Current Applied Science and Technology Vol. 20 No. 3 (September-December 2020)

A Simple and Green Approach for Colorimetric Ammonia Determination by Combining Pervaporation with Paper Impregnated Anthocyanins Extracted from Red Cabbage

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Received: 21 January 2020, Revised: 9 May 2020, Accepted: 26 May 2020

Abstract

This work reports on the development of a simple, green and inexpensive analytical method utilizing a microwell plate for ammonia determination by combining pervaporation with a paper-based colorimetric sensor. The method is based on the conversion of ammonium ions to ammonia gas by alkalinization of water samples using powdered calcium hydroxide. The generated ammonia gas diffuses freely across the headspace to a paper-based sensor impregnated with natural anthocyanins extracted from red cabbage using deionized water as a non-toxic solvent. The reaction causes the paper sensor to change color from pink to blue, and the sensor is then scanned on a flatbed scanner to quantify ammonia by correlation to the reflectance of the blue spot on the paper sensor. Under the optimal conditions, a working range of 1.0-25.0 mg N l^{-1} was obtained. The limit of detection and the limit of quantification were found to be 0.29 and 0.98 mg N l^{-1}, respectively. The method was successfully applied for determining total ammonia in water samples with recoveries in the range of 89.6-110.5%. No significant difference was observed between this method and the phenate spectrophotometric standard method.

Keywords: ammonia, calcium hydroxide powder, red cabbage anthocyanins, paper-based colorimetric sensor
DOI 10.14456/cast.2020.25

1. Introduction

Ammonia is normally used in fertilizer and animal feedstock production. In agricultural areas, ammonia from fertilizer and animal farming runoff frequently drains into water supplies [1, 2]. The total ammonia nitrogen (TAN) in the form of NH_3 and NH_4^+ is normally analyzed and the results can indicate the water quality [3]. TAN values ranging from 0.53 to 22.8 mg l^{-1} are reported to be initially toxic to freshwater organisms [4, 5].

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Various newly developed methods for the analysis of ammonia, such as a method that involves a fluorescence and colorimetric ammonia sensor based on a metal-organic gel [6], a nondispersive atomic fluorescence spectrometry method based on gas-phase light scattering [7] and a colorimetric detection using smartphones based on localized surface plasmon resonance of silver nanoparticles [8] have been reported, while standard methods for determination of ammonia in waters including titration, ammonia-selective electrode method, spectrophotometry, and flow injection analysis [9] are still being utilized. These spectrophotometry and flow injection analysis (FIA) methods are based on the Berthelot-phenate reaction, which relies on the formation of indophenol blue dye in an alkaline solution from the selective chemical reaction between ammonia and Berthelot’s reagents (phenol, hypochlorite, and sodium nitroprusside). The toxic phenol and unstable hypochlorite reagents have been replaced by salicylate and dichloroisocyanurate in the modified Berthelot reaction [10]. Moreover, 1-naphthol has been reported to have been used instead of both phenol and nitroprusside in spectrophotometric FIA [11]. Alternatively, a sequential injection gas diffusion system [12] and a paper-based platform with gas diffusion [13] have been reported for the determination of ammonia in waters using membrane separation of ammonia gas generated from the reaction before its detection using acid-base indicators. The disadvantages of these methods with a gas diffusion system were the wetting, clogging, and leakage of the membrane. Various paper-based visual color change gas sensors for ammonia detection [14-17] have also been developed. Consequently, a membraneless gas-separation [18, 19] and a micro-distillation microfluidic paper-based analytical device [4] were developed for ammonia monitoring in waters. These methods use a pervaporation technique where the ammonia gas is converted from ammonium ions, diffused through a headspace, and reacted with an acid-base indicator to overcome clogging problems of the membrane. Because the detection zone does not contact directly with the sample surface, a membrane does not need to be used. Colorimetric assay employing a well microplate with gas pervaporation and diffusion for determination of ammonia in swine farming wastewater by using natural indicator immobilized on paper sensors [20] has been investigated. This method allows microliter-scale solution handling and offers environmentally friendly chemical analysis in which anthocyanins extracted from butterfly pea flower are applied as a natural indicator. Recently, an increased number of studies concerned with the creation of pH indicators based on anthocyanins [21-23] have appeared in the literature. Fruits and vegetables with purple colors, such as red cabbage, are found to contain phenolic compounds called anthocyanins. Anthocyanins have increasingly attracted the attention of researchers because they are natural, water-soluble, and non-toxic pigments, and because they respond to a wide range of pH [24].

