 365 nm (Rindet *et al.*, 2005).

This method in addition to having similar problem is the reaction of isoniazid with 6-methyl-2-pyridinecarboxaldehyde (MPA) resulting in a coloured hydrazone with  $\lambda_{\text{max}}$  at 328 nm obeyed Beer's law but detection limit was very high (Khuhawaret *et al.*, 1988).

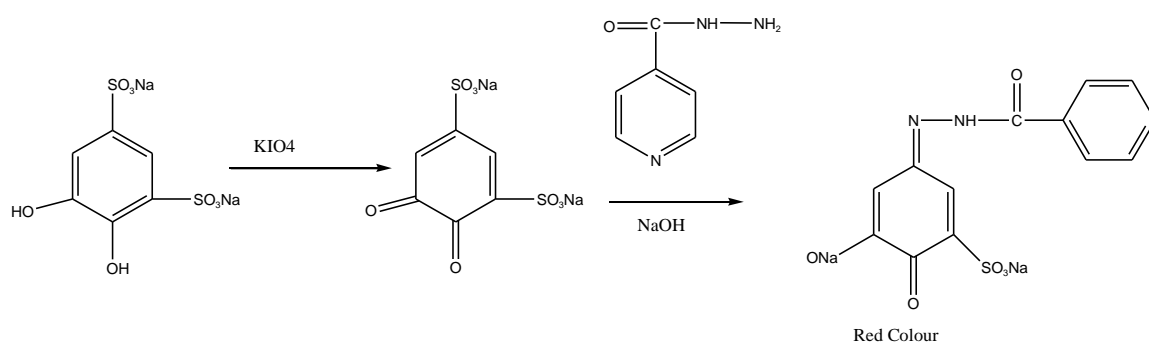


.....equation 2.6



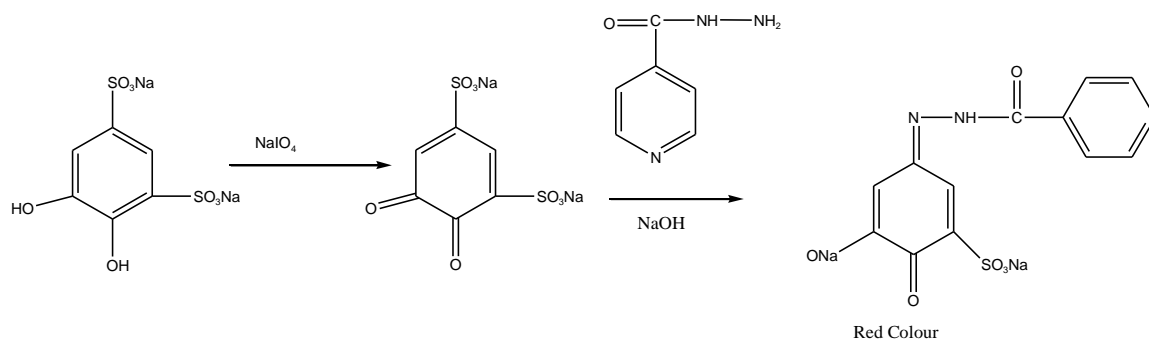
.....equation 2.7

Oxidation of iron with  $\text{KIO}_4$  followed by coupling with isoniazid in alkaline medium to give red colored product with maximum absorbance at 505 nm obeys Beer's law in the concentration of 1.5-18  $\mu\text{g/mL}$  (Gowda *et al.*, 2003)



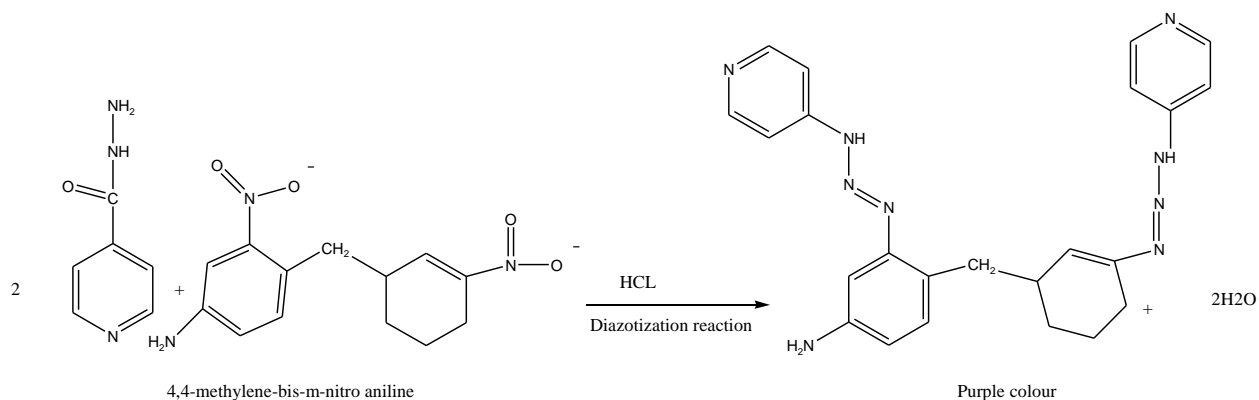
.....equation 2.8

The same authors developed another method based on same reaction but  $\text{NaIO}_4$  was used as oxidizing agent and absorbance of the coupled product was measured at 507 nm (Naidu *et al.*, 2005). This method obeys Beer's law in the concentration of 1.5-18  $\mu\text{g/mL}$  but it was tedious.



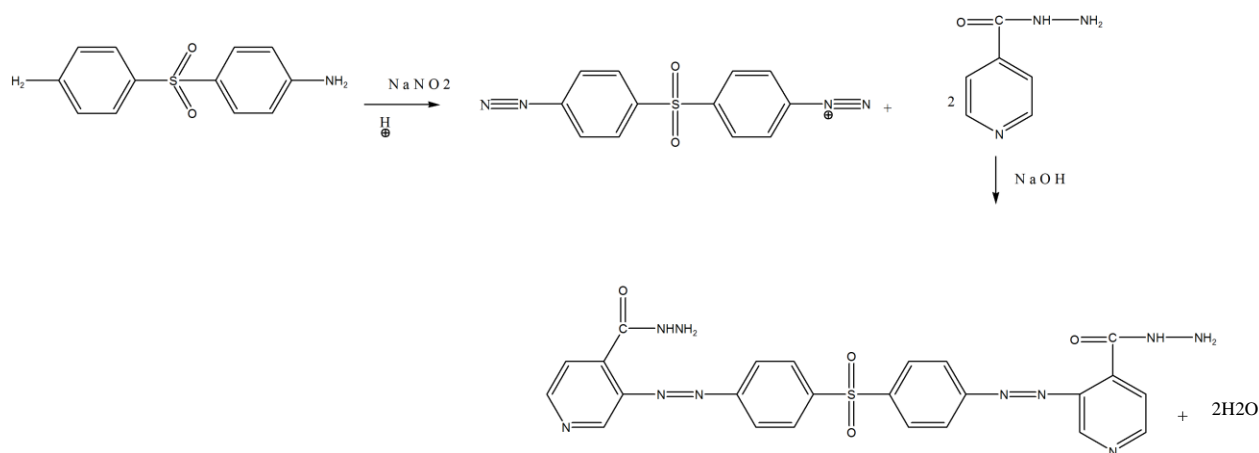
...equation 2.9

Method developed fairly sensitive based on diazotization of 4, 4-methylene-bis-m-nitroaniline followed by the coupling with isoniazid in HCl medium to give purple-colored chromogen having  $\lambda_{max}$  at 495 nm obeyed in the range 0.1-15  $\mu\text{g/mL}$ . This method is indirect and less sensitive



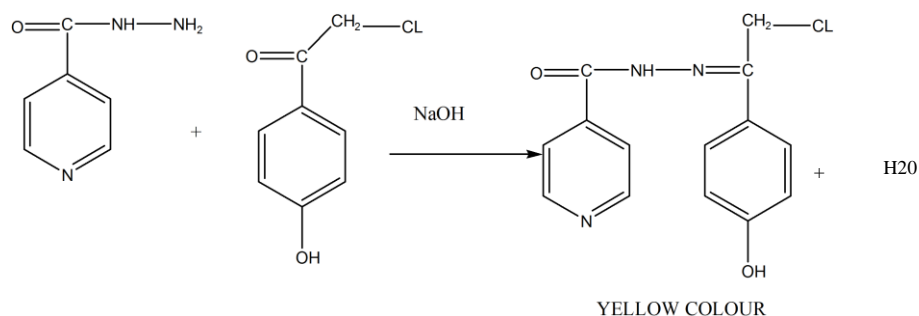
.....equation 2.10

The diazotization of 4, 4 sulphonyldianiline (dapson) followed by coupling with isoniazid in NaOH medium to form diazo-dye with  $\lambda_{max}$  at 460 nm is also complex (Padmarajaihet *al.*, 2002).



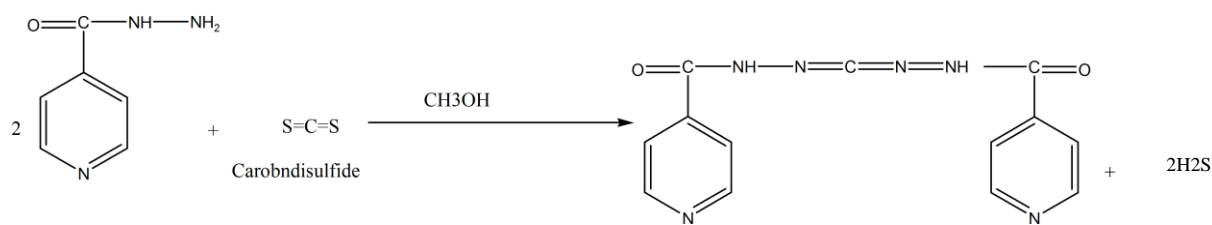
Two other methods were developed based on reaction of isoniazid with epichlorohydrine (EPI) and 4-hydroxyphenacylchloride (HPC) in NaOH medium to form yellow colored chromogen

with  $\lambda_{\text{max}}$  at 405 and 402 nm for EPI and HPC respectively (Divya *et al.*, 2011). This method is also complex.



.....equation 2.12

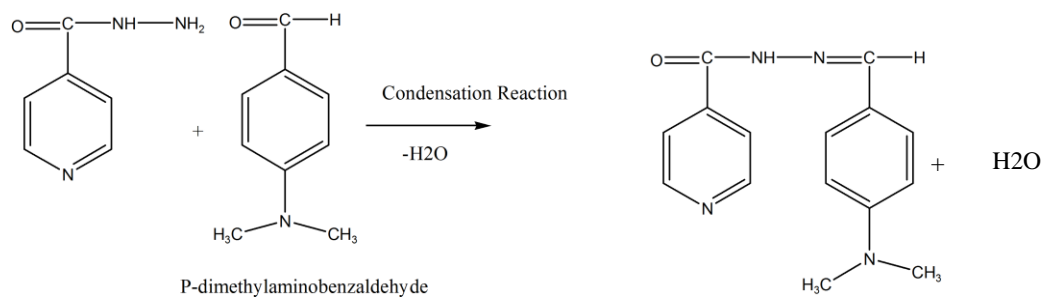
The reaction of isoniazid with carbon disulfide in methanol medium followed by addition of uranyl acetate to form yellow colored uranylisonicotinyldisthiocarbazate complex with  $\lambda_{\text{max}}$  at 410 nm (Kumar *et al.*, 2011). This method lacks readily available reagents.



