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Determination of Total Ammonia Nitrogen by Gas-Diffusion Flow Injection Analysis (GD-FIA)-Spectrophotometry using Minnieroot Flower (*Ruellia tuberosa*) as Natural Reagent

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Abstract

Total ammonia nitrogen (TAN) is a pollutant agent found in water and generated from the metabolisms of living organisms and the decomposition of organic matter. This research aims to develop an efficient, low-cost, and environmentally friendly TAN analysis method. The principle of this method is based on the reaction of TAN injected in the donor stream with NaOH to form alkaline gaseous ammonia. The gaseous ammonia diffuses through a hydrophobic membrane into an acceptor stream containing *Ruellia tuberosa* extract. The presence of NH_3 gas in the acceptor stream changes the extract color from pinkish to green, which is monitored by spectrophotometer at 620 nm. This method was also validated based on linearity, selectivity, and accuracy. Under the obtained optimum conditions of 1 M NaOH, 5% extract, a 120 cm mixing coil, and a 200 µL sample volume, the proposed method showed excellent linearity at 10–1000 ppm (R^2 = 0.993). The selectivity test result shows that this method was selective against sulfite and nitrite compounds up to 600 ppm with a % error value <10%. This method was applied to measure the total ammonia concentration in agricultural water, and satisfying results were shown by high recoveries of 95.03%–98.84%.

Keywords: ammonia, gas-diffusion flow injection analysis, minnieroot flower, selectivity, spectrophotometry

Introduction

Total ammonia nitrogen (TAN) is a pollutant agent found in natural water that is toxic in elevated concentrations [1]. High amounts of ammonia nitrogen can endanger living things and aquatic life by triggering the growth of algae such that it covers water surface and reduces dissolved oxygen. This phenomenon is known as eutrophication [2]. TAN is commonly found in water generated from fish and other living organisms' metabolism and is excreted through feces and the decomposition of organic matter such as feed residues, bacteria, and algae [3]. In water, ammonia is available in two species, ammonium (NH_4^+) and non-ionized ammonia (NH_3), together called TAN [4]. The presence of TAN in water forms an equilibrium reaction (1), where the addition of numerous hydroxide ions causes a change in the NH_4^+ species to NH_3. This result causes pH to affect the specific form of ammonia substantially. High pH will increase NH_3 production, which also increases water toxicity [5].

$$NH_4^+ + OH^- \leftrightarrow NH_4OH \leftrightarrow NH_3 + H_2O \quad (1)$$

TAN detection methods are usually performed using spectrophotometry with Nessler's or indophenol blue reagents, titrimetry, ion chromatography, and ion-selective electrodes [6]. Unfortunately, most of these methods are considered inefficient because of their multiple-step analysis and excessive reagent usage requirements [7]. Therefore, more efficient, inexpensive, and less time-consuming methods for ammonia detection are being developed. One of the developing methods for TAN determination is flow injection analysis (FIA) coupled with a gas-diffusion unit (GD-FIA) [8]. FIA is widely used to detect metal [9] and nonmetal ions [10, 11]. FIA was also developed to detect TAN using synthetic reagents such as indophenol blue and Nessler's reagent [12, 13]. The use of GD-FIA with natural reagents has also been investigated in the determination of ammonia compounds using orchid flower extract [14], and sulfite compounds using rosella extract [15].

FIA equipped with a gaseous diffusion cell (GD-FIA) with spectrophotometric detection has been researched and found to offer high selectivity by converting TAN into gaseous ammonia and separating it from other matrices in a sample by using a hydrophobic membrane [8]. Although this method is up-and-coming, just like other mentioned methods, it still has a drawback: dangerous synthetic reagent use.

Green chemistry has recently become a concern of researchers. Green chemistry is an approach to chemistry focused on raising awareness by reducing the environmental impact of laboratory waste [16]. An application of green chemistry is the use of natural reagents to replace hazardous synthetic reagent use. These natural reagents can be derived from plants or microorganisms [17]. Natural reagents are also widely used in FIA techniques for detecting various compounds, such as *Phyllanthus emblica* Linn extract for detecting Fe(III) and *Morinda citrifolia* root extract for the determination of Al(III) [18, 19]. Natural reagents have also been used to detect TAN through the GD−FIA technique with the natural reagent of orchid flower extract [14]. However, the applicability of *Ruellia tuberosa* flower extract to detecting TAN through the GD-FIA technique has not been reported. Therefore, this research aimed to develop the potential of a natural reagent from *Ruellia tuberosa* flower extract for TAN determination by employing GD−FIA spectrophotometry.