In this work, a simple headspace colorimetric assay based on ammonia gas pervaporation in a microwell plate was investigated. Anthocyanins extracted from red cabbage were applied as a natural indicator immobilized on a paper sensor, and calcium hydroxide was used in the original form of powder solid. Ammonium ions present in the water sample were converted to ammonia gas by reaction with calcium hydroxide in the hole of the microwell plate. Ammonia gas then diffused freely through the headspace and reached to react with the anthocyanins-coated paper covering on the reaction hole, resulting in pink-to-blue color change. A flatbed scanner was used to capture the color images of the tested paper.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals were of analytical grade and all solutions were prepared in deionized water ($\geq$ 18.2 MΩ cm, Millipore Milli-Q). Ammonium chloride and sodium hydroxide were purchased from Loba Chemie (India). Calcium oxide and calcium hydroxide were obtained from Carlo Erba (Canada). Red cabbage was purchased from a local supermarket (Tops, Central Rama II, Bangkok, Thailand).

A stock solution of ammonia (1000 mg N l^{-1}) was prepared from ammonium chloride, and working standard solutions in the range of 1-100 mg N l^{-1} were then prepared daily by stepwise dilution from the stock solution.

An anthocyanins solution (1:1, w:v) as an acid-base indicator was prepared by adding 100 g of chopped red cabbage into 100 ml of deionized water and heated for 30 min, and then the solution was filtered through a filter paper (Whatman No.1).

2.2 Apparatus

Whatman grade 42 filter paper with a diameter of 125 mm was obtained from GE Healthcare UK limited (UK). A 96-microwell plate (Flat bottom shape, Nunc, Denmark) was used throughout the experiments. A CanoScan LiDE210 flatbed scanner (Canon, Thailand) was used for scanning the images of the detection zone on the tested paper.

2.3 Preparation of anthocyanins impregnated paper

A filter paper (Whatman No.42) was cut into 10×10 mm small pieces and immersed in 10 ml of anthocyanins solution (1:1, w:v) contained in a petri dish for 10 min. Then the impregnated paper was taken out from the solution and dried at room temperature. The prepared paper was later used for the headspace colorimetric detection.

2.4 Analytical procedure

2.4.1 Reaction with sodium hydroxide

For the first procedure, sodium hydroxide was tested as a reagent in solution. A syringe was used to inject 0.10 ml of 2 M sodium hydroxide solution into the reaction hole of a 96-microwell plate and into the same reaction hole, 0.10 ml of ammonium chloride standard solution (50 mg N l^{-1}) was introduced by another syringe. The reaction hole was then immediately covered with a piece of anthocyanins impregnated paper that had just been dipped in deionized water. After 20 min, the test paper was scanned on a flatbed scanner (CanoScan LiPE210, Canon), and then an image of the detection zone on the paper was acquired. Image analysis was performed as described in section 2.5. The effect of sodium hydroxide concentration was examined by use of its solutions in the range of 2-10 M.

For the second procedure, sodium hydroxide was tested as a reagent that was coated on paper. The paper coated with sodium hydroxide was prepared as follows. A piece of filter paper (Whatman No.42) was cut into 0.5×0.5 cm pieces, soaked in a petri dish containing 10 ml of sodium hydroxide (8 M) for 10 min, and then dried at 80ºC in an oven for 3 h. A piece of this prepared sodium hydroxide coated paper was placed into the reaction hole of a 96-microwell plate and 0.10 ml of ammonium chloride standard solution (50 mg N l^{-1}) was next introduced by a syringe. This reaction hole was immediately covered with a piece of anthocyanins impregnated paper that had just been dipped in deionized water. After 20 min, the test paper was scanned and an image of the detection zone was acquired by the flatbed scanner. Image analysis was performed as described in section 2.5.

2.4.2 Reaction with calcium oxide

Each portion of calcium oxide (examined in the range of 0.0150-0.0250 g) was weighed and put into the hole of 96-microwell plate. The prepared paper which was impregnated with anthocyanins was dipped in deionized water. Afterwards, a syringe was used to introduce a volume of ammonium chloride standard solution (examined in the range of 0.10-0.20 ml) into the reaction hole and this hole was immediately covered with a moistened prepared paper that was exposed to the sample headspace. After a period of reaction time (examined in a range of 15-25 min), the test paper was scanned on the flatbed scanner and an image of the detection zone was achieved. Image analysis was performed as described in section 2.5.

2.4.3 Reaction with calcium hydroxide

The procedure followed was very similar to that described in section 2.4.2, except for the fact that calcium hydroxide was examined instead of calcium oxide.

2.4.4 Calibration curve

Each portion of approximately 20 mg of calcium hydroxide was scooped with a small-sized spatula and put into a hole of a 96-microwell plate. Two empty holes were left in-between each added portion, as shown in Figure 1A. The prepared paper that was coated with anthocyanins was moistened with a drop (50 μl) of deionized water. Afterwards, a syringe was used to introduce 0.10 ml of a standard or sample solution into each reaction hole and the hole was immediately covered with the moistened prepared paper that was exposed to the sample headspace. After 20 min of the reaction, the test paper was scanned on the flatbed scanner and an image of the detection zone on the paper was captured. The color images of the detection zone were analyzed as explained in section 2.5.

