Sequential Injection Analysis for Determination of Ammonia in Livestock Wastewater Using Acetoacetanilide as Hantzsch Reaction Reagent

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Abstract. Ammonia is a dangerous chemical compound in high concentrations. About 80-90% of ammonia total emissions come from animal fertilizer that produced from livestock, such as cattle farming. Based on the Minister of Environment Regulation Number 5 of 2014, the ammonia level in waste water standards for livestock activities is 25 mg/L. Some of the most common methods that used for determining ammonia are selective spectrophotometry and selective electrode, but the method has a disadvantage such as time consuming and low sensitivity. To overcome this problem, the aim of this study was to determine the dissolved ammonia levels in the liquid waste stream of cattle farms using the Sequential Injection Analysis (SIA) method by UV-Vis spectrophotometry at a wavelength of 307 nm based on the Hantzsch reaction using acetoacetanilide and formaldehyde reagents in acetate solution to form Dihydropyridine derivatives. The optimum condition of product formation occurred in the order of reagents and sample segmentation of formaldehyde (R)-ammonia (S)-AAA(R) acetate with the volume ratio 75µL: 50µL: 100µL, respectively. The optimum conditions of the proposed method are 0.06 M of AAA concentration, 15% of formaldehyde concentration, reaction time of 40 s, and flow rate of 175 µL. High sensitivity (LOD:0.122 mg/L), fast analysis (175 s/sample) and high accuracy (recovery test > 94%), less and harmless waste could be attributed as advantages of the proposed SIA method.

Keywords: Sequential Injection Analysis, ammonia, Acetoacetanilide, Hantzsch reaction, livestock

1. Introduction
Ammonia is a dangerous chemical compound in high concentrations. About 80-90% of ammonia total emissions come from animal fertilizer produced from livestock, such as cattle farming [1]. Ammonia produced from cattle farms comes from solid waste and liquid waste. Cattle farms produce liquid waste of 4.5-11 L/day/cattle on average with a composition of 1.200-2.900 mg/L total nitrogen, 780-2.200 mg/L ammonia and 64-500 mg/L phosphorus [2]. The total nitrogen commonly contained of 69% urea, 7.3% allantoin, 5.8% hippuric acid, 3.7% creatinine, 2.5% creatine, 1.3% uric acid, 0.5% xanthine and hypoxanthine, and 1.3% free amino acids (alanine (3.75%), β-alanine (0.3%), arginine (1.6%), glycine...
(53.3%), histidine (0.8%), 1-methyl histidine (3.3%), 3-methyl histidine (3.45%), isoleucine (0.0%), leucine (0.3%), lysine (1.8%) [3].

The Ministry of Environment Regulation Number 5 of 2014 explains that ammonia levels standards in waste water for livestock activities are 25 mg/L [4]. In high amounts, ammonia can be harmful to the environment, such as decreasing air quality and greenhouse gas emissions. Additionally, it also has a negative effect on human health. If it enters the respiratory tract it can be damaged the upper respiratory tract and may cause eye irritation [5]. The fast and accurate method for determining dissolved ammonia levels in liquid waste is very necessary to handle the high ammonia levels in the aquatic environment.

A common method that can be used to determine ammonia levels is blue indophenol method. However, the measurement condition requires complicated reaction mechanism, time consuming, has detection limit of 0.170 ppm and accuracy of 10.84% [6]. Another method is ion chromatography, which can generate relatively large measurement error of 20% [7]. The ammonia selective electrode in the aquatic environment sample method has the disadvantage of being susceptible to amine disturbance, having a slow equilibrium time and a low sensitivity of 0.07 mg/L [8]. Currently, determination of ammonia levels can be done by using the automatic flow analysis method using Flow Injection Analysis (FIA) and online automatic flow using Sequential Injection Analysis (SIA). Based on Ying Liang's research [9], the determination of ammonia nitrogen levels in water can be done using the Flow Injection Analysis method based on the reaction of NH$_3$-o-phthalaldehyde-Na$_2$SO$_3$ which has a low detection limit 0.007 mmol/L.

