



AN OPTIMAL PROCEDURE FOR AMMONIACAL NITROGEN ANALYSIS IN NATURAL WATERS USING INDOPHENOL BLUE METHOD

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SYNOPSIS

Key words:

ammoniacal nitrogen analysis, contamination, blank absorbance, Bertholet reaction.

The most widely applied method for the determination of ammonia in natural waters or other samples is based on the indophenol dye formation or Berthelot reaction. In this method the samples are treated with phenol and an oxidizing agent, typically alkaline hypochlorite and appropriate catalyst to transform ammonia into indophenol blue dye, which is then determined spectrophotometrically.

Considerable difficulty in obtaining precise results will be encountered if appropriate precautions for eliminating contamination by airborne ammonium are not taken. To effectively reduce blank absorbancies a series of experiments were designed to investigate the source of contaminations. In order to determine ammoniacal nitrogen we examined also the effect of temperature and time on dye formation. The Bertholet color reaction has been investigated with the particular aim of presenting a simple, reliable analytical procedure.

INTRODUCTION

Nitrogen is an essential nutrient for all forms of life in plants, animals and human. Nitrogen is required for the synthesis of amino acids, which are the building blocks of protein. Ammonia is an important source of nitrogen for living organisms and found throughout the environment in the air, soil, and water; and in plants, animals and humans. Exposure to high levels of ammonia can cause irritation and serious burns on the skin, mouth, throat, lungs and eyes. At very high levels, ammonia can even cause death. By reason of its high toxicity and trace level in

natural samples, the determination of ammonia has gained significant importance to a number of applications including environmental protection.

Several methods have been reported for the determination of ammonia including spectrophotometry, solid-phase extraction diffuse reflectance spectroscopy, electrochemical methods, ion-chromatography, spectrofluorimetry and capillary electrophoresis.

Phenol, 2-methylphenol and 2-chlorophenol are currently the most satisfactory reagents for the Bertholet reaction for their high sensitivity involved in the development of indophenol blue. Due to the toxicity of phenol, some people used salicylate as a substitute to measure ammonium, but the sensitivity is significantly decreased (Kempers & Kok, 1989).

Citrate is the most common complex reagent used for the ammonium analysis. Some authors use EDTA as complex reagent (Gibb et al., 1995), while others use EDTA and citrate together (Aminot et al., 1997). It has been argued that EDTA should be excluded from reagents because it may reduce available chlorine (Kempers & Kok, 1989).

Laboratory studies showed that the effective pH at which citrate and EDTA work best is quite different (Gibb et al., 1995). Citrate is effective only in $\text{pH} < 11$ and EDTA works at $\text{pH} > 12$. For ammonium analysis by colorimetric methods, a pH range of 10.5-11.5 was reported to give satisfactory results for the development of indophenol blue (Hansen & Koroleff, 1999; Aminot et al., 1997).

Commercial hypochlorite solutions were a source of variation because of their instability, making necessary a daily check on the concentration of available chlorine. Sodium dichloroisocyanurate (NaDTT) has been used as an alternative hypochlorite donor. This salt has the advantage of being a stable solid, and the yield of hypochlorite on hydrolysis is both rapid and quantitative (Brzezinski, 1987; Darside et al., 1988). However, higher temperatures are required in order to liberate its chlorine (Kempers & Kok, 1989; Bower & Holm-Hansen, 1999).

Without an appropriate catalyst, the reaction rate for the formation of indophenol blue is very slow. Generally, nitroprusside (NP) is used as a catalyst in the IPB method, but in basic medium it becomes nitroferricyanide (NF) and produced aquopentacyanoferrate (AqF). AqF was indeed the actual catalyst, but it usually needs ultraviolet radiation to activate NP and is sensitive to the change of pH (Perry et al., 2005; Zhang et al., 1997). Therefore, sodium nitroferricyanide is used as a catalyst in this study without radiation.

This method has been used to evaluate ammonium concentration in Bovilla reservoir during 2007-2008.

MATERIAL AND METHODS

REAGENTS: The most popular technique for the determination of ammonium in aqueous samples is the colorimetric method based on the formation of indophenol blue (Solorzano, 1969; Hansen & Koroleff, 1999).

The entire chemical used in this study were of analytical reagent-grade.

- Phenol reagent: dissolve 10 g of reagents grade phenol in 100 ml of 95 % v/v ethyl alcohol.

- Citrate reagent: dissolve 50 g of trisodium citrate in 100 ml of water.