Minnieroot flower (*Ruellia tuberosa* L.) (Figure 1) is a wild plant easily found around Southeast Asia. *Ruellia tuberosa* comes from Central America, spreading to Southeast Asia. *Ruellia tuberosa* grows in dry areas starting from 150 m above sea level [20]. The bluish-purple color of the minnieroot flower is caused by a pigment compound called anthocyanin [21]. Anthocyanin (Figure 2) is a water-soluble colored phenolic compound. Anthocyanins are responsible for the red, purple, and blue colors in fruits and vegetables. Anthocyanins are widely used as natural dyes because their properties can provide a variety of colors. Most plant pigments are in the form of inter-glucosylated anthocyanins [22]. Of the many types of anthocyanins, at least six are commonly found in nature. These six types of anthocyanins include pelargonidin, delphinidin, cyanidin, petunidin, peonidin, and malvidin. Each type of anthocyanin has a different chemical structure from its R_1 and R_2 bond according to the structure below [23].

Figure 1. *Ruellia tuberosa* Plant

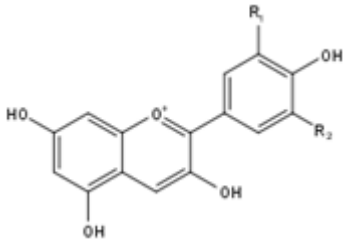

Figure 2. General Structure of Anthocyanin

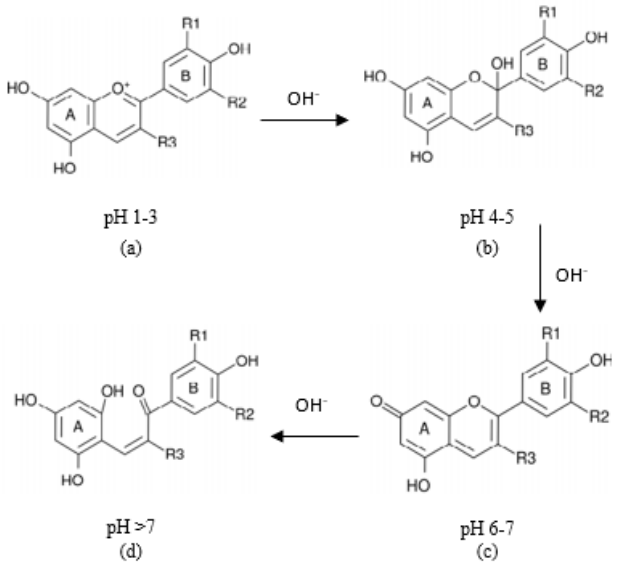

Figure 3. Structure of Flavylium Cation (a), Carbinol Pseudobase (b), Quinoidal Base (c), and Cis-Chalcone (d)

Anthocyanin is an unstable compound. Solution pH dramatically influences the stability of the structure and color of anthocyanins. In solution, anthocyanin rearranges to form an isomeric structure from the basic anthocyanin structure. Anthocyanins are stable at acidic pH, and present as flavylium cations in red color at pH 1–3. An increase in solution pH changes the flavylium cations into carbinol pseudobase, which is colorless at pH 4–5. Carbinol pseudobase undergoes a structural change to become a blue-green quinoidal base at pH 6–7. At a more alkaline pH (>7), the quinoidal base undergoes a structural change to yellowish cis-chalcone [24]. The influence of pH on the structure of anthocyanin is shown in Figure 3 [25].

This research focused on developing a natural reagent, minnieroot flower extract, as a color-changing indicator of ammonia in water by using the GD-FIA spectrophotometry to fulfill the green chemistry system and reduce the effects of synthetic reagent usage. The results of this study should be used as a reference in developing efficient, low-cost, and environmentally friendly TAN analysis methods.