In the Sequential Injection Analysis method, this system is consisted of a syringe pump, holding coil, reaction coil, multiposition valve, and detector, controlled by a computer. All components are connected by PTFE capillary pipes. The advantages of the Sequential Injection Analysis method are that it requires a very little amount of reagent, quick and automatic analysis, and small waste product [10]. Based on Giakisikli's study [11], the determination of ammonia levels using the Sequential Injection Analysis method combined with a fluorimetric detector using the reaction of NH$_3$-o-phthalaldehyde-Na$_2$SO$_3$ under the alkaline conditions (pH 11), has a detection limit between 0.06 - 4 mg/L.

In the Sardesai study [12], it was explained that the determination of ammonia levels can be conducted by fluorometric method using the Hantzsch reaction. The mechanism of the Hantzsch reaction occurs between β-keto ester, formaldehyde, and ammonia which react to form a complex solutions in the form of 3,5-diacetyl-1,4-dihydrolutidine [13].

![Hantzsch Reaction](image)

**Figure 1.** Hantzsch Reaction [15].
Based on the Qiong Li’s research [14], the β-keto ester used in Hantzsch reaction was methyl acetoacetate, ethyl acetoacetate, n-propyl acetoacetate, n-amyl acetoacetate, malonic acid, dimethyl malonate, acetylacetone, and acetoacetonilide. Acetoacetonilide can be used as a new reagent in the Hantzsch reaction because it has a high selectivity with the largest molar extinction value at 25°C of 6100 dm$^3$mol$^{-1}$ compared to the others. In the Hantzsch reaction, two acetoacetonilide molecules are involved in the reaction, one molecule will react with formaldehyde and another molecule will react with ammonia to form an enamine type intermediate, followed by cyclodehydration to produce the dihydropyridine derivative.

In this study, the determination of ammonia levels was carried out using the Sequential Injection Analysis (SIA) method by UV-Vis spectrophotometry combined with acetoacetonilide reagents and acetate-formaldehyde reagents in the Hantzsch reaction. The UV-Vis spectrophotometer was used to measure the formed product at wavelength 307 nm. Some optimizations parameter performed include the sequence of reagents and samples segmentation, volume comparison of reagents and samples segmentation, acetoacetonilide concentration, formaldehyde concentration, reaction time, and flow rate. Tests on the effect of matrix disturbances in the form of amino acids (histidine), creatine and urea as other total nitrogen products contained in wastewater from cattle farms were carried out in ammonia levels determination to find out how is the other total nitrogen disturbances were against ammonia measurements. In addition, the sensitivity and validity of the method is based on the value of LOD and% recovery for the ammonia determination in the liquid waste samples of the livestock area.

2. Experimental

2.1. Apparatus
The tools used are glassware, Mettler's analytical balance, spray bottles, and a set of SIA (laboratory made SIA systems) consisting of a syringe pump (SP; Hamilton, Reno, Nevada, USA) with a volume of 2500 µL, eight valve selection valve (SP; Hamilton, Reno, Nevada, USA), detectors in the form of UV-Vis spectrophotometers and computer-controlled RGB-LED colorimeter cuvette detectors which controlled by using Visual Basic Program base home-made software (MPV LITE 2), capillary tubing (PTFE 0.75 mm ID), capillary tubing for holding coil (PTFE 1.8 mm i.d) and pH meter (Horiba).

2.2. Reagent
The ingredients used were 95% ethanol (Merch), 40% formaldehyde (Smartlab), absolute acetic acid (100%) (Merch), ammonia (25%)(Merch), acetoacetonilide solids (TCI-gr), sodium acetate solids (p.a), solid urea (Merch), creatine solids (Sigma Aldrich), histidine solids (Sigma Aldrich), aquades, liquid waste samples from livestock area (taken from the waste stream of Brawijaya University Faculty of Animal Science Field Laboratory, livestock waste stream in Karangploso District, Malang Regency, livestock waste stream in Pujon District, Malang Regency).

2.3. Procedure
SIA system preparation is divided into 3 stages. The first step is washing the capillary pipe (line), reaction coil, and detector using distilled water. The second stage is filling the capillary pipe (line) to be used by flowing acetoacetonilide reagent solution, formaldehyde acetate reagent, and ammonia from each post used as in Fig 2.

