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# Fluorescent microplate analysis of amino acids and other primary amines in soils

Anthony Darrouzet-Nardi*, Mallory P. Ladd, Michael N. Weintraub

*Department of Environmental Sciences, University of Toledo, 2801 W. Bancroft St., Mail Stop 604, Toledo, OH 43606, USA*

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ABSTRACT

In studies of soil nitrogen (N) cycling, there is growing demand for accurate high-throughput analyses of amino acids and other small organic N compounds. We adapted an existing fluorometric amino acid method based on *o*-phthaldialdehyde and β-mercaptoethanol (OPAME) for use in 96-well microplates, and tested it using standards and field samples. While we started with an existing protocol, we made one critical change: instead of using a 1-min incubation period, we used a 1-h incubation period to deal with differences in reaction timing among microplate wells and to reduce interference from ammonium. Our microplate method is similar in sensitivity to existing protocols and able to determine leucine standard concentrations as low as ~0.5 μM. Finally, we demonstrate that the OPAME reagent fluoresces in the presence of primary amines other than amino acids, such as amino sugars and tyramine. Because of this broad sensitivity to primary amines, descriptions of the measured pool should be revised from total free amino acids (TFAA) to total free primary amines (TFPA).



## 1. Introduction

Amino acids and other small organic nitrogen (N) compounds are increasingly recognized as important constituents of labile N in soils (Schimel and Bennett, 2004; Hobbie and Hobbie, 2012). Thus, analytical methods are needed to rapidly assay concentrations of these compounds in soil solution samples and soil extracts. Historically, analysis of total free amino acid (TFAA) concentrations in aqueous samples has been done using the colorimetric ninhydrin assay, which can be used as a more rapid alternative to HPLC techniques (Moore and Stein, 1954). More recently, a reaction based on *o*-phthaldialdehyde and β-mercaptoethanol (OPAME) has been adopted for TFAA determination due to higher amino acid sensitivity and lower interference from non-amino acid compounds (Fisher et al., 2001; Jones, 2002). The high sensitivity of OPAME and the need for only one reagent make this assay a good candidate for high-throughput analysis (Jin and Evans, 2007). Our goal in this study was to adapt this method for use on 96-well microplates.

The OPAME reagent reacts with primary amines ($R-NH_2$) such as amino acids, creating products that peak in fluorescence within several minutes, then degrade over several hours (Roth, 1971; Simons and Johnson, 1978). The stability and fluorescence of these products vary among amino acids, with the strongest fluorescence in serine and the lowest in cysteine and proline, which has no primary amino group (Trepman and Chen, 1980; Fisher et al., 2001). Because fluorescence can vary for equimolar concentrations of different amino acids, estimates of amino acid concentrations based on fluorescence in the presence of OPAME are not equivalent to true amino acid concentrations. These variations in fluorescence require that we adopt a standard with a representative fluorescence level to report the results of the OPAME assay. In this study, we evaluate the use of leucine as such a standard.

Existing protocols (Fisher et al., 2001; Jones, 2002) recommend conducting measurements near the time of peak amino acid fluorescence by using a 1-min incubation time between reagent addition and fluorescence measurement. In our early attempts to use the OPAME protocol in 96-well microplates, we observed that leucine standards on the right-hand side of the microplate were ~5% higher than the same standards on the left-hand side. This cross-plate effect was even more pronounced for ammonium. We hypothesized that this effect was due to deviations from the recommended 1-min incubation period caused by our inability to pipette reagents and read all 96 microplate wells with a precise 1-min incubation period.

The OPAME reagent reacts not only with amino acids but also with ammonium and other primary amines such as peptides and amino sugars (Carroll and Nelson, 1979; Chen et al., 1979). In polymers, even those as small as dipeptides, the fluorescence of

* Corresponding author.
*E-mail address:* anthony@darrouzet-nardi.net (A. Darrouzet-Nardi).

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these products is greatly quenched (Chen et al., 1979; Fisher et al., 2001). However, ammonium, amino sugars, and other small monomers can still interfere with measurements of amino acids. Because ammonium is typically measured separately in soil analyses, existing protocols recommend correcting amino acid concentrations by subtracting the fluorescence of ammonium (Jones, 2002). In soils, amino sugars such as glucosamine and galactosamine can be present (Fischer et al., 2007), though these compounds are likely less abundant than the secondary amine N-acetylglucosamine, which is produced in many soils by N-acetylglucosaminidases as they break down chitin and peptidoglycan (Sinsabaugh et al., 2008). Amino acid derivatives are also common in nature: compounds such as tyramine, serotonin, and phenethylamine are known to occur widely in plants (Smith, 1977; Ly et al., 2008; Kang et al., 2009) and might be expected to also occur in soils. Other amines, particularly of microbial origin, may still be unknown. However, no OPAME-based studies of soil samples that we are aware of have addressed the fluorescence of amino sugars or amino acid derivatives.

