Determination of Ammonium Ion by Fluorometry or Spectrophotometry after On-Line Derivatization with o-Phthalaldehyde

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A fast, sensitive, simple, and highly reproducible method for routine assay of ammonium ion (NH$_4^+$) was developed by using HPLC equipment. The method is based on the reaction of NH$_4^+$ with o-phthalaldehyde (OPA) in the presence of 2-mercaptoethanol. After an on-line derivatization, the resulting NH$_4^+$-OPA product was quantified by using fluorometric or spectrophotometric detection. For fluorometric detection, the excitation and emission wavelengths were 410 and 470 nm, respectively. The spectrophotometric detection was made by measuring absorbance at 410 nm. Results on the effects of OPA reagent composition and pH, reaction temperature, sample matrix, and linearity of the assay are presented. Even though it took about 2 min from the time of sample injection to the appearance of sample peak, sample injections could be overlapped at an interval of about 1 min. Thus, the actual time needed for analysis was about 1 min per assay. The method can be used in a fully automated mode by using an autosampler injector.

Historically, the methods for quantitative assay of ammonium ion have been relatively insensitive and time-consuming and have poor reproducibility. Most of the earlier methods were based on Nessler's reagent, with which many other inorganic ions interfere. The method currently used by most laboratories is based on the phenyl hypochlorite reaction (Weatherburn, 1967). The Technicon AutoAnalyzer (Technicon Instruments, Tarrytown, NY) and Lachat Quikchek (Lachat Chemicals, Inc., Mequon, WI) are commercially available equipment used for NH$_4^+$ analysis using the phenyl-hypochlorite reaction.

Cohn and Lyle (1) first reported the reaction of o-phthalaldehyde (OPA) with glutathione in alkaline pH, resulting in the formation of an OPA-glutathione fluorophore. Subsequently, Roth (2) showed that OPA reacted with all primary amines and NH$_4^+$, provided a reducing agent (2-mercaptoethanol) was included in the reaction mixture. This reaction has become popular for assaying picomole levels of amino acids by using HPLC techniques (3-6). The reaction has also been used in a few specialized situations to assay NH$_4^+$ (7, 8); however, no focused effort has been made to exploit the potential of this chemistry for measuring NH$_4^+$. The reaction of NH$_4^+$ with OPA in the presence of 2-mercaptoethanol is easily carried out and its subsequent measurement by fluorometry makes it a very sensitive method; however, the reaction needs to be characterized. Potentially, it can replace all other methods at NH$_4^+$ measurement.

This paper describes a method for assaying NF$_4^+$ based on the NH$_4^+$-OPA reaction, which can be used routinely as a fully automated method requiring only about 1 min per assay. The method is simple, highly reproducible, flexible, and very sensitive and has a large linear concentration range, which eliminates the need for sample dilution. So far, the OPA-NH$_4^+$ primary amine product has only been measured by fluorometry (2-8). This study reports the possibilities of using spectrophotometry for situations where a fluorometer may not be available. The molar response factor of NH$_4^+$-OPA fluorophore formed in alkaline pH (9.0-10.0) is at least an order of magnitude lower (2.5) and higher (7), respectively, than for primary amines and amino acids. Thus, the latter was used in this study. Special care is required since a slight contamination of samples with primary amines and/or amino acids could result in significant overestimation of NH$_4^+$ if the reaction is run in alkaline pH. However, NH$_4^+$ could be separated by using high-performance liquid chromatography (HPLC) in which interference from primary amines and/or amino acids would not be a problem (5). But the HPLC procedure usually requires, at least, 20-25 min per sample. The method reported here is presently proposed for nonphysiological fluids, e.g., water samples, acid digests of organic materials, nutrient solutions, soil extracts, inorganic industrial waste, and others. Extension of this method for use with physiological fluids, e.g., blood, urine, green plant extracts, etc., is currently under development and will be reported shortly. Our preliminary results (unpublished data) show that representative primary amines and amino acids did not interfere with NH$_4^+$ determination in the reported procedure.

EXPERIMENTAL SECTION

Equipment. The equipment system included a modular HPLC (high-performance liquid chromatography) unit consisting of a pump (Laboratory Data Control, Riviera Beach, FL; Model, Constametric IIIG), a fluorescence detector (Shimadzu Scientific Instruments, Inc., Columbia, MD; Model, RF-530), a UV-vis detector (Shimadzu; Model, SPD-6AV), an injector (Rheodyne, Inc., Cotati, CA; Model 7125; for 0.5-mL injections, Model 7413), an integrator/data processor (Shimadzu; Model, C-R3A), and a constant-temperature water bath (Fisher Scientific, Inc., Santa Clara, CA; Model 730). A coil of 0.5 mm i.d. Teflon tubing (total volume, 4 mL) immersed in the constant temperature bath was used as reaction coil. The flow diagram of the equipment used is shown in Figure 1.