- Alkaline dichloroisocynurate reagent: dissolve 1 g of dichloroisocyanuric acid sodium salt dehydrate ($C_3Cl_2N_3NaO_3 \cdot 2 H_2O$, Fluka Chemika, 35915) and 3.6 g sodium hydroxide in 100 ml of water. This reagent should be freshly prepared.

- Nitroprusside reagent: dissolve 0.5 g of sodium nitroprusside in 100 ml of water. The reagent should be stored in an opaque bottle.

- Ammonium stock standard solutions were prepared from analytical reagent-grade pre-dried ($105^{\circ} C$ for 2 h) ammonium sulfate $[(NH_4)_2SO_4]$ and stored at $4^{\circ} C$ in a refrigerator. Working standard was prepared with serial dilution of stock solutions with deionized water.

All the reagents were prepared fresh daily except citrate reagent that was prepared each week.

APPARATUS:

(i) Filtration equipment with filter holder and vacuum pump (Fisher Brand model FB 70150); Whatman GF/C glass-fibre filter (1.0 μm pore size, 47 mm diam);

(ii) Spectrophotometer UV-VIS, SHIMAZDU 2401PC;

PROCEDURE: Water samples were filtered through a Whatman GF/C glass-fibre filter. Filtered samples has been kept in a freezer (-4°) until ready to proceed with the following procedure. To 25 ml of sample was added sequentially without delay, and mixed thoroughly, 1 ml of phenol reagent, 1 ml of citrate reagent, 1 ml of alkaline dichloroisocynurate reagent and 1 ml of nitroprusside reagent. The absorbance was measured at 630 nm. Following colorimetric measurement, the final pH is also checked, and should be 10.5 ± 0.1 . The samples were heated at a known temperature and successively the sample was taken out at an appropriate interval and the absorbance of the development was read at 630nm with a spectrophotometer.

RESULTS AND DISCUSSION

The chemical reaction of the colorimetric method based on the formation of indophenol blue takes place in two steps. Firstly, the addition of hypochlorite to the ammonium samples results in the formation of mono-chloroamines. Secondly,

phenol reacts with the mono-chloramines to produce an indophenol blue dye (Fig. 1).

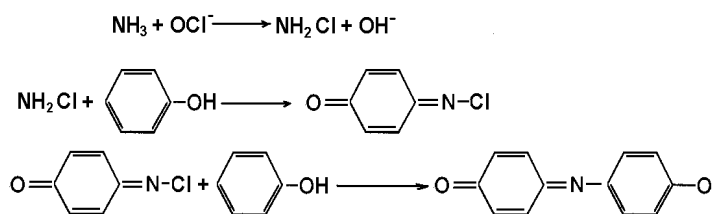


Figure 1: The chemical reaction of the colorimetric method based on the formation of the indophenol blue.

Considerable difficulty in obtaining precise results will be encountered if appropriate precautions for eliminating contamination by airborne ammonium are not taken. During ammoniacal nitrogen determination we obtained anomalous results and after a series of experiments this procedure give us consistent results. Initially we found that the blank absorbancies were high and erratic. To effectively reduce blank absorbancies a series of experiments were designed to investigate the source of contaminations.

In order to test the reliability of this method, it was applied to the determination of ammoniacal nitrogen in water of Bovilla reservoir. This reservoir is the main source of drinking water for the city of Tirana (more than 700.000 inhabitants). In table 1 are presented the blank and sample absorbancies analyzed during 2007-2008.

Table 1: The blank and sample absorbancies recorded in the determination of ammoniacal nitrogen during 2007-2008.

Time of analysis	Blank absorbancies	Interval of samples absorbancies
January, 2007	0,036	0,045 – 0,104
March, 2007	0,033	0,072 – 0,096
May, 2007	0.035	0,112 – 0,183
July, 2007	0,138	0,245 – 0,602
September, 2007	0,138	0,214 - 0,642
November, 2007	0,043	0.207 - 0.321
January, 2008	0,024	0.054 - 0.095
March, 2008	0,014	0.019 - 0.035
May, 2008	0,014	0.021 - 0.041

Ammonium contamination resulted not only for blanks but also for standard solutions of ammonium. Glass cups without stoppers were found to be subject to ambient ammonium contamination, which might be caused by the adsorption of ammonium on the glass walls. Use of volumetric glass with stoppers instead of erlenmeyer flask eliminates the major part of contamination. Blank absorbancies were 0.057 unit higher in the case of using erlenmeyer flask instead of volumetric glass with stoppers. The same fact resulted even for standard solutions. For the determination of ammoniacal nitrogen only volumetric glass flask with stoppers were used to avoid ambient ammonium contamination.