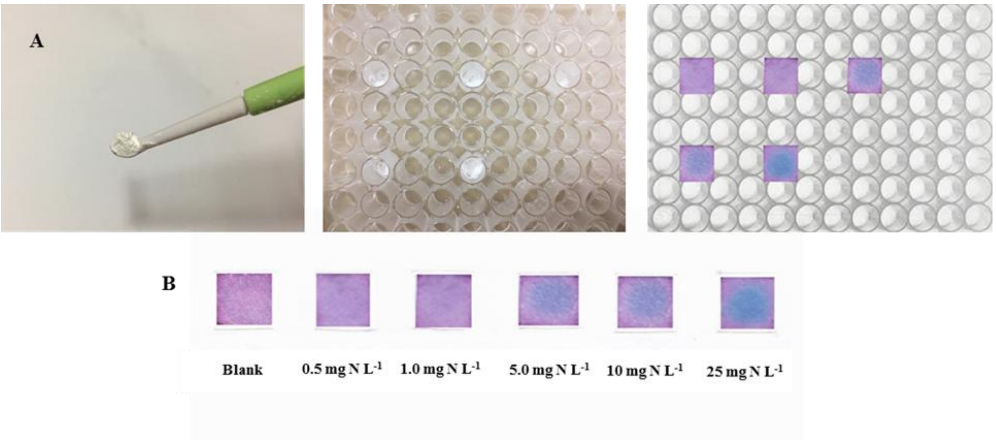

Figure 1. Schematic illustration of the experimental setup (A), and color images of test paper (B)

2.5 Image analysis

Image analysis was performed using ImageJ software (National Institute of Health, USA). The average color intensity was determined within a circular shape (w = 50, h = 50). Red color intensity was chosen as it offered the greatest sensitivity. The intensity values obtained were transformed to pseudo reflectance values: Reflectance = $-\log (I/I_0)$, where I refers to the red intensity of the standard or sample, and I_0 refers to the red intensity of the blank (deionized water) [25].

3. Results and Discussion

In this experiment, the reaction involves the generation of ammonia gas by alkalinization of the sample solution. The generated ammonia diffuses into the headspace to react with anthocyanins on the prepared paper sensor covering the top of the reaction hole. The natural dye anthocyanins from red cabbage were extracted into deionized water, which is non-toxic to the environment and thus considered a green solvent. The major structures of anthocyanins in red cabbage are based on a core structure of cyanidin-3-O-diglucoside-5-O-glycoside [24]. Red cabbage's anthocyanins in solution change from red to blue when brought into contact with ammonia gas. The 96-microwell plate was selected as a tool for this application as it is a very simple apparatus normally found in the laboratory and because ammonia gas generation and pervaporation could possibly be controlled within the reaction hole, which has the small volume of 0.36 ml.

3.1 Reaction with sodium hydroxide

First, a sodium hydroxide solution was selected to be used as a reagent for alkalinizing the sample solutions. The effect of sodium hydroxide concentration was examined in the range of 2-10 M. The procedure was as follows. A portion of 0.10 ml of sodium hydroxide solution was injected into a reaction hole, followed by the addition of 0.10 ml of ammonium chloride standard solution (50 mg N l^{-1}). A piece of the prepared paper impregnated with anthocyanins was then immediately placed over the reaction hole. It can be observed from Figure 2A that higher concentrations of sodium hydroxide provided higher reflectance values. To provide more convenience, the system was modified by using a filter paper coated with sodium hydroxide instead of using sodium hydroxide solution. The paper coated with sodium hydroxide was prepared by soaking a small piece of filter paper (0.5 cm x 0.5 cm) in sodium hydroxide solution (8 M) for 10 min and then drying at 80ºC in an oven for 3 h. As can be seen in Figure 2B, a higher reflectance value was obtained when sodium hydroxide was coated on the filter paper. This may be a result of increased surface area for the reaction. However, the prepared paper coated with sodium hydroxide seemed to absorb more moisture when contacting the air. This may be a disadvantage in actual use.

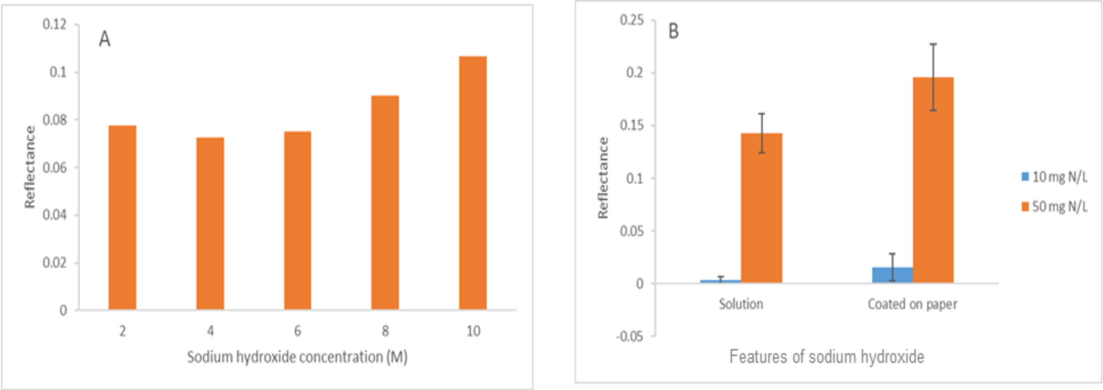

Figure 2. Optimization of reaction with sodium hydroxide: The effect of sodium hydroxide concentration (A), and a comparison of the effect of sodium hydroxide used in solution and coated on paper (B)