The goals of this study were to: (1) Determine whether the OPAME assay is sufficiently sensitive and reproducible for use on soil solutions and extracts when run at a small scale on 96-well microplates; (2) Evaluate leucine as a reference standard for the OPAME assay; (3) Determine if alterations in reaction timing can eliminate the cross-plate effect we observed; and (4) assess the contributions to OPAME fluorescence by ammonium, amino acids, amino acid derivatives, and amino sugars.

## 2. Methods

### 2.1. OPAME reagent

Following Jones (2002), the OPAME working reagent was mixed as follows: 25 mg of OPA was dissolved in 2.5 mL methanol. OPA was dissolved in methanol, then added to 100 mL 0.02 M potassium tetraborate buffer, adjusted to pH 9.5 with NaOH. Finally, 50 μL β-mercaptoethanol was added to the working reagent. The reagent was then allowed to sit for at least 2 h to allow background fluorescence to subside (Molnár-Perl and Bozor, 1998). In addition to buffer pH, fluorescence levels of the reaction product can also be affected by temperature and concentration of salt ions in solution (Trepman and Chen, 1980; Goyal et al., 1988). Because of these known effects, all of our incubations were done at room temperature (23 °C) and we matched the reaction matrix (i.e., water or salt solution) of the standard with that of the sample. Assessment of the working reagent after storage at 4 °C for up to 48 h indicated that slight reductions (<5%) in raw OPAME fluorescence occurred consistently in both standards and samples, thus causing no measurable effect on assay results. These reductions were also uniform across amino acid type and concentration, indicating that storage of the OPAME reagent for 48 h is feasible.

### 2.2. Reaction kinetics

To examine the reaction rate over time, we assayed standard concentrations of leucine, ammonium, glucosamine, N-acetylglucosamine, galactosamine, tyramine (a derivative of tyrosine that lacks a carboxyl group), and a mixed amino acid standard (Thermo Scientific #20088) containing 17 of the 20 proteinogenic amino acids[1] (alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine·HCl, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine). All standards were diluted to the following concentrations to create standard curves: 0, 0.5, 1, 2, 5, 10, 15, 20 μM. In each well of a black 96-well microplate (USA Scientific 5665-5076), 50 μL of standard and 100 μL of working reagent were combined and read in a Bio-Tek Synergy HT microplate reader (Bio-Tek Inc., Winooski, VT) with an excitation wavelength of 360 nm, an emission wavelength of 460 nm, and a sensitivity setting of 50. The plate reader was set to kinetic reading mode, with each plate read every 1.5 min for 3 h. Five runs were done in total to assess variation in the relative sensitivities of leucine and ammonium. In separate runs, we compared leucine with glucosamine, galactosamine, N-acetylglucosamine, and tyramine. We performed a parallel run of leucine and ammonium at a single timepoint (3 h) to ensure that the repeated readings were not affecting fluorescence values; this run showed no indication of repeated measurements altering fluorescence values.

### 2.3. Method assessment

To assess sources of error in our microplate procedure, we mixed two identical leucine standard curves (0, 0.5, 1, 2, 5, 10, 15, 20 μM) from separately prepared stock solutions and ran them in triplicate (three identical microplate wells per sample) on each of two separate plates at the same time with the same OPAME reagent. Based on data from the kinetic reads, an incubation time of 60 min was used for these samples. An incubation time of 60 min was chosen because it was the shortest time in the window in which ammonium interference is minimized (discussed below). After regressing the fluorescence values against the standard concentrations, we then calculated the relative importance of the standard, the plate, and unexplained sources of error in accounting for residual variation. Relative importance was calculated by dividing the sum of squares for each source of variation (standard, plate, or unexplained errors) by the total sum of squares (Gromping, 2006).

To test our modified protocol on field samples, we analyzed 62 water extracts of arctic moist acidic tundra soils. The extractions were done on 5-cm-diameter 10-cm-deep cores of organic soil. The soils were homogenized and roots were removed before extracting 5 g subsamples by shaking at ~125 rpm for 1 h in 25 mL of Nanopure water and vacuum filtering them through Pall A/E glass fiber filters (1 μm pore size). These samples were analyzed for primary amines using our modified OPAME procedure with an incubation time of 60 min. Two identical analyses on the same samples using the same standards were performed on separate days with two different batches of OPAME reagent. Samples were also assayed for: (1) ammonium concentration using a modified Berlethot reaction (Rhine et al., 1998) to assess any potential contribution of ammonium to fluorescence levels; and (2) autofluorescence by using borate buffer alone instead of working reagent in a separate microplate. Final analyte concentrations were calculated as follows:

$$\text{Total Free Primary Amines}(\mu\text{M}) = (O - B - A - \text{NH}_4{}^{*}m_{\text{NH}_4})/m_{\text{Leu}}$$

where $O$ is the OPAME fluorescence value of a sample (in fluorescence units, FU, which are unitless, relative numbers that are specific to the plate reader and sensitivity setting used); $B$ is the 0 μM standard—the background fluorescence (FU) of only OPAME reagent and sample matrix (e.g., water or salt extractant); $A$ is the autofluorescence (FU) of the sample with borate buffer but no OPAME reagent minus the fluorescence (FU) of the buffer and

[1] All amino acid standards used in this study were L-isomers except glycine, which is not chiral.