OPA Reagent. The reagent was composed of potassium phosphate buffer, OPA, and 2-mercaptoethanol. The concentrations of various components studied were as follows: potassium phosphate, 50, 100, 150, and 200 mM; OPA, 0, 1, 3, 5, 10, 15, 20, 25, and 30 mM; 2-mercaptoethanol, 0.5, 1.25, 2.5, 5.0, 7.5, 10.0, and 12.5, mM. The potassium phosphate buffer (K-PO$_4$) was prepared by adding equimolar amounts of KH$_2$PO$_4$ and K$_2$HPO$_4$. This resulted in a final pH close to the desired (ca. 7.0). The pH of the reagent was adjusted and then filtered through 0.2-μm filter prior to adding mercaptoethanol. The reagent was generally prepared about one day ahead of use, covered, and left at room temperature and normal laboratory light conditions. A freshly prepared reagent could also be used but resulted in a slight continuous drop of baseline, presumably due to decay of the NH$_4^+$-OPA product in the reagent itself.

Procedure. The OPA reagent was pumped through the HPLC pump and the NH$_4^+$ samples (as NH$_4$Cl) were injected into the stream. The sample, mixed with the OPA reagent, passed through
the reaction coil immersed in a water bath before entering the detector. For most studies the flow rate of the OPA reagent was adjusted such that the time interval between the injection and appearance of the peak (referred to as residence time, hereafter; since no column was used, it should not be called retention time) was 2 min. For 0.5-, 5-, and 20-μL injections a complete loop fill technique was used. A gas-tight syringe, with a 100-μL top on the HPLC injector, was used when varying volumes of sample were injected.

The excitation and emission wavelengths for fluorescence measurements were 410 and 470 nm, respectively. For spectrophotometric studies, the absorbance was measured at 410 nm. The results were based on peak areas except that the absorbance data, reported as "absorbance units" were based on peak heights. For residence time vs. response studies, various residence times were achieved by adjusting the flow rate of the OPA reagent while the reaction coil volume was constant. The response was calculated from peak heights. Ammonium chloride was used as the source of NH₄⁺.

At the end of each day, the entire system was flushed with deionized water (filtered through 0.2-μm filter). To date we have not seen any unusual wear on the system, despite the high salt content of the liquids being pumped.

Statistical Analysis. The NH₄⁺ concentration vs. response data were subjected to regression analysis with an IBM (XT) microcomputer and a curve fitting program, "Curve Fitter-PC" (Interactive Microwave, Inc., College Park, PA). Based on the linear regression analysis, "useful linear ranges" were calculated. The useful linear range is defined as that range of NH₄⁺ concentration over which any single data point deviated by no more than 5% from linear regression. In some cases, the useful linear range may be slightly larger than those reported due to the resolution in NH₄⁺ calibration standards. The standard errors in all cases were much smaller than the size of the data points shown in the figures. Thus, the standard error bars are contained within the data points. In general, the maximum variability experienced was always less than ±0.5%.

Limit of Quantitation. The lower limits of quantitation (LOQ), not the detection limit, were calculated under different conditions. The definition of LOQ as suggested by Keith et al. (9) was followed in this study.

RESULTS AND DISCUSSION

Effect of OPA Reagent Composition on Fluorescence. The fluorescence of the OPA–NH₄⁺ product increased as the OPA concentration of the reagent increased up to 10 mM. Further increase in the OPA concentration caused a decrease in fluorescence (Figure 2). Taylor et al. (7) also observed an increase in fluorescence with increased OPA concentration; however, the response did not level off even at the highest OPA concentration tested (37.5 mM).

The lowest concentration of 2-mercaptoethanol (0.5 mM) tested in this study gave nearly the maximal fluorescence
(K2SO4) was chosen as a representative salt of OPA reagent (7).

The fluorescence increased with increasing time allowed for the reaction up to 36°C:

![Figure 5. Effect of reaction temperature on the fluorescence of NH4+-OPA product with 1.5 min residence time: OPA, 3.75 mM; 2-mercaptoethanol, 12.5 mM; pH 6.8; K-PO4, 50 mM.]

![Figure 6. Effect of reaction temperature on the fluorescence of NH4+-OPA product with 2 min residence time. The other conditions were same as in Figure 5.]

![Figure 7. Effect of residence time on the fluorescence of NH4+-OPA product: OPA, 3.75 mM; 2-mercaptoethanol, 12.5 mM; temperature, 63 °C; pH 6.8; K-PO4, 50 mM.]

Hence, it should be possible to further reduce the reaction time required for maximal fluorescence by further increasing the reaction temperature. Conversely, it should be possible to reduce the temperature required by increasing the residence time.