3.2 Reaction with calcium oxide

To solve the problem of too much moisture absorbed on the sodium hydroxide coated paper, sodium hydroxide was replaced with calcium oxide in its powdered form, that is without coating it on the filter paper. The experiment was then carried out with the same steps as described above, except for the fact that powdered calcium oxide was used instead of the prepared paper coated with sodium hydroxide. The effect of calcium oxide content, ammonium chloride solution volume, and reaction time were investigated to reach the maximum sensitivity.

Calcium oxide amount was optimized in the range of 0.0150-0.0250 g. From Figure 3A, it can be seen that the signal increased with increasing calcium oxide amount up to 0.0200 g and then slightly decreased at higher calcium oxide amount. When calcium oxide amounts were applied at 0.0150 g and 0.0200 g, the added ammonium chloride solution was able to contact all the powdered calcium oxide and make a thorough reaction. Excessive use of calcium oxide (0.0250 g) caused a thick layer of solid powder to form at the bottom of the reaction hole, and the added ammonium chloride solution was evidently able to contact and react with calcium oxide on the surface of the solid layer. This contact surface was less than that which was available when calcium oxide was applied at 0.0150 g and 0.0200 g, and thus less ammonia gas was generated. Based on the obtained data, a calcium oxide content of 0.0200 g was selected for further experiments.

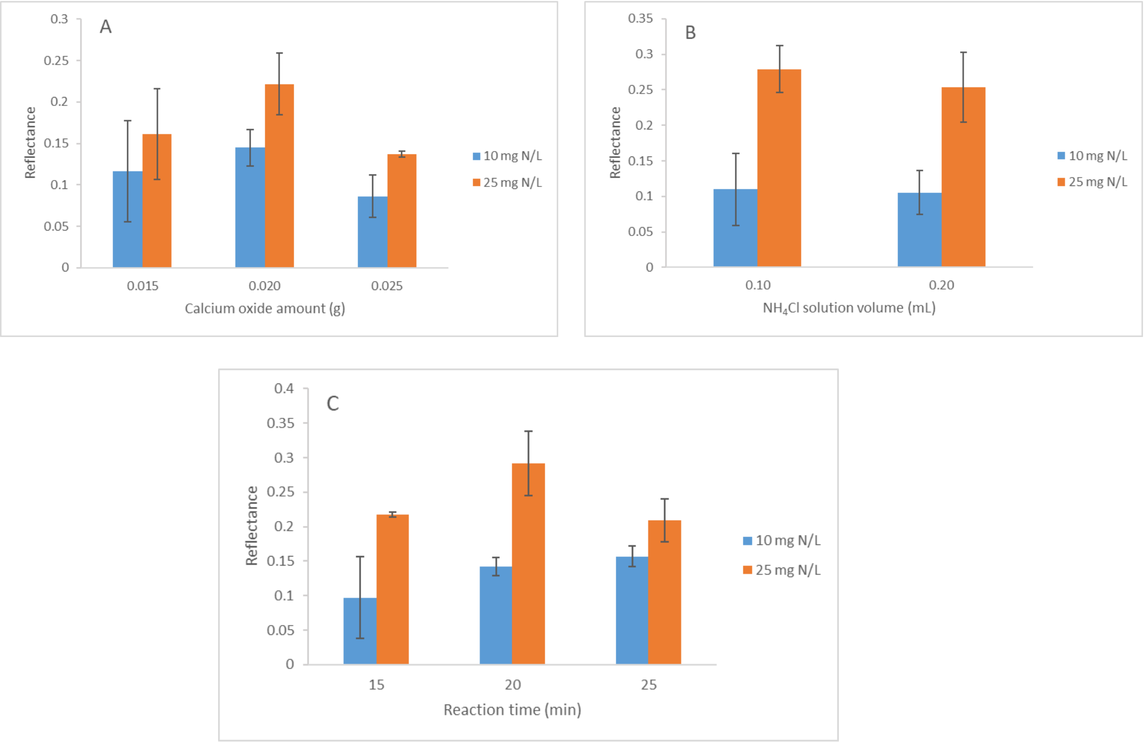

Figure 3. Optimization of reaction with calcium oxide: The effect of calcium oxide amount (A), ammonium chloride solution volume (B), and reaction time (C)