Materials and Methods

Chemicals. The solution used for the acceptor stream contained minnieroot flower extract, whereas the donor stream solution was 1 M sodium hydroxide (NaOH, Sigma Aldrich). Ammonium chloride (NH_4Cl, Sigma Aldrich) was used as the standard solution. Sodium nitrite ($NaNO_2$, Sigma Aldrich) and sodium sulfite (Na_2SO_3, Sigma Aldrich) were used as the interfering compounds. A 96% ethanol and demineralized water solution was used as the solvent. The water sample used for validation test was agricultural water obtained from Malang, East Java.

GD-FIA-spectrophotometry manifold. The GD-FIA spectrophotometry system (Figure 4) employed a donor stream of NaOH solution and acceptor stream of minnieroot flower extract solution, which flowed through capillary tubes through a peristaltic pump. A syringe was used to inject the TAN sample to fill the sample loop at the "load" injector position. Then, the injector was moved to the "inject" position to allow NaOH solution carrying while reacting with the TAN sample to the gas-diffusion (GD) unit. The gaseous NH_3 formed from the equilibrium shift of NH_4OH due to adding alkaline NaOH (reaction 1) [14] was then diffused through the hydrophobic PTFE membrane to the acceptor stream. The presence of gaseous NH_3 in the acceptor stream changed the pH of the extract, resulting in a color change from pinkish to green (Figure 5). This color change caused an increase in the absorbance at 620 nm, which was detected using the UV–vis spectrophotometer.

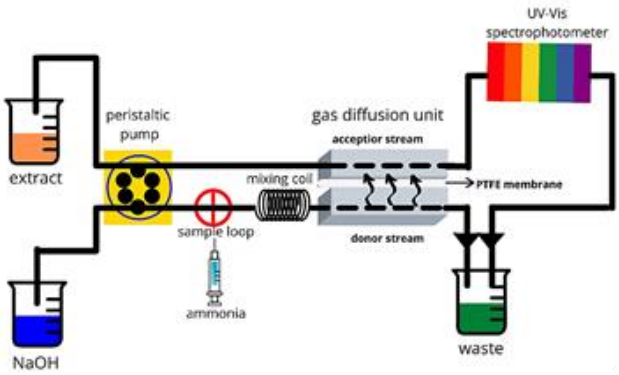

Figure 4. GD-FIA spectrophotometry manifold

Figure 5. Color Series of Minnieroot Flower Extract at pH 6-13

Minnieroot flower extraction. Extraction was performed using the maceration method. Minnieroot flower petals were weighed to 25 g and then soaked in 100 mL of ethanol for 24 h. Then, the extract was filtered using filter paper [26].

pH dependence test of minnieroot flower extract. The color-changing activity test of minnieroot flower extract was performed by preparing solutions with pH values of 6–13. Then, 1 mL of 10% minnieroot flower extract was mixed with the solution. The pH was adjusted to the desired value using 1 M HCl and 1 M NaOH. The absorbance of each solution was then observed using a UV–vis spectrophotometer (Shimadzu) at 380–800 nm [14].

Optimization of GD-FIA-spectrophotometry. The GD-FIA-Spectrophotometry was optimized towards chemical and operational parameters in order to achieve high sensitivity of measurements. These optimizations were conducted based on GD-FIA-spectrophotometry procedure (Figure 4).

Chemical optimization. Chemical optimization was performed by optimizing the NaOH and minnieroot flower extract concentrations. In this work, conditions such as 75 cm of mixing coil length, 2.8 mL/min of flow rate, 75 µL of sample volume, 5% minnieroot flower extract, and 1000 ppm of standard NH_4Cl were applied. To optimize NaOH concentration, it was varied from 0.1 to 1.5 M. This work aimed to obtain the optimum TAN conversion into NH_3 gas, which strongly affects the proposed method's sensitivity. The optimum NaOH

solution was then used to optimize minnieroot flower extract under the same conditions. The extract concentration was 1 to 15% (w/v) [26].