.....equation 2.13

Another method was developed from aliquots of standard stock solution of different concentrations ranging from 100-600  $\mu\text{g/mL}$  transferred to a series of 50 mL volumetric flasks. To each flask 5 mL of ethanolic p-dimethylaminobenzaldehyde (p-DAB) solution was added. The volume was then made up with distilled water and the absorbance of the yellow colored Chromogen was measured at 395 nm against the reagent blank. The amount of isoniazid was computed from the calibration curve obtained by plotting concentration versus absorbance. Pure powder and tablet of isoniazid was successfully analyzed by this method (Niraimathiet *al.*, 2014). These methods are indirect, less sensitive and use expensive chemicals, absorbance measured at shorter wavelength and reagents.





Other methods include those of Niramathiet *al.*, 2013, Safaviet *al.*, 2004, Diabet *al.*, 2008, Prabet *al.*, 2008, Prabet *al.*, 2012 among others.

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Isoniazid analytical grade powder and tablet

Standard isoniazid powder was purchased from Sigma Aldrich Germany while the tablet was purchased from registered pharmacy. Information on tablet was recorded.

##### 3.1.2 Chemicals and reagents

All chemicals are of analytical grade. The methanol, ethanol, distilled water, Concentrated HCl, 2,4-dinitrophenyl hydrazine, Thiourea, Sodium nitrite, Sulfamic acid, Paratoluidine, Potassium bromide were purchased from BDH chemical, England.

##### 3.1.3 Glass wares and accessories

10 mL, 100 mL measuring cylinders (Pyrex England)

100 mL and 250 mL conical flasks (Pyrex England)

25 mL and 50 mL beakers (Pyrex England)

10 mL test tubes (Pyrex England)

10 mL, 50 mL and 100 mL volumetric flasks (Pyrex England)

10 mL Centrifuge tubes (Pyrex England)

1 mL, 10 mL pipette

Filter paper

Capillary tube

Glass rod

### **3.1.4 Equipment**

Electronic weighing balance (MettlerPr 63 Switzerland) Centrifuge machine (Gallenkamp, England) UV double beam spectrophotometer model (MNF, Helios Zeta, Model 164617.

Thermo Scientific England)

Stop watch (Smith England clock system)

Melting point apparatus(Gallenkamp, England)

Infra-red spectrophotometer (Model. Cary 630, Agilent Technology Germany)

pH meter (Fisher scientific, Singapore)

Water bath (modelBJE 750 AGallenkamp, England)

Thermometer (Mc Donald Scientific International, England)

## **3.2Methods**

### **3.2.1Quality control of isoniazidstandard powder and tablet**

#### *3.2.1.1Identificationof pure isoniazid powder*

##### *Melting point determination*

A capillary tube was filled with the standard isoniazid powder and carefully tapped to about 3.5 mm, placed into the melting point apparatus and machine set at high temperature for rapid determination of melting point. The melting process was observed with magnifying lens and the melting point recorded was compared with reference standard (BP, 2009)

##### *FTIR analysis*

A portion of pure isoniazid powder was directly subjected to FTIR analysis and the spectrum obtained was compared with the spectrum of a reference.

### *3.2.1.2 Assay of standard isoniazid powder*

The assay was carried out by dissolving the standard isoniazid powder (0.250 g) in 100 mL of distilled water. A portion (20 mL) of the solution was withdrawn and transferred into a beaker. Water (100 mL), hydrochloric acid (20 mL), potassium bromide (0.2 g) and methyl red solution (0.05 mL) were added. The resultant solution was then titrated with 0.0167 M potassium bromate with continues shaking until the red colour disappeared.

Isoniazid content was determined using relation 1 mL of 0.0167 M potassium bromate is equivalent to 3.429 mg of isoniazid (BP, 2009).

### *3.2.1.3 Identification of isoniazid tablet*

#### *FTIR analysis*

Ten tablets of isoniazid were weighed and powdered in pestle and motor. A quantity of powder equivalent to 0.1 g of isoniazid was taken and shaken with 10 mL of ethanol (96 %v/v) for 15 minutes then centrifuged and supernatant liquid was decanted. The residue was further extracted with two 10 mL portions of ethanol (96 %v/v) and the combine extract was evaporated to dryness. The infrared spectrum of the dried residue was determined and compared with the reference spectrum of isoniazid (BP, 2009).

### *3.2.1.4 Assay of isoniazid tablets*

Twenty tablets of isoniazid were accurately weighed and powdered in pestle and motor. A quantity of powder 0.467 g equivalent to 0.4 g of isoniazid was dissolved in distilled water and filtered. The residue was washed with sufficient water to produced 250 mL To 50 mL of resulting solution, water 50 mL, hydrochloric acid 20 mL and potassium bromide 0.2 g were added and titrated with 0.0167 M potassium bromate with continues shaking until the red colour disappeared. Each one mL of potassium bromate 0.0167 M is equivalent to 3.429 mg of isoniazid

(BP, 2009).

### **3.2.2 Selection of solvents**

The solubility of isoniazid in distilled water, methanol, ethanol, chloroform, ether and benzene at room temperature was tested.

### **3.2.3 Preparation of solutions and reagents**

#### *3.2.3.1 Preparation of isoniazid stock solutions in distilled water and methanol.*

Stock solutions (100 µg/mL) of isoniazid were prepared in distilled water and methanol by dissolving 10 mg isoniazid standard powder in 100 mL distilled water and methanol respectively.

#### *3.2.3.2 Preparation of reagents.*

Preparation of 4.5M HCl, 2%, 85% w/v HCl, 1% w/v sodium nitrite, 2% sulfamic acid, 2, 4-dinitrophenylhydrazine and para toluidine solutions were prepared as described in appendix II.

### **3.2.4 UV spectrophotometric method using distilled water.**

This method was based on using distilled water as solvent.

#### *3.2.4.1 Determination of wavelength of maximum absorption ( $\lambda_{max}$ ).*

A working solution (10 µg/mL) was prepared from the stock solution and then scanned through 200-400 nm against reagent blank on the UV spectrophotometer to determine the  $\lambda_{max}$ .

#### *3.2.4.2 Determination of pH of maximum absorption.*

Portion (5 mL) of isoniazid solution in distilled water (10 µg/mL) was transferred into 10 different test tubes labeled 1 – 10 and adjusted to pH 1 – 10 respectively. The absorbance of each solution was then measured at the obtained  $\lambda_{max}$ .

#### *3.2.4.3 Preparation of calibration curve*

Different solutions of isoniazid of concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 µg/mL were prepared from the stock solution and adjusted to the pH of maximum absorption. The absorbance of

each solution was then measured at the  $\lambda$  max against reagent blank. The absorbances obtained were then plotted against their corresponding concentrations.

#### *3.2.4. Assay of isoniazid tablets by UV spectrophotometric method using distilled water*

A quantity of powder containing equivalent to 10 mg isoniazid was accurately weighed from the previously crushed 20 isoniazid tablets, transferred into a volumetric flask (100 mL) and the volume made up to mark with distilled water. The pH of the mixture was adjusted to the pH of maximum absorption and shaken for 10 minutes. The solution was filtered through a Whatman filter paper no. 41. A portion (1 mL) was transferred into a volumetric flask (10 mL) and made up to the 10 mL mark with distilled water to obtain a concentration of 10  $\mu\text{g/mL}$ . The absorbance of the resulting solution was then measured at the  $\lambda_{\text{max}}$  against the reagent blank.

#### **3.2.5 UV spectrophotometric method using methanol.**

This method was based on using methanol as solvent.

##### *3.2.5.1 Determination $\lambda$ max.*

A working solution (10  $\mu\text{g/mL}$ ) was prepared from the stock solution and then scanned through 200-400 nm against reagent blank on the UV spectrophotometer to determine the  $\lambda_{\text{max}}$ .

##### *3.2.5.2 Determination of pH of maximum absorption.*

Portion (5 mL) of isoniazid solution in methanol (10  $\mu\text{g/mL}$ ) was transferred into 10 different test tubes labeled 1 – 10 and adjusted to pH 1 – 10 respectively. The absorbance of each solution was then measured at the obtained  $\lambda$  max.

##### *3.2.5.3 Preparation of calibration curve*

Different solutions of isoniazid of concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10  $\mu\text{g/mL}$  were prepared from the stock solution and adjusted to the pH of maximum absorption. The absorbance of each

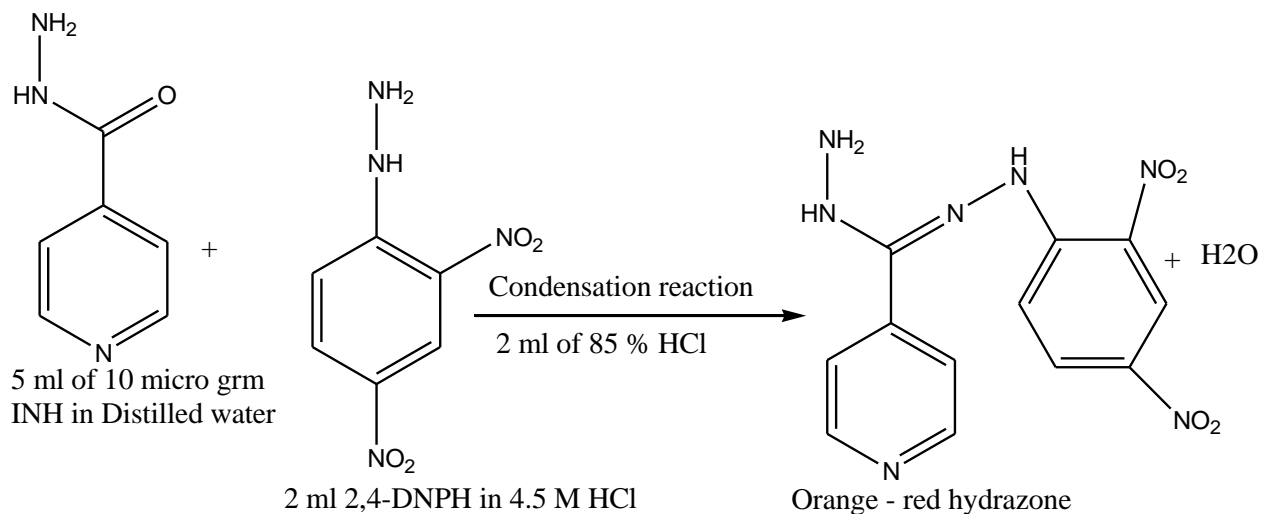
solution was then measured at the  $\lambda$  max against reagent blank. The absorbances obtained were then plotted against their corresponding concentrations.

#### 3.2.5. 4 Assay of isoniazid tablets by UV spectrophotometric method using methanol

A quantity of powder 12 mg equivalent to 10 mg isoniazid was accurately weighed from the previously crushed 20 isoniazid tablets, transferred into a volumetric flask (100 mL) and the volume made up to mark with methanol. The pH of the mixture was adjusted to pH of maximum absorption and shaken for 10 minutes. The solution was filtered through a Whatman filter paper no. 41. A portion (1 mL) was transferred into volumetric flask (10 mL) and made up to the 10 mL mark with methanol to obtain a concentration of 10  $\mu$ g/mL. The absorbance of the resulting solution was then measured at the  $\lambda$ max against the reagent blank.

#### 3.2.6 Development of colorimetric method using 2,4-dinitrophenylhydrazine and optimization.

This method is based on formation of Schiff's base between isoniazid and 2,4-dinitrophenylhydrazine under acidic condition (equation 3.1).