To study the effect of ammonium chloride solution volume on method sensitivity, ammonium chloride standard solution with the volume of 0.10 or 0.20 ml was injected via a syringe into the reaction hole where calcium oxide was initially placed. As can be seen in Figure 3B, the reflectance values were similar when the volume of ammonium chloride solution was either 0.10 or 0.20 ml. When ammonium chloride solution volume was applied at 0.20 ml, the initial volume introduced into the reaction hole was able to mix thoroughly with the presence of calcium oxide, but the volume that was introduced later in the addition caused such a high level of the solution in the reaction hole that some of ammonium chloride at the top could hardly react with calcium oxide. Therefore, some of ammonium chloride was not converted to ammonia gas. Moreover, ammonia gas is highly soluble in water, and this means that the loss of ammonia gas depends on the solution volume. Consequently, an ammonium chloride solution volume of 0.10 ml was selected for further experiments to save the solution and reduce the generated waste.

The effect of reaction time was studied from 15 to 25 min. The initial reaction time was recorded when the moistened anthocyanins coated paper was placed on top of the reaction hole. From Figure 3C, the signal increased with increasing reaction time from 15 min to 20 min and then tended to slightly decrease when the reaction time was 25 min. As a result, 20 min was selected as the optimum reaction time for color development. The decrease in the signal values at longer times was possibly due to the drying up of the anthocyanins coated paper.

The comparison of using sodium hydroxide and calcium oxide as a reagent is shown in Figure 4. The sensitivity was maximum when calcium oxide was used in the form of a powder, while sodium hydroxide that was used as a solution or coated on the filter paper resulted in less sensitivity. For sodium hydroxide used as a solution, the higher concentration caused the higher density. Because of the large difference of density between sodium hydroxide solution and ammonium chloride solution, the injected ammonium chloride solution hardly mixed with the dense under-layer of sodium hydroxide solution and subsequently less ammonia gas was generated. Moreover, when sodium hydroxide coated onto filter paper was used, the sensitivity slightly improved. The disadvantage of using this feature was that sodium hydroxide readily absorbs water. In a later experiment, sodium hydroxide was replaced by calcium oxide in powder form, and better sensitivity was obtained. Even though calcium oxide was less soluble than sodium hydroxide, for the reaction with ammonium chloride solution in this experiment, the use of a little amount of powder calcium oxide resulted in more thorough mixing than did the use of a high-density solution of sodium hydroxide.

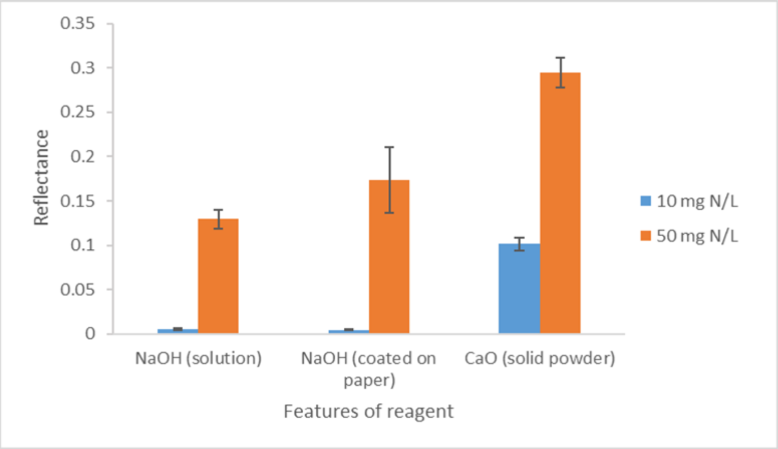

Figure 4. The signal comparison of using sodium hydroxide and calcium oxide as a reagent

3.3 Reaction with calcium hydroxide

Although the use of powdered calcium oxide can help the experiment to proceed more easily, calcium oxide is relatively expensive, so replacing it with calcium hydroxide, which is cheaper, seems to be a good choice. The same procedure as used for the reaction with calcium oxide was conducted except for the point that calcium oxide was replaced by powdered calcium hydroxide. To reach the maximum sensitivity, the influence of calcium hydroxide content, ammonium chloride solution volume, reaction time, and anthocyanins concentration was studied. In all experiments with calcium hydroxide, a portion of 0.10 ml of ammonium chloride standard solution, either at the concentration level of 10 or 25 mg N l^{-1} was used.