Operational optimization. The result of chemical optimization was then used for operational optimization. This work was done by optimizing the mixing coil length and sample volume under the same conditions as chemical optimization. To optimize the mixing coil length, it was varied from 35 to 160 cm. This work aimed to obtain a complete mixing of TAN with NaOH to produce NH_3 gas. The optimum length was then used for optimizing the sample volume. This work was done by varying the volume of the sample loop from 50 to 250 µL Under the same conditions, the measurement was performed also by the same procedure [14].

Linear detection range test. The linear detection range test was performed using the GD–FIA spectrophotometry method under the obtained optimum conditions. This test was performed by injecting standard NH_4Cl at 10, 50, 100, 300, 500, 700, 1000, and 5000 mg/L. The effect of the standard ammonia concentration on absorbance was observed, and a linearity curve was made. The curve showing the best linearity was used as the TAN calibration curve [14].

Determination of TAN in a real samples. The determination of TAN through GD–FIA was accomplished by measuring the agricultural water collected from Malang, Indonesia in three areas (A, B, and C). Before the measurements, the sample was filtered to separate dirt and impurities [26]. The sample solution was then injected into the sample loop with the GD–FIA system under the obtained optimum condition results. The absorbance of the resulting FIA-gram was then interpolated into the calibration curve equation to calculate the TAN concentration available in the samples [14].

Selectivity test. Selectivity test was used o determine the interfering compounds that are available in agricultural water along with ammonia. The interfering compounds studied in this test are nitrite and sulfite. The selectivity test was performed by mixing $NaNO_2$ or Na_2SO_3 (300, 600, and 1500 ppm) with 300 ppm of NH_4Cl in 100 mL volumetric flask. The selectivity of the method was determined through the % error value by comparing the absorbance of ammonia in the absence and presence of interfering ions [14, 26].

Accuracy test. An accuracy test was performed by adding standard NH_4Cl (300 and 700 ppm as ammonia) to an agricultural water sample in a 100 mL volumetric flask. The measurement was performed according to the method, and the %recovery was calculated. An additional accuracy test was performed by comparing the results of TAN determination through the proposed method to those obtained from standard spectrophotometry using indophenol blue. A paired t-test was performed to evaluate the significant difference between both results [26].

Result and Discussion

pH dependence of minnieroot flower extract. Anthocyanin is a flavonoid group compound that easily changes its color with a change in solution pH [27]. Therefore, a pH dependance test of anthocyanin color in minnieroot extract was used to determine the color series of minnieroot extract at pH 6–13. Based on Figure 5, this extract is pinkish at pH 6–7, green at pH 8–10, and yellow at pH 10–13. This color-change test of minnieroot extract shows a similar result to a previous report (Putri, 2019) in which the minnieroot extract was pink and orange in acid pH (1-6) and blue, green, and yellow in alkaline pH [28]. Each solution was scanned from 380–750 nm using a UV–vis spectrophotometer to obtain the spectral profile to determine the maximum absorbance wavelength for measurements. Figure 6 shows that minnieroot flower extract has a maximum absorbance wavelength of 521 nm at pH 6–7, 620 nm at pH 8–10, and 382 nm at pH 382 nm. The change in wavelength is caused by a rearrangement reaction of the anthocyanin structure (Figure 3). The further addition of ammonia to minnieroot flower extract (pH 6) caused a color change from pinkish to green due to the increasing pH of the extract up to pH 10. The wavelength of 620 nm was then for further measurements, and based on Figure 7, the absorbance increased with the pH until reaching a maximum value at pH 9. This result indicates that the detection of TAN using minnieroot flower extract may only be carried out up to a concentration that gives $pH \leq 9$.

Optimization of NaOH concentration. The NaOH concentration must be optimized to obtain an adequate amount of NaOH to convert all NH_4^+ ions in the sample solution into NH_3 gas, which is then passed through the hydrophobic membrane into the minnieroot flower extract in the acceptor stream. The more NH_3 gas formed, the greener the extract became, and the higher the absorbance of the solution at 620 nm. The NaOH concentration was optimized by varying it from 0.1 to 1.5 M.