The third stage is the detection stage of ammonia solution with various concentrations. The reaction results in the reaction coil are flowed towards RGB colorimeter to find out the absorbance value of the product formed at the wavelength of 307 nm using a UV-Vis spectrophotometer equipped with a flow cell.
3. Result and Discussion

3.1. Optimization of Reagent and Sample Segmentation Sequences

There are three components that were injected in the optimum segment sequence conditions in the determination of ammonia using SIA system, namely, ammonia/sample solution, AAA reagent solution, formaldehyde acetate reagent solution. Therefore, there are six injection ways as shown in Table 1, in order to form a different segmentation in the reaction coil. The results of segmentation optimization are shown in Figure 3.

Table 1. Segmentation Type.

| Type | Segmentation Sequences                     |
|------|-------------------------------------------|
| 1    | Ammonia- acetate-formaldehyde - AAA       |
| 2    | Ammonia - AAA- acetate-formaldehyde      |
| 3    | acetate-formaldehyde - ammonia -AAA       |
| 4    | acetate-formaldehyde - AAA - Ammonia      |
| 5    | AAA- acetate-formaldehyde -ammonia        |
| 6    | AAA - ammonia- acetate-formaldehyde       |

Based on Fig. 3 it can be seen that the order of different segments gives different absorbance values. The highest absorbance value is obtained in segmentation type 3, when the formed segmentation is acetate-formaldehyde-ammonia-AAA. Based on the Hantzsch reaction mechanism, two moles of acetoacetanilide are used, one mole of acetoacetanilide will react first with ammonia, while one other mole of acetoacetanilide will react with one mole of acetate-formaldehyde. Furthermore, the two intermediate types formed will experience cyclodehydration to form the dihydropyridine derivative. With the order of the acetate-formaldehyde-ammonia-AAA segment, ammonia which first get in contact with AAA will react faster to form the first intermediate product. Then, because AAA is made excessively, the remaining AAA which does not react with ammonia will react with acetate-formaldehyde to form the second intermediate product. Then, the two intermediate products will undergo a cyclodehydration reaction to form the Hantzsch reaction product in the form of dihydropyridine derivatives.

If there is AAA between ammonia and acetate-formaldehyde among the segmentation formed, then the reaction formation of the two intermediates will happens at the same time. Thus it can not be assured that the ammonia in the sample are all reacted, so that the product formed can not be used to interpret the total amount of ammonia in the sample. It was revealed by the absorbance measurement in the segment condition where AAA is between ammonia and acetate-formaldehyde. Meanwhile, if among the formed segmentation, AAA and ammonia are separated by acetate-formaldehyde, then the measured...
absorbance will become lower. Acetate-formaldehyde which first contacts AAA will react to form the first intermediate product. Because the amount of acetate-formaldehyde in the reaction system is made excessive, then AAA will react with a number of available acetate-formaldehyde, so the remaining AAA reacts with ammonia only in small quantities or even none so that the Hantzsch reaction product formed will be very small or not even formed.

**Figure 3.** Effect of the segmentation (see Table 1) and absorbance of Hantzsch reaction products.

### 3.2. Segmentation Volume Optimization of Reagent and Sample

In the determination of ammonia using this SIA system, the volume of segmentation between reagents and samples injected with the segmentation sequence of acetate-formaldehyde-ammonia-AAA is shown in Table 2. The results of segmentation volume optimization are shown in Figure 4.

**Table 2. Segmentation Volume Type.**

| Type | Segmentation Volume Acetate-formaldehyde : Ammonia : AAA (µL)* |
|------|-------------------------------------------------------------|
| 1    | (50:100:75)                                                |
| 2    | (100:50:75)                                                |
| 3    | (75:50:100)                                                |
| 4    | (50:75:100)                                                |
| 5    | (75:100:50)                                                |
| 6    | (100:75:50)                                                |