The effect of the reagent amount was studied by varying the amount of powdered calcium hydroxide from 0.0150 to 0.0250 g. As can be seen in Figure 5A, the maximum response was obtained when the amount of calcium hydroxide was 0.0200 g. A higher amount of calcium hydroxide (0.0250 g) gave a solid layer at the bottom of the reaction hole, which limited the surface area available to come into contact with ammonium chloride solution, and therefore the production of ammonia gas decreased. For this reason, powdered calcium hydroxide at the amount of 0.0200 g was selected for further experiments. The ammonium chloride solution volume was investigated by applying various amounts of ammonium chloride standard solution. From Figure 5B, the signal increased with decreasing ammonium chloride solution volume from 0.14 ml to 0.10 ml. Increasing the volume of ammonium chloride solution gave a higher solution level in the reaction hole, making it difficult for the solution injected later to react with calcium oxide. Also, the generated ammonia gas dissolved more into the solution with higher volume. Consequently, an ammonium chloride solution volume of 0.10 ml was selected as the optimum volume. The effect of reaction time was also studied from 15 to 25 min. Considering the data in Figure 5C, 20 min was the optimal reaction time for the test paper to reach the maximum color intensity, and a paler color intensity was observed after longer reaction time because of excessive drying up of the test paper. As a result, 20 min was selected as the optimum reaction time. These three parameters, calcium hydroxide amount, solution volume, and reaction time, provided the same trends as in the reaction with calcium oxide.

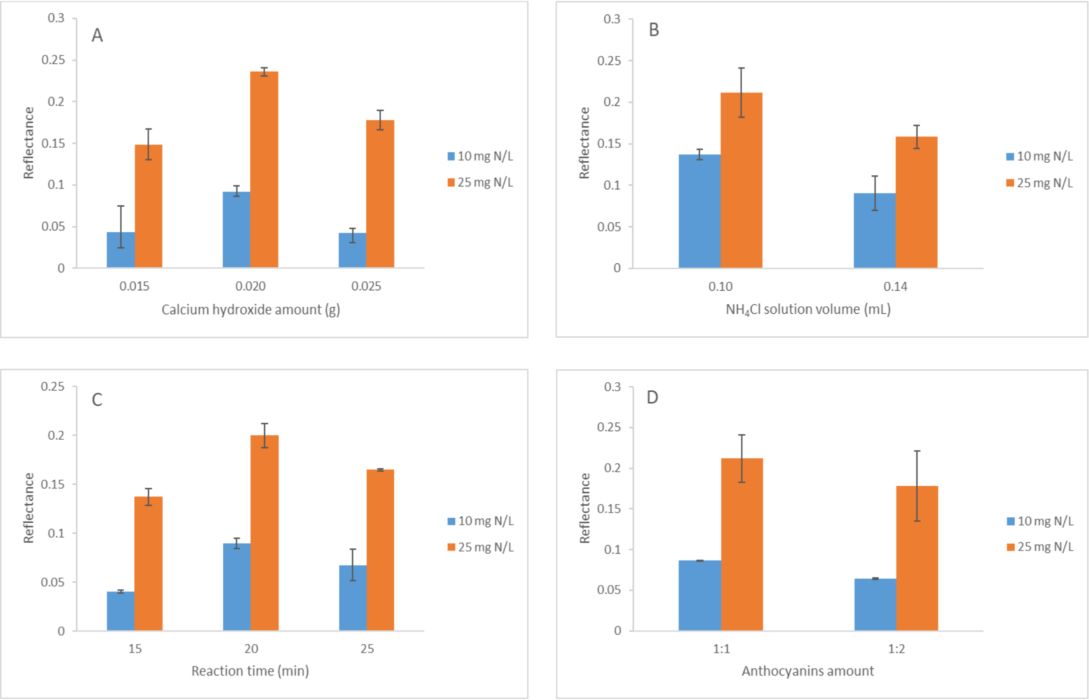

Figure 5. Optimization of reaction with calcium hydroxide: The effect of calcium hydroxide amount (A), ammonium chloride solution volume (B), reaction time (C), and anthocyanins amount (D)

To study the effect of anthocyanin amount on method sensitivity, prepared paper impregnated with anthocyanins that had been extracted from red cabbage in the w:v ratio of 1:1 and 1:2 was examined. As shown in Figure 5D, a higher signal was obtained when anthocyanins extracted with a ratio of 1:1 (w:v) was applied to the paper. Anthocyanins are water-soluble pigments; however, increased deionized water at 1:2 (w:v) makes the extract too dilute. Therefore, extracted anthocyanins in deionized water at the ratio of 1:1 (w/v) were selected for further experiments.

Various conditions of use of the prepared paper impregnated with anthocyanins for the detection process were examined to reach the maximum sensitivity. These conditions included (1) immediate use of the paper after soaking in extracted anthocyanins for 10 min, (2) use after drying the anthocyanins coated paper overnight, (3) use after dropping deionized water [(a) 10 μl and (b) 50 μl] on the dried anthocyanins coated paper, and (4) use after soaking the dried anthocyanins coated paper in deionized water before placing on top of the reaction hole. According to the results shown in Figure 6, the use of dried anthocyanins coated paper with a drop of deionized water (50 μl) resulted in the best sensitivity. When condition (1) and condition (2) were compared, the decrease in the reflectance values was probably due to the drying up of the anthocyanins coated paper. When the prepared paper that had been dried overnight was used, the lowest sensitivity was detected. To improve the sensitivity, deionized water was added on the dried paper before it was placed on the reaction hole. Reflectance values increased when the volume of deionized water was increased from 10 to 50 μl. Even though a higher volume of deionized water (50 μl) would seem to make more dilution, it appears that at this optimized reaction time (20 min), the test paper with a lower volume of deionized water (10 μl) dried more quickly and thus produced a paler color. Immersing the dried anthocyanins coated paper in deionized water caused dissolution of the anthocyanins from the coated paper, so the reflectance value decreased due to the dilution effect. As a result, the dried anthocyanins coated paper with a drop of deionized water (50 μl) was selected as the optimum condition of use.