Figure 8 shows that the extract absorbance gradually increases from 0.1 to 0.75 M, dramatically increased at 1 M, and then remained relatively constant at higher concentrations (1.5 M). This increase indicates that the greater the NaOH concentration was, the more ammonia gas was produced. Therefore, 1 M NaOH was chosen as the optimum concentration and used in subsequent measurements since it gave high absorbance, which leads to high sensitivity. In comparison, Sukaram in 2018 used only 0.1 M NaOH for ammonia detection with the GD–FIA method. This difference in concentration resulted from the different natural reagent used, which led to different behavior and sensitivity [14].

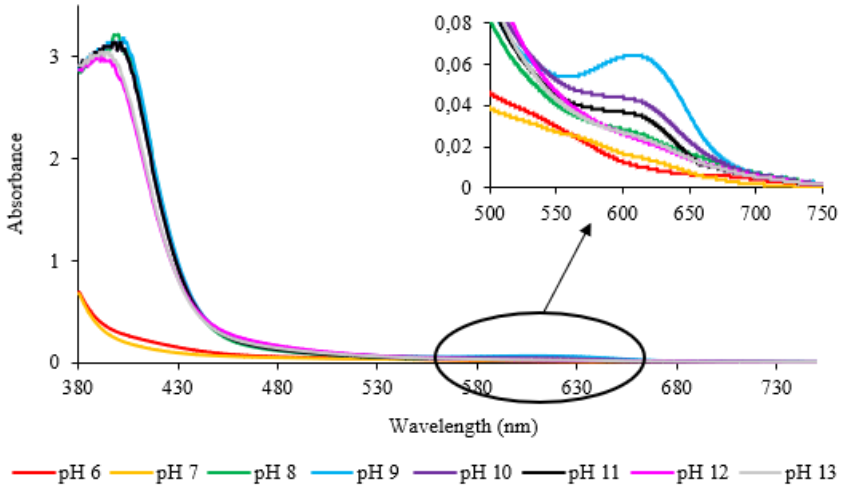

Figure 6. UV–vis Spectra of Minnieroot Flower Extract Against Changes in pH

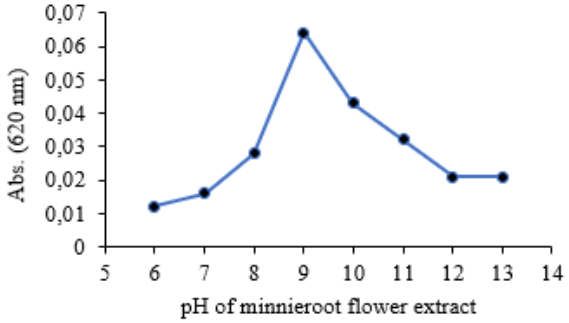

Figure 7. Effect of pH on Extract Absorbance (620 nm)

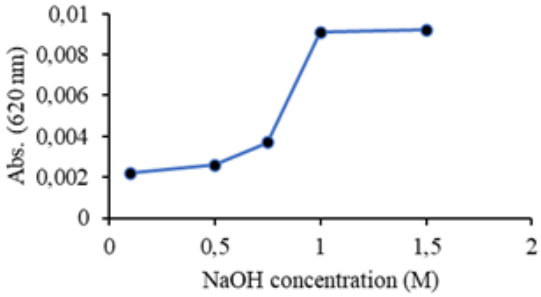

Figure 8. Effect of NaOH Concentration on Extract Absorbance (620 nm)

Optimization of minnieroot flower extract concentration. The optimization of minnieroot flower extract aimed to obtain the highest absorbance of the extract to increase method sensitivity. The more concentrated the extract was, the greater the absorbance of the extract. The extract concentration was optimized by varying concentration from 1 to 10% (w/v). The effect of extract concentration on absorbance at 620 nm is shown in Figure 9.