.....equation 3.1

### *3.2.6.1 Determination of $\lambda$ max.*

A portion (5 mL) of isoniazid solution (10  $\mu\text{g}/\text{mL}$ ) in distilled water was treated 2,4-dinitrophenylhydrazine in the presence of 4.5 M HCl (2 mL). The resulting solution and also the reagent blank (containing 5 mL distilled water, 2 mL reagent and 2 mL of chilled 85 % HCl) were heated at 100°C in a thermostatic water bath for 10 minutes. Thereafter, the solution containing isoniazid was treated with 2 mL of chilled 85 %v/v HCl to form an orange-red coloured hydrazone (equation 3.1). The hydrazone formed was allowed to stand for 15 minutes for complete color development then scanned through a wavelength range 400 – 750 nm against the reagent blank of 5 mL distilled water, 2 mL 2,4-dinitrophenylhydrazine, 2 mL 85% HCl on the UV spectrometer to obtain the  $\lambda$  max.

### *3.2.6.2 Optimization of analytical working conditions.*

*Determination of effective concentration of the reagent. 2,4-dinitrophenylhydrazine (1-20  $\mu\text{g}/\text{mL}$ )*

Different concentrations (1-20  $\mu\text{g}/\text{mL}$ ) of 2, 4-DNPH (2 mL each) were added to separate 5 mL solutions of isoniazid (10  $\mu\text{g}/\text{mL}$ ) in distilled water in twenty different flasks and treated as described earlier to form orange-red coloured hydrazone. Absorbances were recorded at the fixed  $\lambda$  max to determine the effective concentration of reagent.

*Determination of effective volume of the reagent.*

Different volumes (1,2,3,4 and 5 mL) of 2,4-DNPH at effective concentration were condensed with 5 mL of 10  $\mu\text{g}/\text{mL}$  isoniazid in distilled water and absorbance of the hydrazine formed were measured at  $\lambda$ max to determine the effective volume.



### *Determination of effective time for complete color development*

A solution of 5 mL of 10 µg/mL Isoniazid in distilled water was reacted with 2,4-DNPH at the effective concentration and volume, the absorbance of coloured hydrazone formed was measured at  $\lambda$  max at time intervals of 5, 10, 15, 20, 25 and 30 minutes to determine the time at which maximum absorbance is observed. This is the effective time for complete colour development.

### *3.2.6.3 Preparation of calibration curve for concentration of the reagent.*

#### *2,4-dinitrophenylhydrazine (1-20 µg/mL)*

Different solutions of isoniazid in distilled water of concentrations 2, 4, 6, 8, and 10 µg/mL were treated with 2,4-DNPH at the effective concentration and volume. The colours of the hydrazone formed were allowed to stand up to the effective time for complete development. The absorbance of each solution was measured at the  $\lambda$ max against reagent blank. The calibration curve was constructed by plotting the absorbance against their corresponding concentrations.

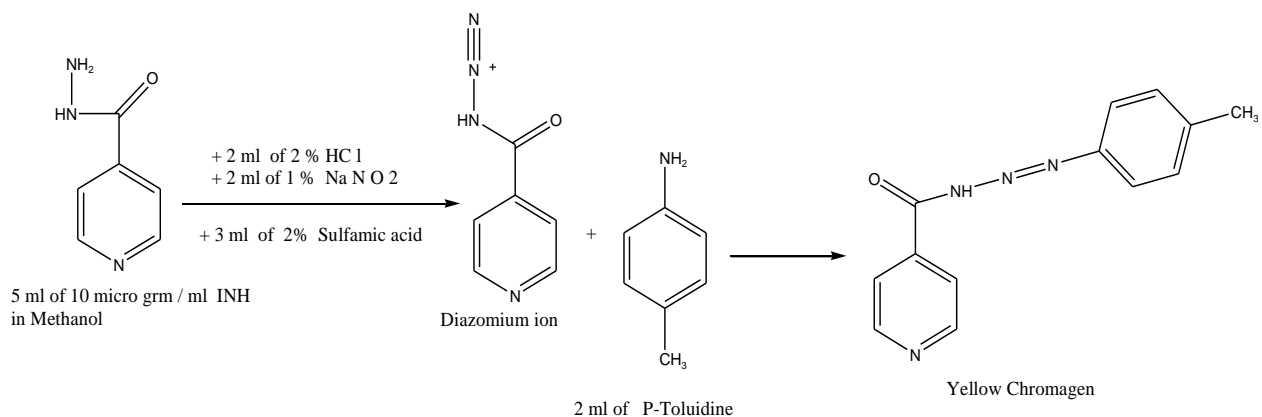
### *3.2.6.4 Assay of isoniazid tablets using colorimetric method using 2,4-dinitrophenylhydrazine and optimization.*

A quantity of powder tablet 12 mg, equivalent of 10 mg isoniazid was accurately weighed from the previously crushed 20 isoniazid tablets, transferred to volumetric flask (100 mL) and the volume made up to mark with distilled water and shaken for 10 minutes. The mixture was filtered through a Whatman filter paper no. 41. A portion (1 mL) was transferred to a volumetric flask (10 mL) and the volume made up to mark with distilled water to obtain a 10 µg/mL solution. A portion (5 mL) of this solution was treated with 2, 4 DNPH at effective concentration and volume, then heated at 100 °C in a thermostatic water bath for 10 minutes. Thereafter, 2 mL of chilled 85 % v/v HCl was added to form orange-red coloured hydrazone which was allowed to

stand up to the effective time for complete colour development. The absorbance was then measured at  $\lambda$  max determined against reagent blank.

### 3.2.7 Development of colorimetric method by using paraaminotoluidine and optimization.

This method is based on the formation of a diazo dye from the reaction of isoniazid with p-toluidine.



..... Equation 3.2

#### 3.2.7.1 Determination of $\lambda$ max

A portion (5 mL) of isoniazid solution 10  $\mu$ g/mL in methanol was treated with 2 mL of 2 % HCl followed by 2 mL of 1 % sodium nitrite solution (NaNO<sub>2</sub>), stirred with glass rod and allowed to stand for 5 minutes. Thereafter 3 mL of 2 % w/v sulfamic acid was added, stirred and also allowed to stand for 5 minutes to distribute the residual sodium nitrite. Finally 2 mL of p-toluidine solution was added and heated for 10 min. The yellow coloured chromagen formed was allowed to stand for complete colour development then scanned through wavelength range 400–750 nm against reagent blank (containing 5 mL methanol, 2 mL 2 % HCl, 2 mL of 1 % sodium nitrite, 3 mL of 2 % sulfamic acid 2 mL of p-toluidine) on the UV spectrometer and the  $\lambda$  max recorded.

### 3.2.7.2 Optimization of analytical working conditions.

#### *Determination of effective concentration of the reagent p-toluidine (1-20 µg/mL)*

Different concentrations (1-20 µg/mL) of p-toluidine (2 mL each) were added to separate 5 mL solutions of isoniazid (10 µg/mL) in methanol in twenty different flasks and treated as described earlier to form yellow-coloured chromagen. Absorbances were recorded at the fixed  $\lambda$  max to determine the effective concentration.

#### *Determination of effective volume of the reagent.*

Different volumes (1, 2, 3, 4 and 5 mL) of p-toluidine at effective concentration were coupled with 10 µg/mL isoniazid in methanol and absorbance of the yellow chromagen formed were measured at  $\lambda$  max to determine the effective volume.

#### *Determination of effective time for complete color development*

A solution of 5 mL of 10 µg/mL isoniazid in methanol was reacted with p-toluidine at the effective concentration and volume, the absorbance of coloured chromagen formed was measured at  $\lambda$  max at time intervals of 5, 10, 15, 20, 25 and 30 minutes to determine the time at which maximum absorbance is observed. This is the effective time for complete colour development.

### 3.2.7.3 Preparation of calibration curve for colorometric method by using paraaminotoluidine

Different solutions of isoniazid in methanol of concentrations 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 µg/mL were treated with p-toluidine at the effective concentration and volume. The yellow coloured chromagen formed were allowed to stand up to the effective time for

complete development. The absorbance of each solution was measured at the  $\lambda_{\text{max}}$  against reagent blank. Then calibration curve was constructed by plotting the absorbance against their corresponding concentrations.

#### *3.2.7.4 Assay of isoniazid tablets using colorometric method by using paraaminotoluidine*

A quantity of powder containing equivalent of 10 mg isoniazid was accurately weighed from the previously crushed 20 isoniazid tablets, transferred into a volumetric flask (100 mL) and the volume made up to mark with methanol and shaken for 10 minutes. The mixture was filtered through a Whatman filter paper no. 41. A portion (1 mL) was transferred to a volumetric flask (10 mL) and the volume made up to mark with methanol to obtain a 10  $\mu\text{g/mL}$  solution. A portion (5 mL) of this solution was then treated with 2 mL of 2% HCl, 2 mL of 1% NaNO<sub>2</sub> and stirred with glass rod for 5 minutes followed by addition of 3 mL 2 % sulfamic acid and stirred for another 5 minutes. The solution of p-toluidine at effective concentration and volume was added to the content of the flask then gently heated in a thermostatic water bath for 10 minutes. The yellow coloured chromagen formed was allowed to stand up to the effective time for complete colour development. The absorbance was then measured at  $\lambda_{\text{max}}$  determined against reagent blank.

### **3.2.8. Validation of the analytical methods**

All the four developed analytical methods were validated according to ICH Q2R1 (1996) guidelines for validation of analytical procedures in order to determine the linearity, precision, accuracy and percentage recovery, limit of detection and limit of quantification.

#### *3.2.8.1 Linearity*

The linearity of each developed method was evaluated by analyzing different concentrations of the standard isoniazid from the stock solution. Linearity was examined by plotting the

absorbance against concentration. Beer's-Lamberts was obeyed at a particular concentration range to give a straight line graph (concentration is directly proportional to absorbance). Coefficient of correlation ( r ) indicate linearity. The closer the r value to 1, the more the linearity of the calibration curve.

### 3.2.8.2 Precision

The precision was carried out to ascertain the reproducibility of the developed methods.

#### *Intraday*

A solution of isoniazid(10 µg/mL) solution was prepared from the stock solution and absorbance measured at interval of one hour for six hours within the same day after treatment using each of the methods developed.

#### *Interday*

A solution of isoniazid (10 µg/mL) was prepared from the stock solution and absorbance measured daily for three consecutive days, after treatment using each of the methods developed. From the result, then mean, standard deviation (SD) and percentage relative standard deviation or percentage coefficient of variance( % CV) were calculated.

$$\% CV = S/X \times 100 \dots\dots\dots\text{Formula 1}$$

Where S is the standard deviation and X is the mean

### 3.2.8.3 Accuracy and percentage recoveries

Accuracy is expressed as percentage relative error (%Er). Accuracy of this method was checked by standard addition method. A solution of 10µg/mL solutions of isoniazid in distilled water was prepared in four separate 10 mL volumetric flasks and labeled A, B, C and D. Test tube A was left unspiked while test tubes B, C and D were spiked with 80% (8 µg/mL), 100% (10 µg/mL)

and 120% (12 µg/mL) from the stock solution (100 µg/mL) and made up to the mark with distilled water and methanol for methods 1, 3 and 2, 4 respectively. Absorbances were measured and substituted in the regression equation to determine the concentrations. Percentage relative error (%Er) was calculated as follows:

$$\%Er = \frac{X - \mu}{\mu} \times 100 \dots \dots \dots \text{Formula 2}$$

Where X is the actual value (concentration) and  $\mu$  is the expected value (concentration).

$$\% \text{ recovery} = \text{measured concentration} / \text{expected concentration} \times 100 \dots \dots \text{Formula 3}$$

The average of these determinations was taken as the percentage recovery.