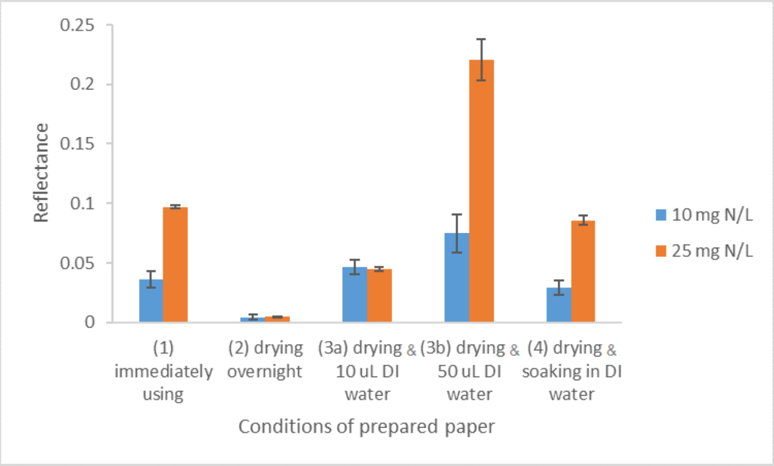

Figure 6. A comparison of signal strength resulting from various conditions of use of prepared paper impregnated with anthocyanins

3.4 Method validation and analytical performance

A validation of the method involving the reaction with calcium hydroxide was conducted, and its analytical characteristics such as the linear range, the limit of detection (LOD), the limit of quantification (LOQ), precision, and recovery were observed when the optimum conditions were applied. The method linearity was explored using various concentrations of ammonium chloride standard solutions. The developed method showed a linear dynamic range of 1.0-25.0 mg N l^{-1} by a calibration curve's equation $y = 0.0082x - 0.0086$ with the determination coefficients (R^2) of 0.9999 as shown in Figure 7. The LOD and the LOQ of this proposed method defined as 3(SD)$_{\text{blank}}$ and 10(SD)$_{\text{blank}}$ divided by the slope of the linear equation were 0.29 and 0.98 mg N l^{-1}, respectively. This sensitivity of detection may be enhanced if the anthocyanins impregnated paper is fabricated by use of a hydrophobic barrier to match the diameter of well and then a controllable color change area established for higher color intensity. The precision of the method was measured by performing replicate experiments (n = 11) of the standard solution at a concentration of 10 mg N l^{-1} and the result was reported by a relative standard deviation of 4.2%. The effect of inter bathes of extracted anthocyanins was also examined by constructing inter-calibration curves and analysis of ammonium chloride standard solution (10 mg N l^{-1}) and the results were presented in Table 1. The precision of the standard solution analysis was reported with %RSD in the range of 5.8-8.0. As extracted anthocyanins from red cabbage may vary from batch to batch, the construction of the calibration curve and analysis of samples should be performed using anthocyanins impregnated paper prepared from the same batch of red cabbage.

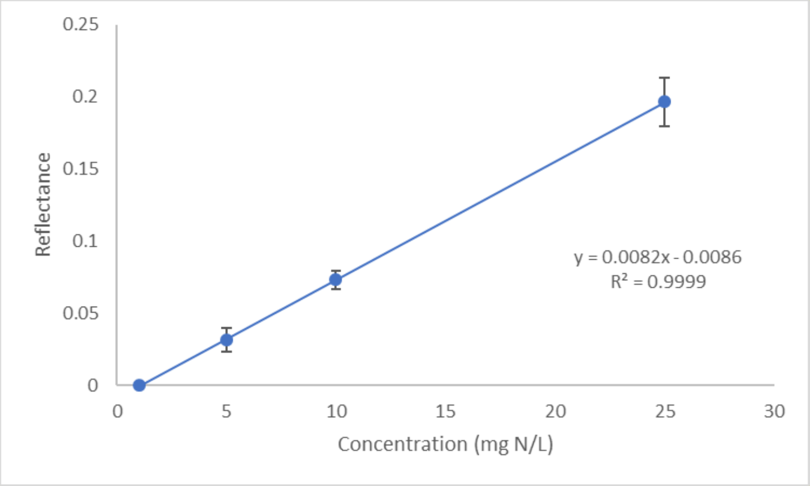

Figure 7. Calibration curve of ammonium chloride (1.0-25.0 mg N l^{-1})