*Total volume of segmentation: 225 µL

Based on Fig.4 it can be seen that the volume of segmentation between reagents and samples in the segmentation sequence of acetate-formaldehyde-ammonia-AAA with a volume ratio of 75; 50; 100 µL gives the highest absorbance value. Based on the Hantzsch reaction of two moles of acetoacetanilide, one mole of acetoacetanilide will react first with ammonia, while another one mole of acetoacetanilide will react with one mole of acetate-formaldehyde. At a volume ratio of 75; 50; 100 µL, acetate-formaldehyde and AAA reagents were produced more than the ammonia. So that in the reaction, ammonia will react completely in order to form the intermediate products, when reacting with AAA, the remaining AAA will then react with acetate-formaldehyde to form the second intermediate product. At the reagent and sample volume ratio of 50; 75; 100 µL AAA reacts more with ammonia to form the first intermediate product, and AAA which reacts with acetate-formaldehyde forms a small intermediate product because the acetate-formaldehyde reagent is not made excessively. At a volume ratio of 50; 100; 75 µL and 75; 100; 50 µL ammonia is made the most excess, when reacting the AAA reagent will completely react with ammonia before reacting with formaldehyde acetate so that the reaction does not react perfectly and causes a low absorbance value. While at the reagent and sample volume ratio of 100; 50; 75 µL and 100; 75; 50 µL, acetate-formaldehyde is made excessively, during this reaction before
AAA reacts with acetate-formaldehyde, AAA which reacts with ammonia has completely reacted. In this reaction, only the first intermediate product is formed and the absorbance value is low.

![Graph](image.png)

**Figure 4.** Effect of the segmentation volume type (see Table 2) and absorbance of Hantzsch reaction products.

### 3.3. Acetoacetanilide Concentration Optimization

Determination of the optimum AAA concentration was carried out to determine the AAA concentration needed to react with ammonia and acetate-formaldehyde so that the Hantzsch reaction product can be formed completely. The optimum AAA concentration can be seen from the peak height or absorbance value which shows the intensity of the Hantzsch reaction product formed. The AAA concentration used is 0.01 - 0.1 M. The results of the AAA concentration optimization are shown in Fig. 5. Based on the absorbance values measured at various concentrations of AAA, optimum conditions for AAA concentration of 0.06 M were obtained which were indicated by high absorbance values. At small ammonia concentrations, AAA is not enough to change the Hantzsch reaction product. At the concentrations above 0.06 M it did not increase because the Hantzsch reaction had reached equilibrium and all the ammonia contained in the system had reacted.

![Graph](image.png)

**Figure 5.** Effect of the concentration of AAA and the absorbance of the Hantzsch reaction product.

### 3.4. Optimization of Formaldehyde Concentration

Determination of the optimum formaldehyde concentration was conducted to determine the concentration of formaldehyde that was needed to react with AAA, so that the Hantzsch reaction product can be perfectly formed. The formaldehyde concentration used was 5, 15, 25, 35%. The results of the optimization of formaldehyde concentration are shown in Fig.6. Based on Fig.6 the optimum concentration of formaldehyde is 15%. At the concentrations of 5-15% the absorbance value increases because the concentration of formaldehyde is sufficient to convert all ammonia in the system to the
product of the Hantzsch reaction. At concentrations of more than 15%, the absorbance value decreases. This means that the Hantzsch reaction has reached equilibrium and more AAA is used to react with acetate-formaldehyde.

![Figure 6](image1.png)

**Figure 6.** Effect of the concentration of formaldehyde and absorbance of the Hantzsch reaction product.

3.5. **Optimization of Reaction Time**

Determination of the optimum reaction time is carried out to determine the most optimum time needed for the complete formation of the Hantzsch reaction product. The reaction time used are 10, 20, 30, 40, 50 and 60 seconds. The results of the optimization of reaction time are shown in Fig. 7. Based on Fig 7 the highest absorbance which shows the optimum condition is obtained at a reaction time of 40 seconds. At the reaction time of 10-20 seconds the increase in absorbance tends to be small. Then, in the reaction time of 30-40 seconds, the absorbance increases significantly. In reaction time of more than 40 seconds or at the reaction time of 50-60 seconds the absorbance of the measured product is not constant and decreases. It is caused by the decomposition of the Hantzsch reaction product by sunlight because of the tip mixing where the reaction has a translucent or transparent surface. The reaction time of 40 seconds is the optimum condition because at that time, the Hantzsch reaction goes perfectly and all of the ammonia contained in the sample completely reacted with acetate-formaldehyde and AAA.