#### 3.2.8.4 Limit of detection

The detection limit (LOD) was determined by dividing the standard deviation of y-intercept over slope of the calibration curve.

$$LOD = 3.3\sigma / S \dots \dots \dots \text{Formula 4}$$

Where  $\sigma$  is the standard deviation of y-intercepts and S is the slope of the calibration curve.

#### 3.2.8.5 Limit of quantification

The quantitation limit (LOQ) was determined using the expression:

$$LOQ = 10\sigma / S \dots \dots \dots \text{Formula 5}$$

Where  $\sigma$  is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

### **3.2.9 Statistical analysis.**

Results were expressed as mean  $\pm$  SD using IBM statistical package for social sciences (SPSS)summer 2013 version 2.0 software at  $p < 0.05$ . Difference between the mean of the developed methods and that of official standard was insignificant.

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Quality Control of isoniazid Standard Powder and Tablet

The labeled information of the isoniazid tablet sample used for this study is presented in table

**4.1.1**The melting point of both isoniazid standard powder and tablet sample are shown in table

**4.1.2**while their respective IR spectrums are presented in figure 4.1 and 4.2 respectively.

**Table 4.1: Information of isoniazid tablet used in the research**

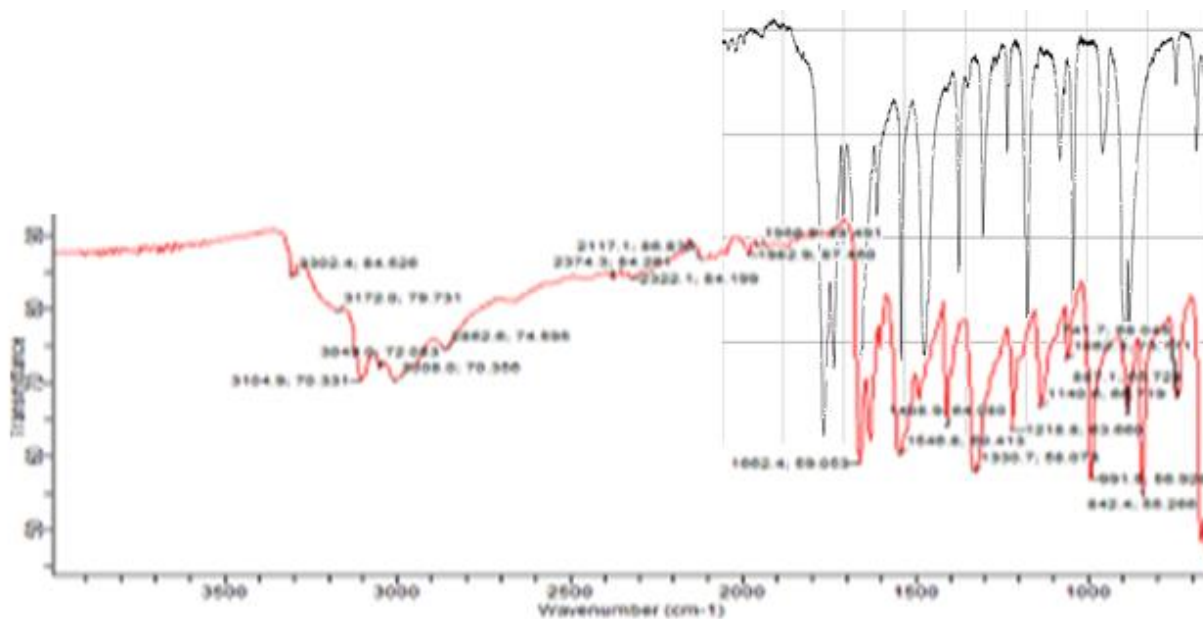
Drug	Source	INH content (mg)	Batch No.	Man date	Exp. date	NAFDAC No.
INH	India	300	TQ27	06/2015	05/2020	A4-0763



**Table 4.2: Melting point determination of isoniazid standard powder and tablet**

<b>Sample</b>	<b>Melting point</b>
Standard INH Powder	170 – 172 °C
Tablet	172 – 174 °C

Official reference standard is 170 – 174 °C



**Figure 4.1: FTIR Spectra of isoniazid standard powder and reference, superimpose at fingerprint region (600-1500 cm<sup>-1</sup>)**

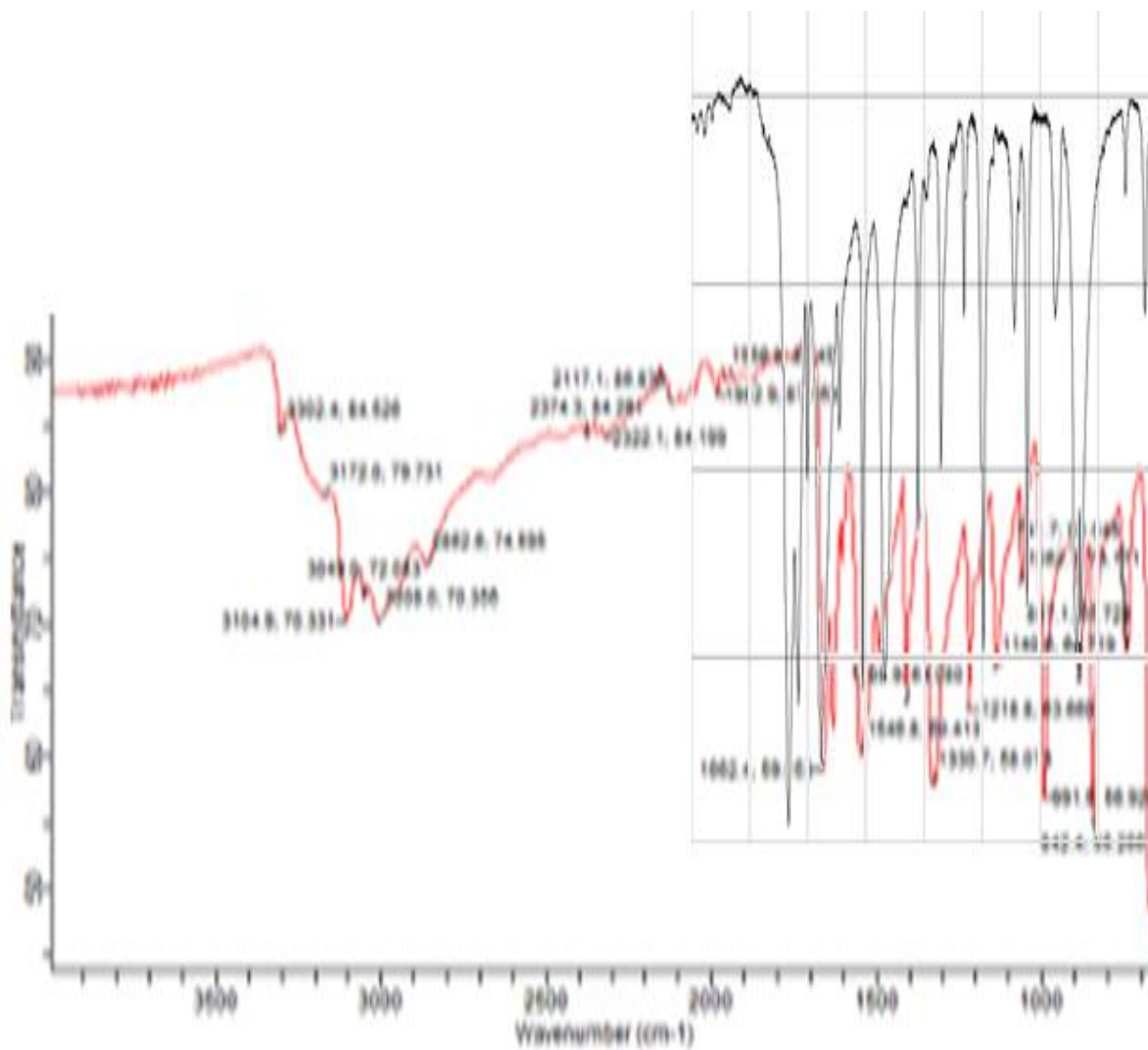


Figure 4.2: FTIR spectra of isoniazid tablet and reference superimposed

## 4.2 Methods Development

### 4.2.1 UV spectrophotometric methods using water and methanol

The solubility of isoniazid in various solvents is presented in table 4.3. The method and calibration parameters of method 1 and 2 are presented in table 4.4, figure 4.3 and figure 4.4 respectively. The validation parameters are shown in table 4.5.

### 4.2.2 Colorimetric methods using 2,4-dinitrophenylhydrazine and by diazotization.

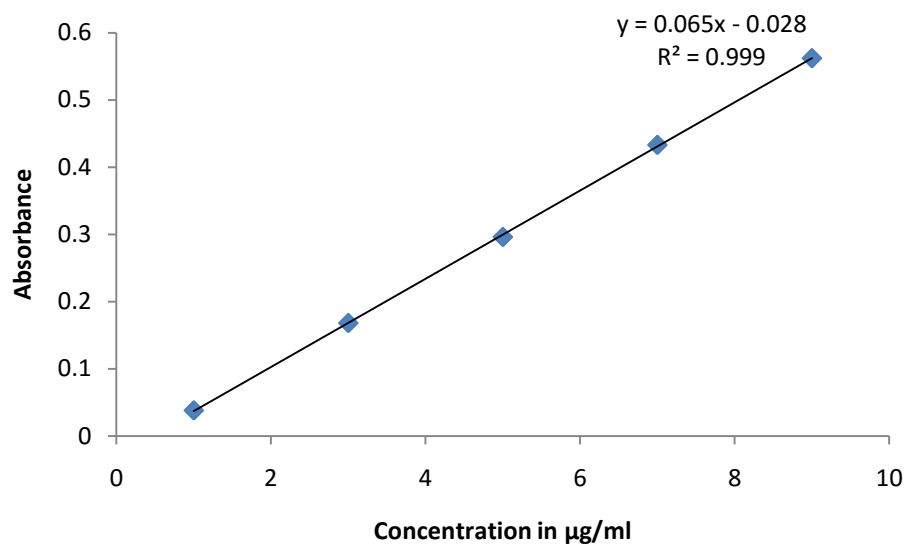
The method and calibration parameters of method 3 and 4 are presented in table 4.6, figure 4.5 and figure 4.6 respectively. The validation parameters are shown in table 4.7