When the reaction temperature was held constant at 63 °C, the fluorescence increased with increasing time allowed for the reaction up to 3 min and remained essentially constant up to 4 min (Figure 7). A further increase in time led to a slight decrease in fluorescence. At room temperature, a 30-min reaction time was required to achieve maximal fluorescence (7).

Effect of OPA Reagent pH on Fluorescence. The effect of OPA reagent pH on fluorescence of NH4+-OPA product is shown in Figure 8. The highest fluorescence was obtained at a pH of 6.8. Unfortunately, no apparent plateau for the pH effect occurred; hence pH control in this assay becomes very important. In contrast, Taylor et al. (7) reported the maximal fluorescence at pH 7.3 with a plateau of ±0.1 pH units. Hence, for samples with high acid content, the volume of the sample injected should be minimized and the buffer concentration should be increased to provide higher buffering capacity.

Effect of Sample Matrix on Fluorescence. The effect of sample matrix (background ionic content) of samples. Other salts were also tested for specific ionic effects and the results are reported later in this section. An increased concentration of K2SO4 in the sample decreased fluorescence dramatically, up to about 20 mM, and leveled off at 50 mM. This was a rather perplexing observation, especially considering that the OPA reagent (which serves as a reactant as well as a carrier) had up to 200 mM K-PO4. The effect seemed to be related to three factors:

A. The NH4+ concentration of the sample to be analyzed; the lower the NH4+ concentration, the greater was the effect. For example, addition of 1 mM K2SO4 to a 0.1 mM NH4+ solution decreased the fluorescence by about 72% (5-µL injection using a 200 mM K-PO4 OPA reagent) whereas the response of 1 mM NH4+ solution decreased only by about 30% when analyzed under the same conditions.

B. The volume of the sample injected; the larger the sample, the lower the effect and vice versa (Figure 9). For instance, addition of 1 mM K2SO4 to a 1 mM NH4+ solution decreased the response by 29% and 17%, respectively, when 5- and 20-µL samples were injected.

C. The K-PO4 concentration of the OPA reagent; the higher the concentration, the greater the effect and vice versa (Figure 9).

The mechanism by which the ionic content of the sample might be affecting the fluorescence is not clear; however, two possibilities exist: (1) A partial loss of absorbed energy through collisions with ions (10) such that the energy emitted as light is lowered and/or (2) The ions interfere in the synthesis of the NH4+-OPA fluorophore. Tests showed that the addition of K2SO4 to NH4+ solutions decreased the absorbance (A410) of the OPA-NH4+ product in the same proportion as it did the fluorescence (results not shown). Hence, the ionic content of sample interfered with the synthesis of the NH4+-OPA fluorophore and not the emission process.

As to why the salt concentration of the sample lowered the synthesis of the fluorophore, it was possible that the sample...
band did not fully mix with the OPA reagent. Thus, the ionic concentration of the sample band remained different from that of the OPA reagent surrounding it on both sides. Due to the osmotic effects, then, the salt content of the sample band controlled the mixing of the sample with the reagent. When a mixing chamber was inserted in the fluid stream (to force complete mixing), the results remained unchanged. Moreover, the addition of sucrose instead of K$_2$SO$_4$ did not decrease the fluorescence (results not shown). These observations show that the effect of sample ionic concentration on fluorescence was not due to mixing or osmotic reasons. Instead, it was probably related to the decreased activity of NH$_4^+$ ion in the sample due to ionic strength.

The effect of sample ionic concentration on the formation of OPA-NH$_4^+$ fluorophore has not been reported previously. It is possible that the OPA-primary amines/amine acids product may also be affected; however, it is difficult to assess at this time whether the effect was caused only under the conditions used in this study. Taylor et al. (7) carried out the reaction in a test tube at room temperature. Judging from the similarities in the effect of ionic concentration of the OPA reagent on the fluorescence observed in this study and those reported by Taylor et al. (7), the effect of the ionic concentration of the sample remains a strong possibility under many conditions involving OPA reagent.

Effect of Various Inorganic Salts in Sample on the Fluorescence. Four salts, out of six tested, namely K$_2$SO$_4$, Na$_2$SO$_4$, NaCl, and KCl, affected the fluorescence of OPA-NH$_4^+$ chromophore similarly. This shows that there were no specific ionic interactions involved in the formation of NH$_4^+$-OPA chromophore. The other two, CaCl$_2$ and Ca(NO$_3$)$_2$, had slightly greater effect than any one of the other four, at equimolar concentrations (Table I). The effect of the latter two salts was about the same as the former four at half the concentration (Table I). This suggests that the anions contributed more to the observed salt effect.

Ammonium Concentration vs Fluorescence. The NH$_4^+$ concentration of the sample (from 0 to 20 mM) vs fluorescence (5- and 20-μL injections at 63 °C) is shown in Figure 10. Basically, the response was curvilinear and represented a second-order function. Such a response is generally expected of fluorometric methods over a large concentration range (11).