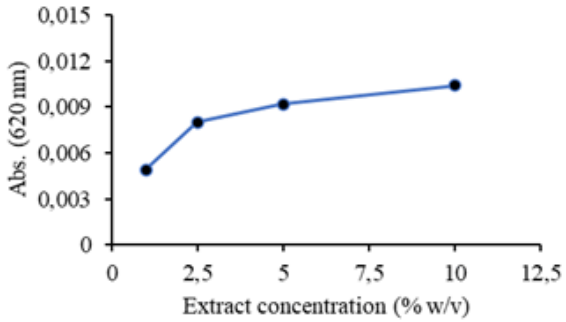

Figure 9. Effect of Extract Concentration on Absorbance at 620 nm

Figure 9 shows that a moderate increase from 1 to 10% occurs in the extract absorbance. The optimum concentration of minnieroot flower extract was chosen as 5%, at which the extract gave a relatively high absorbance and high repeatability (0.0095 ± 0.0003). This choice was supported by the absence of a significant difference between absorbances of 2.5, 5, and 10%. A significant test was used to evaluate the difference between the 5 and 10% test results. The results show that the t-value (2.944) is smaller than t-table (3.182) indicating no significant difference between the results of *Ruellia tuberosa* flower 5% and 10% extracts. Apart from producing a fairly high absorbance, the choice of a 5% concentration also aimed to reduce minnieroot flower use for greater efficiency and to conserve reagents.

To standardize the natural reagent, the 5% extract was controlled by measuring its absorbance at the maximum absorption wavelength of 521 nm before use. The extract should have an absorbance of 0.039 Au at its maximum absorption wavelength (521 nm) to ensure the same quality and anthocyanin levels in each measurement; thus, errors can be minimalized. The optimum extract of the 5% concentration is lower than the 15% of red rose extract

obtained by Puspita in 2022 for ammonia detection in pond water using the ML-GS µPAD method with the same detection principle. This comparison shows that using minnieroot flower is more efficient in terms of the extract concentration used [26].

Optimization of mixing coil length. The length of the mixing coil (MC) affects the reaction of NH_4^+ with NaOH to form NH_3 gas optimally. The MC was coiled to allow turbulent flow which increases the collisions between the sample and reagent so that the reaction occurs completely. However, a MC that is too long can also cause dilution of the sample and increase dispersion. Therefore, the optimum length of the MC must be determined.

The MC length was optimized by varying it from 35 to 160 cm. Figure 10 shows that the absorbance reached a peak at 120 cm and slightly dropped at the longer length (160 cm). The highest absorbance was achieved using a 120 cm MC with an absorbance value of 0.0109 ± 0.0001. This result shows that in using a 120 cm MC, the mixing of NH_4^+ and NaOH ran optimally, so large amounts of NH_3 gas were formed. The larger amount of NH_3 formed caused a higher absorbance of extract at 620 nm. Therefore, 120 cm of MC length was used for further measurements. Mana in 2000 used smaller lengths of 53 and 10 cm to determine ammonium by fluorimetric FIA. This difference was caused by the different detection mode and reagents used [29].

Optimization of sample volume. The amount of the injected sample greatly affects the measurements. The higher sample volume leads to higher absorbance and sensitivity of the measurements. However, the usage of sample volume must be controlled because an excessive sample volume occupies a longer sample zone; thus inhibits the availability of reagent in the entire sample zone length to react with sample. The lack of reagent in the center of the sample zone can result in the deterioration of peak absorbance. In this study, the sample volume was optimized for use in determining TAN levels in samples through GD-FIA by varying it from 50 to 250 µL.

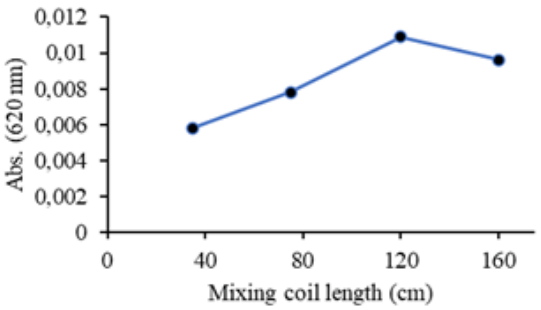

Figure 10. Effect of Mixing Coil Length on Extract Absorbance (620 nm)

Figure 11 shows a substantial increase in extract absorbance from 50 to 200 µL and then a slight decrease at 250 µL. The highest absorbance was achieved at 200 µL with an absorbance value of 0.0131 ± 0.0004. This result indicates that at 200 µL, the reaction of NH_3 gas formation occurred optimally. The decrease in absorbance at 250 µL is possibly due to an oversized sample volume, causing a large dilution of the TAN with NaOH, resulting in lower concentration of NH_3 gas in the donor stream. Therefore, a sample volume of 200 µL was chosen and used in the subsequent measurements. This result differs from the study by Sukaram in 2018 that showed an optimum volume sample of 100 µL for ammonia determination, as it used a different source of natural reagent (orchid flower instead of minnieroot) [14].