**Table 4.3: Solubility of isoniazid in different solvents**

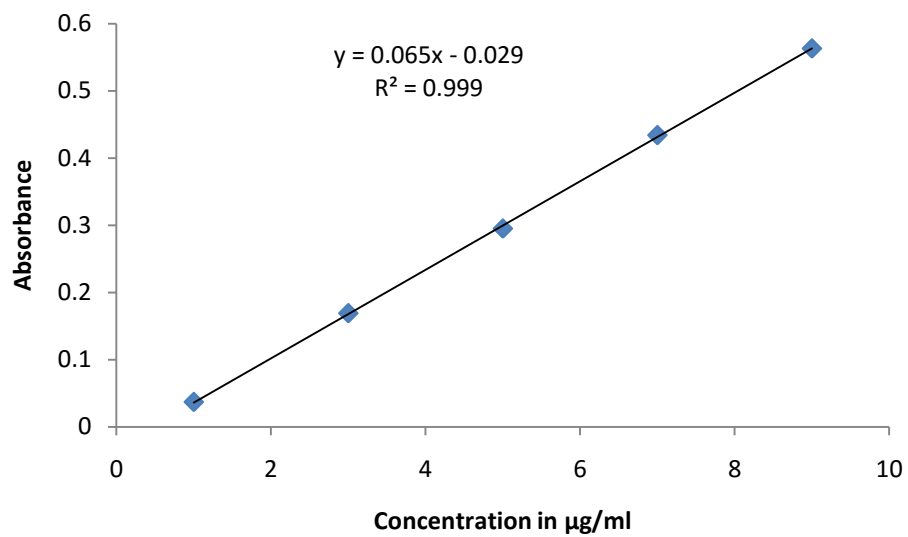
<b>Sample</b>	<b>Water</b>	<b>Methanol</b>	<b>Ethanol</b>	<b>Chloroform</b>	<b>Ether</b>	<b>Benzene</b>
INH Std Powder	Soluble	Soluble	Soluble	Sparingly soluble	Insoluble	Insoluble

**Table 4.4: Validation parameters for UV spectrophotometric methods using water and methanol.**

<b>Parameter</b>	<b>Water</b>	<b>Methanol</b>
$\lambda$ max	264 nm	264 nm
Beer's law range	1 – 11 $\mu\text{g/mL}$	1 – 11 $\mu\text{g/mL}$
Regression equation	$y = ax - b$	$y = ax - b$
Slope (a)	0.0657	0.0658
Intercept (b )	0.0288	0.0296
Coefficient. of determination $R^2$	0.9999	0.9998
Coefficient. of correlation ( r )	0.9999	0.9999
Effective pH for max. absorption	4	4



**Figure 4.3: Calibration curve of isoniazidin distilled water**



**Figure 4.4: Calibration curve of isoniazid in methanol**

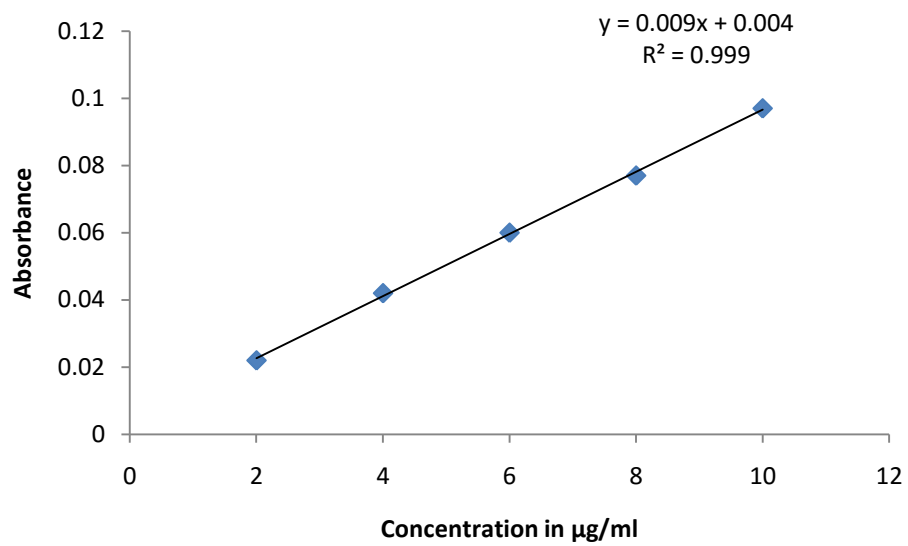
**Table 4.5: Validation parameters for UV spectrophotometric methods using water and methanol.**

<b>Parametr</b>	<b>Water</b>	<b>Methanol</b>	<b>Acceptable limit</b>
Precision (% CV)	0.630	0.168	< 15 %
Accuracy ( % Er )	4.130	0.251	< 5 %
% recovery	102.01	99.8	98-102 %
LOD ( $\mu\text{g/mL}$ )	0.113	0,145	
LOQ ( $\mu\text{g/mL}$ )	0.342	0.438	

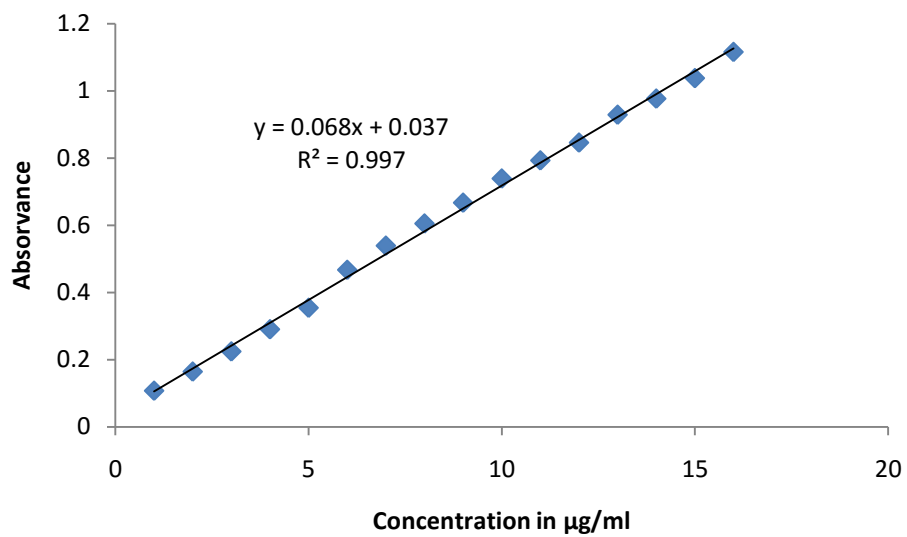


**Table 4.6m Validation parameters for calorimetric methods using 2,4-dinitrophenylhydrazine and by diazotization.**

<b>Parameter</b>	<b>Hydrazine</b>	<b>paratoluidine</b>
$\lambda$ max	464 nm	420 nm
Beer's law range	1 – 18 $\mu\text{g/mL}$	1 – 18 $\mu\text{g/mL}$
Regression equation	$y = ax + b$	$y = ax + b$
Slope (a)	0.0093	0.068
Intercept (b )	0.0041	0.0376
Coefficient. of determination $R^2$	0.9992	0.9971
Coefficient. of correlation ( r )	0.9996	0.999
Effective concentration of the reagent	18 $\mu\text{g/mL}$	18 $\mu\text{g/mL}$
Effective volume of the reagent	3 mL	4 mL
Effective time for complete colour development	15 min	20 min



**Figure 4.5: Calibration curve of isoniazid solution in 2,4-dinitrophenyl hydrazine.**



**Figure 4.6: Calibration curve of isoniazid solution in paraaminotoluidine.**

**Table 4.7: Validation parameters for calorimetric methods using 2,4-dinitrophenylhydrazine and by diazotization.**

<b>Parametr</b>	<b>Hydrazine</b>	<b>Paratoluidine</b>	<b>Acceptable limit</b>
Precision (% CV)	1.570	0.182	< 15 %
Accuracy ( % Er )	0.328	0.670	< 5 %
% recovery	99.83	100.06	98-102 %
LOD ( $\mu\text{g/mL}$ )	0.355	0.185	
LOQ ( $\mu\text{g/mL}$ )	1.076	0.559	

**Table 4.8: Percentage content of isoniazid assayed.**

<b>Methods</b>	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>BP, 2009</b>
Standard powder	99.9	99.5	99.7	99.8	99-101
Tablet	98.3	98.0	98.1	98.2	98-102

Official range for standard powder and tablet is 99 - 101 % and 98 – 102 % (BP, 2009).

## CHAPTER FIVE

### 5.0 DISCUSSIONS

The melting point purity agreement with what was reported in the literature. 170 – 172 °C and 172 – 174 °C as compared to official standard of 170 – 174 °C (table 4.2) while their IR spectra was found to be superimposable with the reference standard of isoniazid spectrum therefore the sample is likely to be isoniazid. (figures 4.1 to 4.2).

Solubility studies showed that isoniazid is soluble in polar solvents such as water, methanol and ethanol and insoluble in non-polar solvents such as benzene, ether and chloroform (table 4.3). This is what informed the use of water and methanol as solvent for preparing stock solutions of isoniazid. This is in agreement with BP 2009.

The four developed methods have maximum absorption ( $\lambda_{max}$ ) at 264 nm for 1 and 2 with 464 nm and 420 nm for methods 3 and 4 respectively. The calibration curves for quantitative determination of isoniazid in all the four developed methods obeyed Beers-Lamberts law within concentration range of 1-11  $\mu\text{g/mL}$  for methods 1 and 2 and 1-18  $\mu\text{g/mL}$  for methods 3 and 4 respectively. The coefficient of correlations ( $r$ ) of the calibration curve is a measure of the linearity of the methods developed. In all four methods, their value was very close to unity, indicating the linearity of the methods. It clearly shows the direct proportional relationship and high correlation between the absorbance ( $y$ ) and the respective concentration ( $x$ ) used for this determination in the regression equations for the four developed methods (tables 4.4 and 4.6) was compared to 0.996, 0.96, 0.9998 reported by Padmarajaiah *et al.*, (2002), Pratap *et al.*, (2012) and Arifa *et al.*, (2013) the  $r$  value in our methods were better. Both methods 1 and 2 were found to have effective pH at 4. This is the pH at which the drug absorbs maximally. Methods 3 and 4

were found to have effective concentrations of 18 µg/mL and effective volumes of 3 and 4 mL for methods 3 and 4 respectively. The yellow coloured hydrazine formed takes 15 minutes for the colour to completely develop while it takes 20 minutes for method 4 which indicates the methods are rapid..

Precision which is express as Percentage of Coefficient of Variation (% CV). Both the intraday and interday precisions of the four methods developed were found to be very good. This is indicated by the very low % CV (table 4.5 and 4.7) recorded in all the methods which were within the acceptable limit of < 15 % CV.(Padmarajaiah *et al.*, (2002),Pratapet *al.*, (2012) andArifaet *al.*, (2013) reported the precisions of 0.35, 1.2, 1.37 % respectively. Precision of the developed UV/Vis spectrometric methods are satisfactory. This is in accordance with the

accuracy of the four developed methods which were presented as the percentage relative error (% Er) and computed to be 4.13, 0.251, 0.328 and 0.670 % UV spectrophotometric methods using water and methanol.and calorimetric methods using 2,4-dinitrophenylhydrazine and by diazotization respectivelyPadmarajaiah *et al.*, (2002), Pratapet *al.*,(2012) and Enoche,(2010) reported respectively 0.4908, 0.05 and 2.47 %. They were all within the range of (< 5 %) for moderately accurate procedure (Harvey, 2000). Percentage recoveries determined for the four developed methods (102.02, 99.8, 99.83 and 100.06) were in the range of percentage recoveries of 100.63, 99.0 and 100.95 % reported by Enoche, (2010), Pratapet *al.*,(2012) andArifaet *al.*, (2013). LOD is the lowest quantity of drug a method can detect limit of detection in the solution but not necessarily quantify..