In this study at attempt was made to enlarge the linear concentration range by varying different parameters. The response for 0-20 mM NH$_4^+$ concentration was essentially linear, when the assay was done at 37 °C, 5-μL injections (y = 0.04 + 1.749X, r = 0.99997; y = -0.009 + 1.786X - 0.0022X$^2$, r = 0.9999), and at 63 °C, 0.5-μL injections (y = -0.0292 + 1.305X, r = 0.99995; y = 0.010 + 1.273X + 0.0018X$^2$, r = 0.99998) (data not shown). Useful linear concentration ranges (see Experimental Section), under different conditions, are listed in Table II. In general, the linear concentration range was extended by changing a parameter such that it resulted in decreased intensity of fluorescence (response). For example, a decrease in the volume of sample injected from 20 to 5 μL resulted in extending the linear range from 0.0002-2 mM to 0.0014-4 mM (Figure 10 and Table II). Injection of a 0.5-μL sample yielded a linear range of 0.002-13 mM (Table II). Similarly, with a decrease of the temperature at which reaction was carried out from 63 to 37 °C (5-μL injections), the linear range increased from 0.0014-4 mM to 0.002-10 mM (Figure 10, Table II). Thus, the system is quite flexible and the individual investigators may choose the conditions suited for their application. A LOQ of 2 pmol of NH$_4^+$ was possible under the conditions of maximal sensitivity (K-P0$_4$, 200 mM; OPA, 10 mM; mercaptoethanol, 12.5 mM; temperature, 63 °C; residence time, 2 min). The fluorescence sensitivity of NH$_4^+$-OPA product relative to quinine sulfate was calculated to be about 1.25 at 63 °C.

**Ammonium Concentration vs Absorbance.** Photometric response (A$_{410}$) of the OPA-NH$_4^+$ product was similar to the fluorometric response in characteristics and was curvilinear (over a concentration range of 0-20 mM), even though the maximum absorbance was well below a value of 2.0 (Figure 11). The useful linear concentration ranges were also similar to the fluorescence method (Table III). A change in conditions that resulted in lower absorbance increased the linear concentration range. The nonlinearity in concentration vs response (fluorometric or absorbance) may be related to the efficiency of derivatization or OPA-NH$_4^+$ reaction kinetics.
Reproducibility. The analysis of a 1 mM NH$_4^+$ solution 40 times in 1 h gave a relative standard error (RSE) of ±0.0015. When the assay was done at 37 °C, the RSE was slightly higher (±0.0023, results not shown). This was expected because a slight fluctuation in reaction temperature at 37 °C caused relatively a greater change in response, than at 63 °C (Figures 5 and 6).

General Comments and Suggested Procedure. An OPA reagent composed of 100 mM K-PO$_4$, 3 mM OPA, and 10 mM 2-mercaptoethanol, pH 6.8, is suggested for routine purposes. For higher sensitivity, the OPA concentration may be increased up to 10 mM. If greater buffering is needed (depending on the sample pH), the K-PO$_4$ concentration of the OPA reagent may be increased to 200 mM. For samples with high acid content (e.g., acid digests of organic materials), injection of small volumes (0.5 μL) would eliminate the need for an additional step of sample dilution. We have analyzed acid digests of plant materials (ca. 1.25 M H$_2$SO$_4$) with high reproducibility (±0.5%), without any sample dilution, by injecting 0.5-μL samples. High correlation coefficients ($r^2 > 0.94$) were found when the present method was compared to the conventional methods (distillation of NH$_4^+$ into boric acid solution followed by titration and phenol–hypochlorite method) of NH$_4^+$ detection, by using acid digests of several plant materials (an assay for total nitrogen). If an injector capable of injecting small volumes is not available, the acid digests must be diluted such that the sample pH does not affect the response. For samples with low salt background (<50 mM), a small aliquot (about 1% of the sample volume) of a concentrated salt solution (e.g., 5 M Ca(NO$_3$)$_2$) to an approximate sample volume, can be added to alleviate the salt effects. More rigorous volumetric adjustments should be made for greater accuracy. The volume of the sample to be injected would largely depend on factors such as sensitivity, linear range required, and the sample pH. A minimum of 2 min residence time is recommended. Overlapping injections at an interval of about 1 min can be made such that 50–60 samples can be analyzed per hour.

Registry No. OPA, 643-79-8; NH$_4^+$, 14798-03-9; K$_2$SO$_4$, 7757-90-5; Na$_2$SO$_4$, 7757-82-6; NaCl, 7447-40-7; CaCl$_2$, 10043-52-4; Ca(NO$_3$)$_2$, 10124-37-5; 2-mercaptoethanol, 60-24-2.

LITERATURE CITED