Linearity of measurement. A linearity test was used to determine the minimum and maximum limits of detection (LODs) of this method. The linearity test was performed under the previously obtained optimum conditions. The ammonia used varied from 10 to 5000 ppm. The results are shown as a plot of the absorbance against the ammonia concentration in Figure 12, and the FIA-gram profile is shown in Figure 13. Figure 12 shows a satisfying linearity (R^2 = 0.9933) with linear equation y = 10^{-05}x + 0,0001 and LOD value of 7.88 ppm.

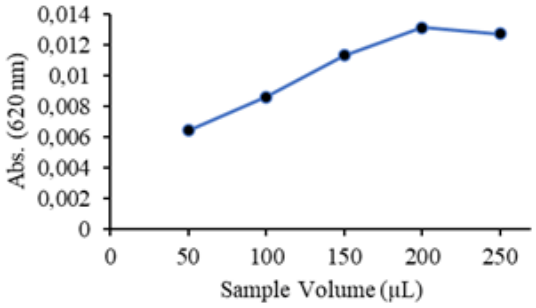

Figure 11. Effect of Sample Volume on Extract Absorbance (620 nm)

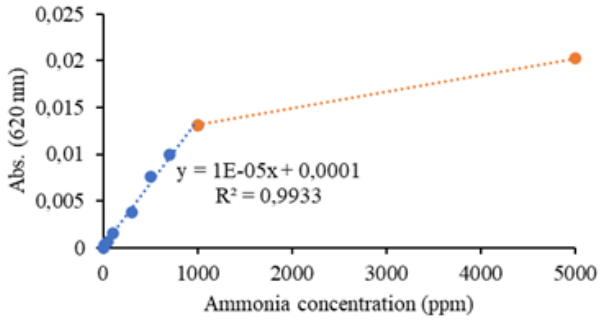

Figure 12. Ammonia Concentration Relationship Curve with Absorbance in the Linearity Test

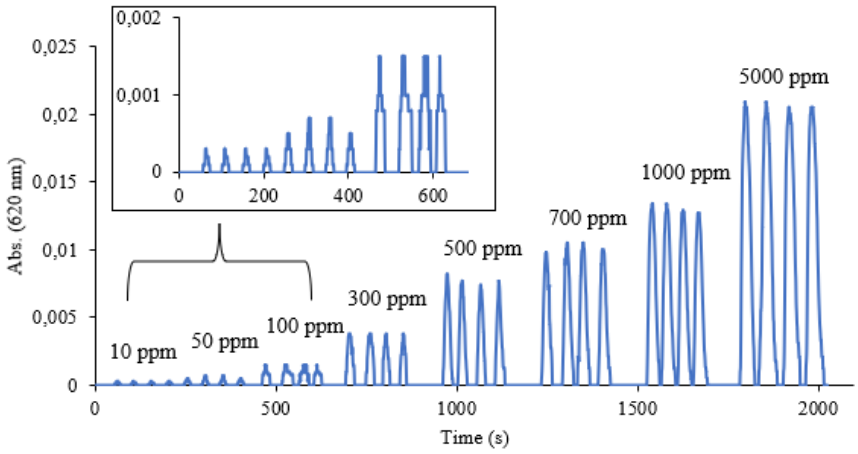

Figure 13.FIA-gram Profile of Standard Ammonia at 10–5000 ppm for the Calibration Curve

Table 1. Selectivity Test Data Results of Ammonia Against Nitrite Interfering Compounds

[NH_4Cl] (ppm)	Interfering Compound	Added (ppm)	Found (ppm)	Error (%)
300	Nitrite ($NaNO_2$)	0	297.11	0.98
		300	289.03	3.65
		600	297.11	0.98
		1500	385.15	28.38
	Sulfite (Na_2SO_3)	0	297.11	0.98
		300	281.03	6.35
		600	313.09	4.36
		1500	321.07	7.02