LOD for the four methods were 0.113, 0.145, 0.355 and 0.185 µg/mL for UV spectrophotometric methods using water and methanol.and calorimetric methods using 2,4-dinitrophenylhydrazine a

nd by diazotization respectively indicating better sensitivity of the methods as compared to 0.3243, 0.481, 80 and 0.585  $\mu\text{g/mL}$  reported by Padmarajaiah *et al.*, (2002), Pratapet *et al.*, (2012), Nagarajuet *et al.*, (2012) and Arifaet *et al.*, (2013) respectively. The limit of quantification (LOQ) is the small quantity of drug a method can detect and quantify in a solution. LOQ for the four methods UV spectrophotometric methods using water and methanol and calorimetric methods using 2,4-dinitrophenylhydrazine and by diazotization respectively which were 0.342, 0.438, 1.076 and 0.559  $\mu\text{g/mL}$  respectively were higher than 1.08, 1.59, 240, 1,772  $\mu\text{g/ml}$  as reported by Padmarajaiah *et al.*, (2002), Pratapet *et al.*, (2012), Nagarajuet *et al.*, (2012) and Arifaet *et al.*, (2013) respectively. The limit of detection (LOD) and limit of quantification (LOQ) are the function of sensitivity of the methods detect and quantify within acceptable precision and accuracy under the stated condition of tests.

A comparison of the mean percentage content of the isoniazid assayed using the developed methods and by using the official method (BP, 2009) showed that no statistical significant difference ( $p < 0.05$ ) was observed in the mean percentage contents of isoniazid. Therefore, the developed methods can be interchanged with the official method for the routine assay of isoniazid. UV spectrophotometric method using distilled water has the highest recovery of isoniazid compared with the other developed methods (table 4.8).



## **CHAPTER SIX**

### **6.0 CONCLUSIONS AND RECOMMENDATIONS**

#### **6.1 Conclusions**

From results obtained, it can be concluded that four new spectrophotometric methods for isoniazid determination in pure and tablet dosage form were developed and validated. There is no significant difference between the content of isoniazid assayed using the developed methods and that of the BP method, thus the developed methods can be interchanged with BP method.

#### **6.2 Recommendations**

Based on the findings of this research, four new developed spectrophotometric methods for isoniazid determination in pure and tablet dosage form can be used for routine quality control test of isoniazid. There is need for collaborations between the drug regulatory agencies such as NAFDAC that monitor and control drug use. There is also need for further studies to examine and compare the developed methods in biological fluids and compare the methods using advance techniques such as high performance liquid chromatography.

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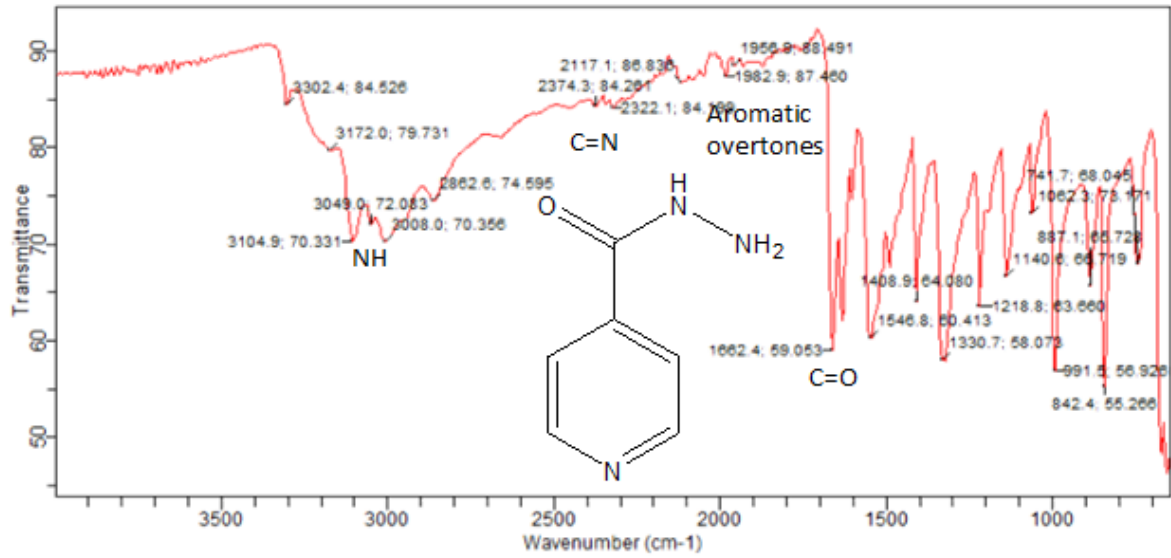
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## APPENDICES

### Appendix I: IR spectrum of isoniazid standard powder



## **Appendix II: Preparation of reagents**

### **Preparation of 4.5 M HCl**

Molarity required = 4.5

Molar mass = 98 gm

Volume of 4.5 M HCl required = 100 mL

Amount required = Molarity x Molar mass x Volume required / 1000 (constant)

$$= 4.5 \times 98 \times 100 \text{ mL} / 1000 \text{ (constant)}$$

$$= 44.1$$

Volume of ConcHCl required = Amount x 100 (constant) / Specific gravity x % purity

$$= 44.1 \times 100 / 1.83 \times 98$$

$$= 24.59 \text{ mL}$$

Therefore 24.59 mL of Conc. HCl was diluted with distilled water to make 100 mL which gave 4.5 M HCl.

For Solids: Molar mass of the solid is dissolved in 1000 mL of distilled water will give 1 M

And 4.5 x Molar Mass dissolved in 1000 mL (1 liter) gives 4.5 M

### **Preparation of 85 %v/v HCl**



A solution of 85 %v/v HCl was prepared by adding 85 mL of Conc. HCl to 15 mL of distilled water.

#### **Preparation of 2 %v/v HCl**

A solution of 2 %v/v HCl was prepared by adding 2 mL of Conc. HCl to 98 mL of distilled water.

#### **Preparation of 2, 4-dinitrophenylhydrazine solution**

This was prepared by dissolving 1 mg of 2, 4-dinitrophenylhydrazine and 2 mg Thiourea in 100 ml of 4.5 M HCl

#### **Preparation of 1 %w/v sodium nitrite**

A solution of 1 %w/v Sodium nitrite was prepared by accurately weighing and dissolving 1 g of sodium nitrite salt in 100 mL of distilled water.

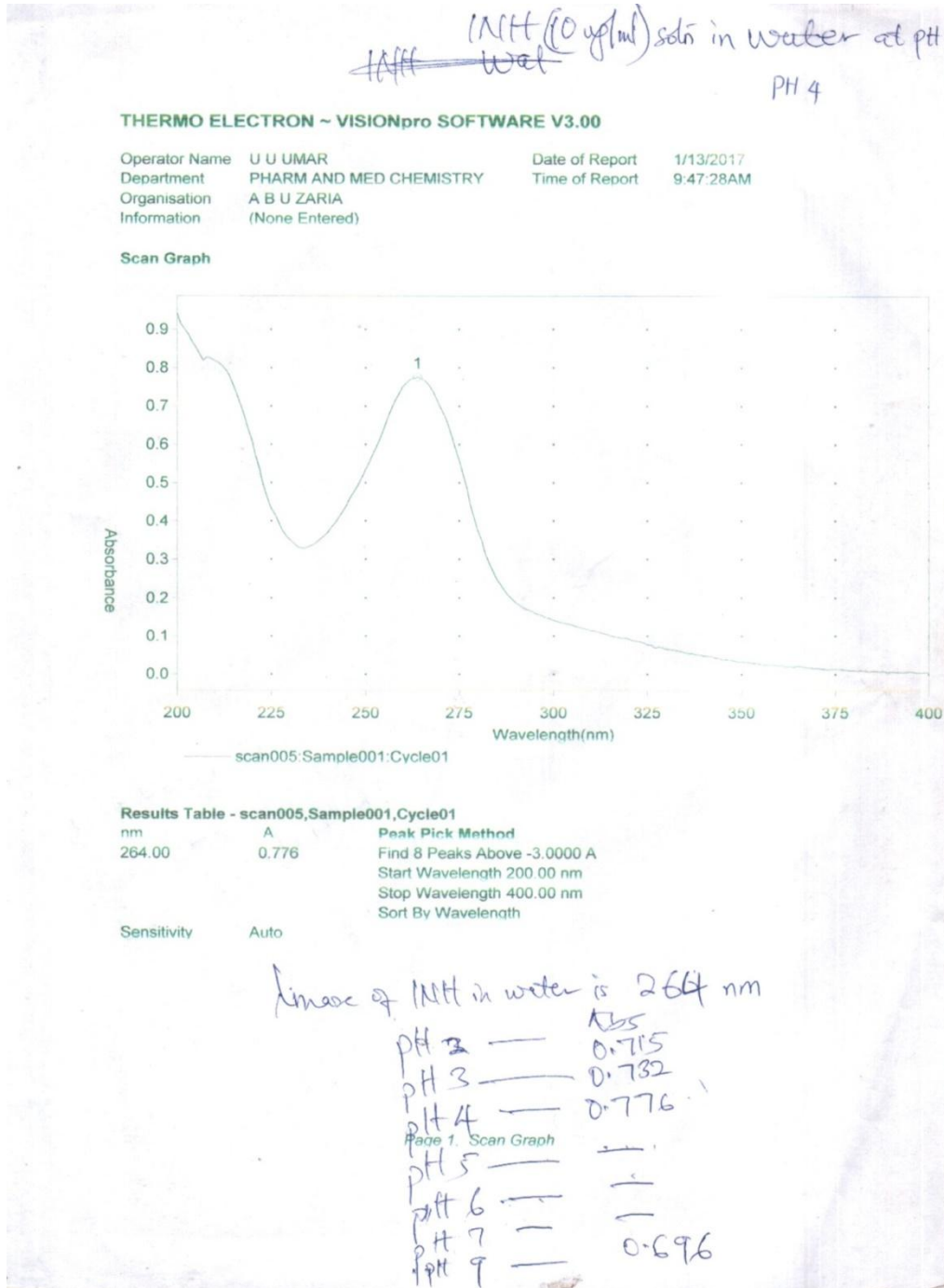
#### **Preparation of 2 %w/v Sulfamic acid**

A solution of 2 %w/v Sulfamic acid was prepared by accurately weighing and dissolving 2 g of Sulfamic acid salt in 100 mL of distilled water.

#### **Preparation of Para toluidine solution**

Para toluidine solution was prepared by accurately weighing and dissolving 1 mg of Para toluidine in 100 mL of methanol.