Table 1. The effect of inter bathes of extracted anthocyanins

No.	**Calibration curve**	**R^2**	**Measured amount[a] (mg N l^{-1})**	
			$\bar{x}^b \pm SD$	**%RSD**
1	y = 0.0076x-0.0013	0.9997	10.1±0.7	7.4
2	y = 0.0083x-0.0067	0.9997	10.2±0.8	8.0
3	y = 0.0079x+0.0019	0.9984	10.0±0.6	5.9
4	y = 0.0086x-0.0049	0.9938	10.1±0.6	5.8

[a] Standard solution (10 mg N l^{-1})
[b] n = 3

The proposed method was applied to determine ammonia in three water samples collected weekly from five sources: agricultural-supplied lagoons A and B (Bang Khun Thian, Bangkok), a canal (Prawet Burirom canal, Ladkrabang, Bangkok), and ponds A and B (Pratep Building Complex, King Mongkut's Institute of Technology Ladkrabang (KMITL), Bangkok). Recoveries were carried out to examine the matrix effect on the analysis of ammonia by the developed method. As reported in Table 2, satisfactory recoveries were found in the range of 89.6-110.5% with good RSDs between 1.7% and 9.8%. As the color change developed on anthocyanins coated paper that covered over the reaction hole and the gas species could only diffuse through headspace and reach the detection paper, possible interferences caused by non-volatile matrices in the sample were also eliminated. According to prior studies, the only interfering compound is methylamine [26]. Its interference is negligible because it is usually present at μg l^{-1} levels in surface waters [13, 27].

Table 2. Determination of ammonia in water samples

Water samples	**Measured amount (mg N l^{-1})**		**Added amount (mg N l^{-1})**	**Spiking recovery (%)**	**RSD[a] (%)**
	Developed method	**Standard method**			
Agricultural supplied lagoon A (Week 1)	4.53±0.16	3.75±0.21	5.00	105.4	1.7
			10.0	89.8	8.1
Agricultural supplied lagoon A (Week 2)	4.64±0.36	5.23±0.13	1.00	92.9	7.6
			10.0	89.6	5.0
Agricultural supplied lagoon B (Week 1)	1.73±0.15	1.10±0.04	1.00	91.8	7.4
			10.0	91.4	3.5
Agricultural supplied lagoon B (Week 2)	2.32±0.08	2.40±0.11	1.00	99.2	8.0
			5.00	96.6	7.7
Canal (Week 1)	8.42±0.34	8.85±0.18	5.00	92.9	2.4
			10.0	98.1	6.9
Canal (Week 2)	8.63±0.28	9.43±0.24	5.00	92.9	5.5
			10.0	91.2	4.3
Canal (Week 3)	4.68±0.50	6.39±0.27	5.00	106.7	9.2
			10.0	95.2	4.1
Pond A (Week 1)	ND[b]	ND	5.00	110.5	6.5
			10.0	107.0	6.5
Pond A (Week 2)	ND	ND	5.00	93.3	7.7
			10.0	92.4	2.3
Pond B (Week 1)	ND	ND	1.00	98.9	9.8
			10.0	103.2	5.1

[a] Relative standard deviation (n = 3)
[b] Not detected

The accuracy of the developed method was further examined using the standard method (4500- NH_3 F. Phenate Method) for ammonia determination in water [9]. This standard method is based on the spectrophotometric measurement of the blue compound indophenol, which is formed by the reaction of ammonia, hypochlorite, and phenol and is catalyzed by sodium nitroprusside. The water samples were filtered before the analysis by the spectrophotometric standard method, whereas the original unfiltered samples were analyzed by the developed method. A paired *t*-test was applied to statistically compare the results obtained by the two methods. The calculated *t*-value was 0.97, while the critical *t*-value was 2.45 (at $p = 0.05$ for a two-tailed test). No significant difference was observed between the two methods.

4. Conclusions

A headspace colorimetric method based on the combination of pervaporation and paper-based sensor was designed to monitor total ammonia in water samples. The most suitable condition selected was reaction with calcium hydroxide. Ammonia gas was generated when 0.10 ml of standard solution or water sample was placed to react with 0.0200 g of calcium hydroxide powder in the reaction hole of 96-microwell plate that was immediately covered with a moistened anthocyanins impregnated paper for the reaction time of 20 min. A flatbed scanner was used to scan the test paper for color images of the detection zone and ImageJ was applied to evaluate the color intensity. The proposed method was simple and cost-effective because no complicated and expensive apparatus was used. Besides, the method used calcium hydroxide in a powder form, which was more stable than the solution form. Using the powder form also saved analysis time as the solution preparation process was not necessary. Moreover, red cabbage is a source of natural anthocyanins that can be extracted into deionized water. Water is inexpensive and the most environmentally friendly solvent that can be used for separation processes. This simple and green method with adequate analytical performance can be successfully applied in the analysis of total ammonia in water samples.

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