EVALUATION OF THE CONTAMINATION LEVELS FROM THE WATER
USED FOR REAGENT PREPARATION

For the evaluation of the contamination levels from the water a series of blanks were prepared, using distilled water; deionized water with osmosis; deionized water with reverse osmosis, ion exchange resin and UV oxidation and different bottled waters. This data are reported in table 2.

Table 2: Blank absorbancies reported during evaluation of ammoniacal nitrogen using different type of water for reagent preparation.

Type of water	Time of analysis	Abs/l (cm ⁻¹)	n
Fresh distilled water	December 2007	0.080	n=3
Deionized water with osmosis	December 2007	0.044	n=3
Qaf Shtama water (bottled water)	December 2007	0,031	n=3
Lajthiza water (bottled water)	December 2007	0,039	n=3
Tepelena water (bottled water)	December 2007	0.045	n=3
Kristal water (bottled water)	December 2007	0.047	n=3
Bureto water (bottled water)	December 2007	0.049	n=3
Deionized water with reverse osmosis, ion exchange resin and UV oxidation	Mars 2008	0.019	n=3

From the data obtained during our experimental work it resulted that deionized water with reverse osmosis, ion exchange resin and UV oxidation represent lower blank absorbancies than fresh distilled water and deionized water with osmosis.

Bottled waters represented also lower levels of ammoniacal nitrogen compared to freshly distilled water. Qaf Shtama and Lajthiza waters represents the lowest levels of blank absorbancies compared to other bottled waters.

EVALUTION OF CLEANING PROCEDURE

Cleaning procedure for vessels during ammoniacal nitrogen analysis played an important rol in the levels of blank absorbancies. Here are reported three method for cleaning the vessel used for this procedure: (i) cleaning procedure with mixture

HNO₃ (1:1) + HCl (1:1) - glass flask; (ii) cleaning procedure with ethanolic solution of NaOH - glass and polypropylen flask (iii) ultrasonicated in 1 M sodium hydroxide solution for 4 h at room temperature - polypropylen flask.

Cleaning procedure with ethanolic solution of sodium hydroxide, rinse with deionized water and drying at 80-90°C represented the lower absorbance value. In table 3 are represented the effect of different cleaning procedure using glasse and polypropylen flask in blank absorbancies.

Table 3: Effect of different cleaning procedure in blank absorbancies.

No	Type of flask	Cleaning procedure	Abs.	A _{mes} (s _R)
1	Volumetric glass flask (25 ml)	HNO ₃ (1:1) + HCl (1:1) - rinsed several times with deionized water	0,024	0.023 7.53 %
2			0,021	
3			0,024	
4	Volumetric glass flask (25 ml)	Ethanolic solution of NaOH (100 g NaOH +100 ml water +200 ml CH ₃ CH ₂ OH 95%) -rinsed several times with deionized water	0.012	0.013 11.46 %
5			0.013	
6			0.015	
7	Volumetric polypropylene flask (25 ml)	Ethanolic solution of NaOH (100 g NaOH +100 ml water +200 ml CH ₃ CH ₂ OH 95%)- rinsed several times with deionized water	0,019	0.020 7.39 %
8			0,022	
9			0,021	
10	Volumetric polypropylene flask (25 ml)	Ultrasonicated in 1 M sodium hydroxide solution for 4 h at room temperature - rinsed several times with deionized water	0,017	0.017 3.46 %
11			0,016	
12			0,017	

As it resulted from table 3, the levels of blank absorbancies decreased and this results are more reproducible. The lowest level of blank absorbancies was obtained from the cleaning procedure with ethanolic solution of sodium hydroxide in glass flask, although even polypropylen flask ultrasonicated in 1 M sodium hydroxide solution for 4 h at room temperature gives also low absorbancies levels. Therefore glass flask were used both for samples and standards.

EVALUATION OF REAGENT CONTAMINATION

Evaluation of reagent contamination on the blank absorbancies was also investigated during our experimental work. For this purpose two series of blanks were prepared using deionized water. In the first serie 1 ml of phenol reagent, 1 ml of citrate reagent, 1 ml of alkaline dichloroisocynurate reagent and 1 ml of nitroprusside reagent was added sequentially without delay, and mixed thoroughly, according to the procedure reported in *Analytica Chimica Acta* 2001 (Pai et al., 2001). In the second serie of blanks the double volume of these reagents was added. The data are presented in table 4.

Table 4: Evaluation of reagent contamination.