**Appendix III: UV Spectrum of isoniazid in distilled water.**



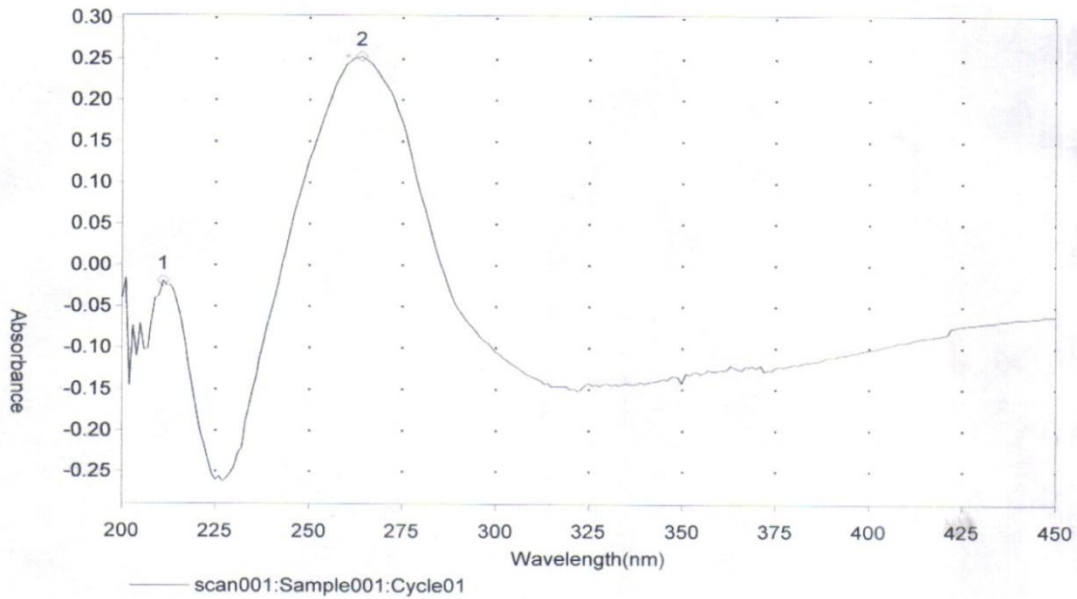
# Appendix IV: UV Spectrum of isoniazid in methanol

4/4/17  
Methanol

## THERMO ELECTRON ~ VISIONpro SOFTWARE V3.00

Operator Name	U U UMAR	Date of Report	2017-03-25
Department	PHARMAND MED CHEM	Time of Report	11:43:14AM
Organisation	A B U ZARI		
Information	(None Entered)		

### Scan Graph



### Results Table - scan001,Sample001,Cycle01

nm	A	Peak Pick Method
211.00	-0.019	Find 8 Peaks Above -3.0000 A
264.00	0.253	Start Wavelength200.00 nm
		Stop Wavelength450.00 nm
		Sort By Wavelength

Sensitivity      Auto

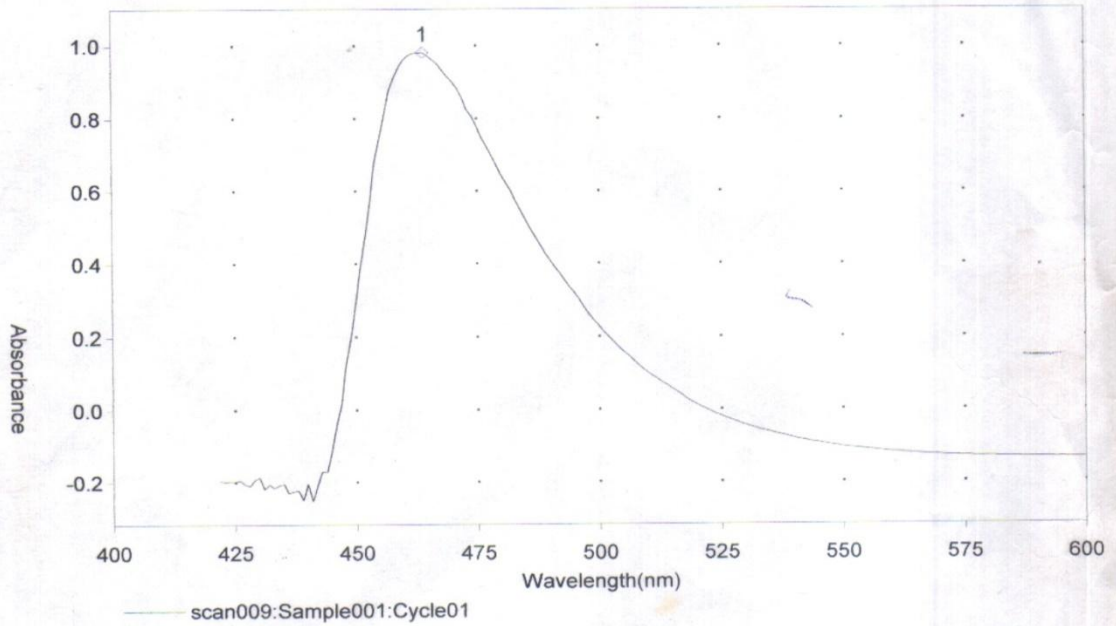
# Appendix V: UV Spectrum of isoniazid Solution with 2,4-dinitrophenylhydrazine

## THERMO ELECTRON ~ VISIONpro SOFTWARE V3.00

Operator Name (None Entered)  
Department (None Entered)  
Organisation (None Entered)  
Information (None Entered)

Date of Report 2017-03-31  
Time of Report 2:36:48AM

### Scan Graph



### Results Table - scan009,Sample001,Cycle01

nm	A	Peak Pick Method
464.00	0.981	Find 8 Peaks Above -3.0000 A Start Wavelength 400.00 nm Stop Wavelength 600.00 nm Sort By Wavelength

Sensitivity  Auto

*INH in 2,4-dinitro  
150 µg/ml Phenylhydrazine*

**Appendix VI: Calibration parameter of isoniazid in distilled water**

Calibration curve of isoniazid in water at pH 4  
 Sample 001 - 010 = 1Ng/ml - 10Ng/ml

14/3/17

A

**THERMO ELECTRON ~ VISIONpro SOFTWARE V3.00**

Operator Name U U UMAR Date of Report 1/18/2017  
 Department PHARM AND MED CHEMISTRY Time of Report 4:40:07AM  
 Organisation A B U ZARIA  
 Information (None Entered)

**Results Table - fixed002**

Data Mode	Absorbance
Sample	264.00nm
1 Sample001	0.038
2 Sample002	0.091
3 Sample003	0.168
4 Sample004	0.238
5 Sample005	0.296
6 Sample006	0.360
7 Sample007	0.433
8 Sample008	0.450
9 Sample009	0.562
10 Sample010	0.634

All calculations have been performed to double precision as defined by ANSI/IEEE STD 754-1985 but have been rounded for display purposes.

Date Collected 1/18/2017 Time Collected 4:35:18AM  
 Operator Name U U UMAR BIOMATE 164312

**Fixed Method - (Untitled)**

Integ. Time 00:00:01 hh:mm:ss Lamp Change 325.00 nm  
 Bandwidth 2.0 nm

0.669  
 - 0.562  
 -----  
 0.107  
 0.038  
 -----  
 0.006

0.445  
 - 0.433  
 -----  
 0.012

# Appendix VII: Maximum concentration of 2,4-dinitrophenylhydrazine

max conc  
1 ml + 2,4-DNPH  
1 mg/ml - 20 mg/ml

## THERMO ELECTRON ~ VISIONpro SOFTWARE V3.00

Operator Name (None Entered) Date of Report 2018-03-06  
Department (None Entered) Time of Report 5:01:02PM  
Organisation (None Entered)  
Information (None Entered)

### Results Table - m.hamza24-DNPh.fre

Data Mode	Absorbance
Sample	464.00nm
1 Sample001	0.012
2 Sample002	0.022
3 Sample003	0.030
4 Sample004	0.042
5 Sample005	0.051
6 Sample006	0.060
7 Sample007	0.067
8 Sample008	0.077
9 Sample009	0.055
10 Sample010	0.097
11 Sample011	0.090
12 Sample012	0.109
13 Sample013	0.113
14 Sample014	0.125
15 Sample015	0.128
16 Sample016	0.126
17 Sample017	0.141
18 Sample018	0.146
19 Sample019	0.136
20 Sample020	0.135

All calculations have been performed to double precision as defined by ANSI/IEEE STD 754-1985 but have been rounded for display purposes.

Date Collected 2018-03-05 Time Collected 2:56:57PM  
Operator Name (None Entered) BIOMATE 164312

### Fixed Method - (Untitled)

Integ. Time 00:00:01 hh:mm:ss Lamp Change 325.00 nm  
Bandwidth 2.0 nm

**Appendix VIII: Maximum time for colour development**

Determination of effective time for max Absorbance  
 (Full colour development) INH is 2.410NPH

**THERMO ELECTRON ~ VISIONpro SOFTWARE V3.00**

Operator Name BALARITI SAIDU Date of Report 2018-05-23  
 Department PHARMACEUTICAL CHEMISTRY Time of Report 10:09:19AM  
 Organisation AHMADU BELLO UNIVERSITY  
 Information (None Entered)

Results Table - et420008  
 Data Mode Absorbance

	Sample	464.00nm
5 min	1 Sample001	0.146
10 min	2 Sample002	0.158
15 min	3 Sample003	0.168
20 min	4 Sample004	0.155
25 min	5 Sample005	0.157
30 min	6 Sample006	0.148
35 min	7 Sample007	0.138

All calculations have been performed to double precision as defined by ANSI/IEEE STD 754-1985 but have been rounded for display purposes.

Date Collected 2018-05-23 Time Collected 10:07:21AM  
 Operator Name BALARITI SAIDUBIOMATE 164312

**Fixed Method - (Untitled)**  
 Integ. Time 00:00:01 hh:mm:ssLamp Change 325.00 nm  
 Bandwidth 2.0 nm

## Appendix IX: Calculation on precision

### METHOD 1

INTRA DAY FOR METHOD 1												
0 (hrs)		1 (hrs)		2 (hrs)		3 (hrs)		4 (hrs)		5 (hrs)		TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	
1	0.186	1	0.184	1	0.184	1	0.184	1	0.184	1	0.186	
2	0.187	2	0.182	2	0.183	2	0.183	2	0.182	2	0.187	
3	0.187	3	0.181	3	0.182	3	0.181	3	0.181	3	0.187	
4	0.186	4	0.180	4	0.186	4	0.180	4	0.180	4	0.186	
5	0.186	5	0.182	5	0.184	5	0.183	5	0.182	5	0.186	
<b>AVERAGE</b>	0.186		0.182		0.184		0.182		0.182		0.186	0.184
<b>SD</b>	0.0049		0.001327		0.001327		0.00147		0.001327		0.0049	0.001072
<b>%RSD</b>	0.262821		0.72973		0.72179		0.806638		0.72973		0.262821	0.585588

INTER DAY FOR METHOD 1							
DAY 1		DAY 2		DAY 3		TOTAL	TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.		
1	0.186	1	0.184	1	0.186		
2	0.187	2	0.183	2	0.187		
3	0.187	3	0.181	3	0.187		
4	0.186	4	0.180	4	0.186		
5	0.186	5	0.183	5	0.186		
<b>AVERAGE</b>	0.186		0.182		0.186	0.555	0.185
<b>SD</b>	0.0049		0.00147		0.0049	0.00245	0.000817
<b>%RSD</b>	0.262821		0.806638		0.262821	1.332279	0.444093



METHOD 2

INTRA DAY FOR METHOD 2												
0 (hrs)		1 (hrs)		2 (hrs)		3 (hrs)		4 (hrs)		5 (hrs)		TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	
1	0.610	1	0.612	1	0.611	1	0.611	1	0.612	1	0.611	
2	0.611	2	0.611	2	0.610	2	0.610	2	0.611	2	0.608	
3	0.610	3	0.608	3	0.609	3	0.609	3	0.608	3	0.609	
4	0.610	4	0.609	4	0.608	4	0.608	4	0.609	4	0.609	
5	0.609	5	0.610	5	0.610	5	0.610	5	0.610	5	0.610	
<b>AVERAGE</b>	0.610		0.610		0.610		0.610		0.610		0.609	0.610
<b>SD</b>	0.000632		0.001414		0.00102		0.00102		0.001414		0.00102	0.001087
<b>%RSD</b>	0.103681		0.231838		0.167291		0.167291		0.231838		0.167346	0.178214

INTER DAY FOR METHOD 2							
DAY I	DAY 2		DAY 3		TOTAL	TOTAL MEAN	
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.		
<b>1</b>	0.610	1	0.611	1	0.611		
<b>2</b>	0.611	2	0.610	2	0.608		
<b>3</b>	0.610	3	0.609	3	0.609		
<b>4</b>	0.610	4	0.608	4	0.609		
<b>5</b>	0.609	5	0.610	5	0.610		
<b>AVERAGE</b>	0.610		0.610		0.609	1.829	0.610
<b>SD</b>	0.000632		0.00102		0.00102	0.002672	0.000891
<b>%RSD</b>	0.103681		0.167291		0.167346	0.438317	0.146106