Table 2. Accuracy Test Data Results of Adding Standard Ammonia to the Sample Solution

Sample	[NH_4Cl] added (ppm)	Found (ppm)	Recovery (%)
A	0	42.93 ± 0.00	-
	300	339.45 ± 12.83	98.84
	700	708.15 ± 16.20	95.03

Table 3. Accuracy Test Data Results of Comparing the Developed and Standard Methods

Sample	TAN found (ppm)		t-test result
	GD-FIA spectrophotometry method	Spectrophotometry blue indophenol method	
B	50.00 ± 11.55	50.69 ± 0.53	0.120
C	42.50 ± 23.69	41.91 ± 4.77	0.048

Selectivity test of the interfering compound. Nitrite and sulfite commonly present with ammonia in water samples which can form a gas under certain conditions. Nitrite can react with acids to form nitric acid and volatile N_2 gas [30] while sulfite can also react with acids to form sulfuric acid, which can decompose into sulfur dioxide gas and water [31]. In addition, Zulfarina (2017) also stated that nitrite can be converted to ammonium with the presence of nitrifying and ammonifying bacteria [32]. Therefore, in this study, the effect of nitrite and sulfite-interfering

compounds was tested because of their abundance in water and their potential to form gases that can affect measurements.

Table 1 shows that adding 300–1500 ppm sulfite does not interfere with measurements, as seen from the small error of less than 10%. This is because sulfite did not form a gas under the condition of this measurements as sulfite only can form gaseous sulfur oxide in an acidic atmosphere. The addition of nitrite at a 300–600 ppm also did not show any disturbance; however, nitrite interferes at very high concentrations. High nitrite concentrations interfere with measurements because nitrite can undergo a denitrification reaction to form NH_3 compounds, increasing ammonia in the sample. Therefore, it can be concluded that sulfites and nitrites do not interfere with measurements up to 600 ppm.

Application and accuracy of GD-FIA-spectrophotometry. The developed method was applied to determine the accuracy of TAN determination in agricultural water sample. This test was done using recovery test and its comparison to standard blue indophenol spectrophotometry. The result of recovery test can be seen in Table 2. The concentration of ammonia in the sample was obtained at 42.93 ppm. This high level of ammonia in agricultural water is caused by the use of organic fertilizers to support plant growth and increase crop yields. The high concentration of ammonia in agricultural water can be a source of pollution for the surrounding waters.

The absorbance from the addition of 300 and 700 ppm ammonia standard solution into the water sample was then interpolated into the calibration curve equation to determine the recovery value. The accuracy test result shows that this method is accurate based on 95.03%–98.84% range recovery values. The result of recovery from this study shows a satisfactory level as reported by Sukaram in 2018 that develop the GD-FIA method for ammonia determination using orchid flower which gave 97.7%–107.6% recovery [14]. An additional accuracy test was performed by comparing the proposed method and indophenol blue as the standard method for TAN determination. This test was performed by comparing the results of real sample measurements by both methods, and a t-test was used to evaluate the methods' accuracies. The accuracy test data results are shown in Table 3.

The results of this comparison were then tested for their significant difference using the paired t-test to determine the suitability of the results of these two methods. The results show that the t-test value is lower than the t-table ($0.120 < 2.314$ and $0.048 < 2.314$) using a significance level of 0.05 (α=0.05). This comparison indicates that the developed method gives an accurate and reliable result for total ammonia determination in agricultural water samples.

Conclusions

The development of a new method for detecting TAN in water by GD-FIA spectrophotometry using minnieroot flower as a natural reagent showed promising results. This method shows good peak reproducibility, selectivity for sulfite and nitrite up to 600 ppm, and satisfactory accuracy at 95.03%–98.84% when the measurements were performed using predetermined optimum conditions (1 M NaOH, an extract concentration of 5%, a MC length of 120 cm, and a sample volume of 200 µL). This method also shows an accurate result compared to the standard indophenol blue method by the absence of a significant difference between their results. This development can be used as a breakthrough for measuring TAN in water in an efficient, accurate, inexpensive, and environmentally friendly manner.

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