![Figure 7](image2.png)

**Figure 7.** Effect of the reaction time and absorbance of the Hantzsch reaction product.

3.6. **Optimization of Flow Rate**

The determination of the product flow rate to the detector is done by varying the flow rate towards the detector. Optimization of flow rate is done because the flow rate of the product towards the detector affects the speed of the analysis process and the shape of the absorption band. The variations in the flow rate used are 50, 75, 100, 125, 150, 175, 200, 115 and 250 µL/s. The flow rate optimization result is shown in Fig. 8. Based on Fig 8 the optimum flow rate is 175 µL/s because the highest absorbance at
the flow rate is produced. If the flow rate of the product goes to the detector too slowly, there will be disperse of the Hantzsch reaction product into the carrier in the form of distilled water resulting in dilution which causes a decrease in the Hantzsch reaction product. As result the absorbance value is low. At flow rates above 175 µL/s, the absorbance decreases because if the flow rate is too fast, the product cannot be detected correctly by the detector and may cause back pressure which can disperse the product so that the concentration of the Hantzsch reaction product decreases.

![Graph showing the effect of flow rate on absorbance.](image)

**Figure 8.** Effect of the flow rate and absorbance of the Hantzsch reaction product.

### 3.7. Interference

In livestock wastewater there are several total nitrogen elements including urea, allantoin, hippuric acid, creatinine, creatine, uric acid, xanthine and hypoxanthine, and free amino acids (alanine, β-alanine, arginine, glycine, histidine, isoleucine, leucine, lysine). In this study, the interference of histidine, creatine and urea on the determination of the ammonia was studied. The effect of the interference matrix was studied by adding interference matrix to the 5 mg/L standard ammonia solution with a ratio of 0 mg/L; 5 mg/L; 25 mg/L and 50 mg/L. The results are shown in table 3.

| Interference Matrix | Tolerance Limits (mg/L) |
|--------------------|-------------------------|
| Histidine          | > 50                    |
| Urea               | > 50                    |
| Creatine           | > 50                    |

Based on the results obtained, it can be seen that the tested histidine, creatine and urea do not cause any interference effect on ammonia detection. Interference limit is large than 50 mg/L.

### 3.8. Calibration graph

The standard curve is made by measuring the absorbance of the Hantzsch reaction product with an ammonia concentration of 0-25 mg/L. Absorbance measurements uses all of the optimum conditions of the predetermined parameters, namely segments sequence, volume ratio between reagents and samples, AAA concentrations, formaldehyde concentration, reaction time and flow rate. The results of the standard curve measurements are shown in Fig. 9.

Based on the standard curve shown in the Fig. 9 linear correlation equation $y = 0.0599x + 0.1180$ with a correlation coefficient ($R^2$) of 0.9816, where $y$ is the absorbance value and $x$ the ammonia concentration obtained. The, the standard curve equation is used for calculating ammonia levels in the sample as well as determining detection limits. Detection limit ($S/N=3$) from blank solution ($n=10$) obtained based on the standard curve that is equal to 0.122 mg/L.
3.9. Applications

In this study, measurement of ammonia levels in samples was carried out using the SIA method. The samples used came from 3 livestock areas in Malang Regency, namely the waste stream of UB's Faculty of Animal Science Field Laboratory (sample 1), community livestock waste streams in Karangploso District, Malang Regency (sample 2), and community livestock waste streams in Pujon District Malang Regency (sample 3). The results of ammonia levels measurement of the sample are shown in table 4.

Table 4. Result of determination of ammonia in livestock wastewater samples.

| Samples | NH₃ found (mg/L) | NH₃ added (mg/L) | Total NH₃ found (mg/L) | Recovery (%) |
|---------|------------------|------------------|------------------------|--------------|
| 1       | 17.94            | 2.0              | 18.97                  | 95           |
| 2       | 17.79            | 2.0              | 18.79                  | 94           |
| 3       | 11.71            | 2.0              | 13.15                  | 95           |

The SIA method can be recommended as a method in ammonia determination because this method is carried out with a fast analysis time, which is only for 175 seconds and has a high sensitivity with very small volumes of samples and reagents. The accuracy of the SIA method with the acetoacetanilide reagent is proven by high recovery %. Recovery % was determined by adding a standard ammonia solution of 2 mg/L as much as 5 μL into each previously measured sample so that the differences in sample concentration before and after the addition of standard ammonia can be measured. Based on the obtained calculation results, % recovery in sample 1 was 95%, in sample 2 was 94% and in sample 3 was 95%.

4. Conclusion

Determination of dissolved ammonia levels in liquid waste in livestock using the Sequential Injection Analysis (SIA) method by UV-Vis spectrophotometry with acetoacetanilide reagents have a low detection limit of 0.122 mg/L and recovery values > 94% in the sample.

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