METHOD 3

INTRA DAY FOR METHOD 3												
0 (hrs)		1 (hrs)		2 (hrs)		3 (hrs)		4 (hrs)		5 (hrs)		TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc	Abs.	Cyc	Abs.	Cyc.	Abs.	Cyc	Abs.	
1	0.098	1	0.097	1	0.099	1	0.097	1	0.099	1	0.099	
2	0.095	2	0.097	2	0.099	2	0.097	2	0.098	2	0.098	
3	0.094	3	0.095	3	0.096	3	0.095	3	0.097	3	0.099	
4	0.096	4	0.096	4	0.094	4	0.096	4	0.095	4	0.099	
5	0.099	5	0.096	5	0.095	5	0.096	5	0.096	5	0.097	
AVERAGE	0.096		0.096		0.097		0.096		0.097		0.098	0.097
SD	0.001855		0.000748		0.002059		0.000748		0.001414		0.0008	0.001271
%RSD	1.923987		0.777891		2.1316		0.777891		1.457952		0.813008	1.313722

INTER DAY FOR METHOD 3							
DAY 1		DAY 2		DAY 3		TOTAL	TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.		
1	0.098	1	0.097	1	0.099		
2	0.095	2	0.097	2	0.098		
3	0.094	3	0.095	3	0.099		
4	0.096	4	0.096	4	0.099		
5	0.099	5	0.096	5	0.097		
AVERAGE	0.096		0.096		0.098	0.291	0.097
SD	0.001855		0.000748		0.0008	0.003403	0.001134
%RSD	1.923987		0.777891		0.813008	3.514887	1.171629

METHOD 4

INTRA DAY FOR METHOD 4												
0 (hrs)		1(hrs)		2 (hrs)		3 (hrs)		4 (hrs)		5 (hrs)		TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.	
1	0.739	1	0.738	1	0.741	1	0.738	1	0.738	1	0.742	
2	0.740	2	0.737	2	0.740	2	0.738	2	0.737	2	0.744	
3	0.739	3	0.739	3	0.736	3	0.737	3	0.739	3	0.743	
4	0.738	4	0.736	4	0.735	4	0.739	4	0.736	4	0.739	
5	0.737	5	0.737	5	0.737	5	0.736	5	0.737	5	0.741	
AVERAGE	0.739		0.737		0.738		0.738		0.737		0.742	0.738
SD	0.00102		0.00102		0.002315		0.00102		0.00102		0.00172	0.001352
%RSD	0.138073		0.138297		0.313793		0.13826		0.138297		0.231931	0.183109

INTER DAY FOR METHOD 4							
DAY 1		DAY 2		DAY 3		TOTAL	TOTAL MEAN
Cyc.	Abs.	Cyc.	Abs.	Cyc.	Abs.		
1	0.739	1	0.741	1	0.742		
2	0.740	2	0.740	2	0.744		
3	0.739	3	0.736	3	0.743		
4	0.738	4	0.735	4	0.739		
5	0.737	5	0.737	5	0.741		
AVERAGE	0.739		0.738		0.742	2.2182	0.740
SD	0.00102		0.002315		0.00172	0.005055	0.001685
%RSD	0.138073		0.313793		0.231931	0.683797	0.227932

## Appendix X: Calculations on accuracy, % recovery, LOD and LOQ

To 10 mL volumetric flask A<sub>1</sub> contains 5 mL of 10 µg/mL isoniazid solution and was kept unspiked. Its absorbance was measured and was recorded as (a = 0.630)

How to prepare 18 µg/L in test tube B<sub>1</sub>.

Concentration of isoniazid solution in 10 mL volumetric flask B<sub>1</sub> = 10 µg/mL (C1)

Volume of solution in 10 mL volumetric flask B<sub>1</sub> = 5 mL (V1)

Concentration of stock solution = 100 µg/mL (C2)

Volume of stock solution to be added to 10 mL volumetric flask B<sub>1</sub> = (V2?)

Final concentration of 10 mL volumetric flask B<sub>1</sub> = 18 µg/mL (C3)

Final volume of test tube B<sub>1</sub> = 10 mL (V3)

$$C1V1 + C2V2 = C3V3$$

$$10 \mu\text{g/mL} * 5 \text{ mL} + 100 \mu\text{g/mL} * V2 = 18 \mu\text{g/mL} * 10 \text{ mL}$$

$$50 \mu\text{g} + 100 \mu\text{g/mL} * V2 = 180 \mu\text{g}$$

$$100 \mu\text{g/mL} * V2 = 180 \mu\text{g} - 50 \mu\text{g}$$

$$100 \mu\text{g/mL} * V2 = 130 \mu\text{g}$$

$$V2 = 130 \mu\text{g} * \text{mL} / 100 \mu\text{g}$$

$$V2 = 1.3 \text{ mL}$$

1.3 mL from the stock solution was withdrawn and added to 10 mL volumetric flask B<sub>1</sub> and made up to 10 mL volume with distilled water and its absorbance was measured and recorded as (b = 1.156)

How to prepare 20 µg/mL in 10 mL volumetric flask C<sub>1</sub>.

Concentration of isoniazid solution in 10 mL volumetric flask C<sub>1</sub> = 10 µg/mL (C<sub>1</sub>)

Volume of solution in 10 mL volumetric flask C<sub>1</sub> = 5 ml (V<sub>1</sub>)

Concentration of stock solution = 100 µg/mL (C<sub>2</sub>)

Volume of stock solution to be added to 10 mL volumetric flask C<sub>1</sub> = (V<sub>2</sub>?)

Final concentration of 10 mL volumetric flask C<sub>1</sub> = 20 µg/mL (C<sub>3</sub>)

Final volume of 10 mL volumetric flask C<sub>1</sub> = 10 mL (V<sub>3</sub>)

$$C_1V_1 + C_2V_2 = C_3V_3$$

$$10 \mu\text{g/mL} * 5 \text{ ml} + 100 \mu\text{g/ml} * V_2 = 20 \mu\text{g/ml} * 10 \text{ ml}$$

$$50 \mu\text{g} + 100 \mu\text{g/mL} * V_2 = 200 \mu\text{g}$$

$$100 \mu\text{g/mL} * V_2 = 200 \mu\text{g} - 50 \mu\text{g}$$

$$100 \mu\text{g/mL} * V_2 = 150 \mu\text{g}$$

$$V_2 = 150 \mu\text{g} * \text{mL}/100 \mu\text{g}$$

$$V_2 = 1.5 \text{ mL}$$

1.5 mL from the stock solution was withdrawn and added to 10 mL volumetric flask C<sub>1</sub> and made up to 10 mL volume with distilled water and its absorbance was measure as (c = 1.235)

How to prepare 22 µg/mL in 10 mL volumetric flask D<sub>1</sub>.

Concentration of isoniazid solution in 10 mL volumetric flask D<sub>1</sub> = 10 µg/mL (C1)

Volume of solution in 10 mL volumetric flask D<sub>1</sub> = 5 mL (V1)

Concentration of stock solution = 100 µg/mL (C2)

Volume of stock solution to be added to 10 mL volumetric flask D<sub>1</sub> = (V2?)

Final concentration of 10 mL volumetric flask D<sub>1</sub> = 22 µ/ml (C3)

Final volume of 10 mL volumetric flask D<sub>1</sub> = 10 mL (V3)

$$C_1V_1 + C_2V_2 = C_3V_3$$

$$10 \mu\text{g/mL} * 5 \text{ mL} + 100 \mu\text{g/mL} * V_2 = 22 \mu\text{g/mL} * 10 \text{ mL}$$

$$50 \mu\text{g} + 100 \mu\text{g/mL} * V_2 = 220 \mu\text{g}$$

$$100 \mu\text{g/mL} * V_2 = 220 \mu\text{g} - 50 \mu\text{g}$$

$$100 \mu\text{g/mL} * V_2 = 170 \mu\text{g}$$

$$V_2 = 170 \mu\text{g} * \text{mL} / 100 \mu\text{g}$$

$$V_2 = 1.7 \text{ mL}$$

1.7 mL from the stock solution was withdrawn and added to 10 mL volumetric flask D<sub>1</sub> and made up to 10 mL volume with distilled water and its absorbance was measure as (d = 1.422)

Then using regression/linear equation, we can calculate as follows:

$$y = 0.0657x - 0.0288$$

For B<sub>1</sub> which was 18 µg/mL:

$$(b-a) = 0.0657x - 0.0288$$

$$0.0657x = (b-a) + 0.0288$$

$$0.0657x = (1.156 - 0.630) + 0.0288$$

$$0.0657x = 0.526 + 0.0288$$

$$0.0657x = 0.5548$$

$$X = 0.5548 / 0.0657 = 8.444 \text{ µg}$$

$$\% \text{ recovery} = 8.444 / 8 \times 100 = 105.55$$

$$\text{Accuracy} = 8.444 - 8 / 8 = 0.0555$$

For C<sub>1</sub> which was 20 µg/mL:

$$(c-a) = 0.0657x - 0.0288$$

$$0.0657x = (c-a) + 0.0288$$

$$0.0657x = (1.235-0.630) + 0.0288$$

$$0.0657x = 0.605 + 0.0288$$

$$0.0657x = 0.6338$$

$$X = 0.6338/0.0657 = 9.650$$

$$\% \text{ recovery} = 9.650/10 \times 100 = 96.5$$

$$\text{Accuracy} = 10 - 9.65/10 = 0.035$$

For  $D_1$  which was  $22 \mu\text{g/mL}$ :

$$(d-a) = 0.0657x - 0.0288$$

$$0.0657x = (d-a) + 0.0288$$

$$0.0657x = (1.422-0.630) + 0.0288$$

$$0.0657x = 0.7920 + 0.0288$$

$$0.0657x = 0.8208$$

$$X = 0.8208/0.0657 = 12.49 \mu\text{g}$$

$$\% \text{ recovery} = 12.49/12 \times 100 = 104$$

$$\text{Accuracy} = 12.49 - 12/12 = 0.04083333$$

Therefore mean % recovery for the three values =  $105.5+96.5+104/3 = 102.01 \%$

Therefore mean Accuracy for the three values =  $0.04083333+ 0.035+0.0555/3 = 0.04377$



**Detection limit:** The detection limit (LOD) was the sensitivity of the method to detect the presence of the analytical drug in the solution- LOD is expressed as the formula:

$$\text{LOD} = 3.3\sigma/S$$

Where  $\sigma$  is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

**Quantitation limit:** The quantitation limit (LOQ) was determined using the expression:

$$\text{LOQ} = 10\sigma/S$$

Where  $\sigma$  is the standard deviation of y-intercepts of the regression lines and S is the slope of the calibration curve.

**Appendix XI: slope, intercept and standard deviations**

Slope	0.06565	Intercept	-0.02885
Standard deviation of slope	0.000391	Standard deviation of intercept	0.002243
R <sup>2</sup>	0.999894	Degree of freedom	0.00247

---

$$\text{LOD} = 3.3\sigma/S = 3.3 \times 0.002243/0.06565 = 0.1127479$$

$$\text{LOQ} = 10\sigma/S = 10 \times 0.002243/0.06565 = 0.341660$$

This was repeated for methods 2, 3 and 4