Blank preparation	Abs/l (cm ⁻¹)	Volume of reagents
Deionized water with reverse osmosis, ion exchange resin and UV oxidation	0.014	First serie of blanks- 1.0 ml of reagents
Deionized water with reverse osmosis, ion exchange resin and UV oxidation	0.026	Second serie of blanks - 2.0 ml of reagents

From the data reported in the table 4 it resulted that reagents gives an important contribute in the blank absorbancies (0.06 absorbance unit in cell with l=5 cm).

EFFECTS OF THE REACTION TIME AND TEMPERATURES ON THE COLOR DEVELOPMENT

The reaction temperature had influence on both the time required for the completion of the color development and the absorbance in completion. When we examined the effects of the reaction time and temperatures two series of standards were prepared. The first serie was kept at room temperature (15°C) and reaction time was 1, 5 and 72 hours, while second serie was kept at an incubation temperature of 40°C with the same reaction times as the first serie. Colling the samples in an ice bath for 5 min after incubation, rather than 1 h at room temperature, does not seem to alter the final results.

In table 5 are presented absorbance values of the first serie of standards kept at room temperature with different reaction time.

Table 5: Evaluation of different time reaction on absorbance values of the standards kept at room temperature (l=5 cm).

First serie of standards	Absorbance (temp. 15°C)		
	Time: 1 h	Time: 5 h	Time: 72 h
Standard 0 (blank)	0.071 (0,000)*	0,086 (0,000)	0,107 (0,000)
Standard 20 µg L ⁻¹ N-NH ⁴	0,139 (0,068)	0,181 (0,095)	0,212 (0,105)
Standard 40 µg L ⁻¹ N-NH ⁴	0,239 (0,168)	0,309 (0,223)	0,333 (0,226)
Standard 80 µg L ⁻¹ N-NH ⁴	0,532 (0,461)	0,634 (0,557)	0,656 (0,549)
Equation of the calibration curve	$y=0.0059 x-0.0314$ $R^2=0.971$	$y=0.0071 x-0.0288$ $R^2=0.9856$	$y=0.0069 x-0.0226$ $R^2=0.9897$

*the net signal of the standards was calculated by subtracting the blank from the value of standards.

From the data presented in table 5 it resulted that absorbance values of the standards were not stable in time; absorbance values increased continuously during the reaction times used for the ammoniacal nitrogen determination.

Calibration curves were not linear for all series kept at room temperature in different reaction time. From this data we concluded that ammoniacal nitrogen analysis realized at room temperature presents unreliable results.

In table 6 are presented absorbancies values of the second series of standards kept at an incubation temperature of 40⁰C with different reaction time.

Table 6: Evaluation of different time reaction on absorbance values of the standards kept at 40⁰C (l=5 cm).

Second serie of standards	Absorbance (temp. 40 ⁰ C)		
	Time: 1 h	Time: 5 h	Time: 72 h
Standard 0 (blank)	0,080 (0,000)*	0,085 (0,000)	0,090 (0,000)
Standard 20 µg L ⁻¹ N-NH ⁴	0,223 (0,143)	0,231 (0,146)	0,235 (0,145)
Standard 40 µg L ⁻¹ N-NH ⁴	0,343 (0,263)	0,349 (0,264)	0,350 (0,260)
Standard 80 µg L ⁻¹ N-NH ⁴	0,620 (0,540)	0,624 (0,539)	0,631 (0,541)
Equation of the calibration curve	$y=0.0067x+0.018$ $R^2=0.9993$	$y=0.0067 x+0.034$ $R^2=0.9997$	$y=0.0067 x+0.0018$ $R^2=0.9988$

*the net signal of the standards was calculated by subtracting the blank from the value of standards.

From the data presented in table 6 it resulted that the higher the temperature, the faster the Berthelot reaction completes. Absorbance values of the standards are relatively stable in time; absorbance values increased significantly during the first hour.

Calibration curves were linear for all the series kept at 40⁰C in different reaction time ($R^2 > 0,999$).

CONCLUSIONS

For the determination of ammoniacal nitrogen only volumetric glass flask with stoppers were used to avoid ambient ammonium contamination. Glass flasks without stoppers were found to be subject to ambient ammonium contamination, which might be caused by the adsorption of ammonium on the glass walls.

We recommend cleaning procedure with ethanolic solution of sodium hydroxide, rinsed several time with deionized water and drying at 80-90⁰C for ammoniacal nitrogen analysis.

The results described here showed that reagents gives an important contribute in the blank absorbancies, so their volumes must be minimized to provide minimal reagents blanks.

The reaction temperature had influence on both the time required for the completion of the color development and the absorbance in completion; Berthelot reaction is performed for 1 h at 40⁰C and the absorbance of the color developed is read at 630 nm.

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Original research article

Received: 31 July 2010

