



# A highly-sensitive microplate fluorimetric method for the high-throughput determination of nitrate ion in aqueous compost extracts

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## ABSTRACT

In this paper, a new spectrofluorimetric method for the determination of nitrate in aqueous compost extracts is presented. The microplate procedure is based on the reduction of nitrate to ammonium by means of Zn powder under acidic conditions and the following derivatization of ammonium with *o*-phthalaldehyde (OPA) and *N*-acetylcysteine (NAC) to give a fluorescent derivative. Optimization work allowed performing the reduction in 30 min on a small sample volume (150  $\mu\text{L}$ ) and using a little quantity of metal (20 mg). The use of strong acids is also avoided. Excellent limit of detection was achieved ( $1.3 \mu\text{M} = 0.08 \text{ mg L}^{-1} \text{ NO}_3^-$ ) along with good precision levels (4.2 and 14.3% for 50 and 5  $\mu\text{M}$ , respectively) and a satisfactory linear dynamic range (4–100  $\mu\text{M}$ ). A good agreement between data coming from our method and from ion-exchange chromatography was found on real compost samples, indicating a good level of accuracy.

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## 1. Introduction

Composting represents a useful way to recycle organic wastes into a fertilizing product. The formation of compost occurs by means of an exothermic and aerobic process promoted by micro-organisms who decompose the organic matter. The physical and chemical changes that occur during the compost formation determine the loss of the putrescibility of the biomass which also undergoes a partial mineralization [1–3]. The industrial importance of compost lies in its capability to enhance the agronomic quality of soil. Its employment in the agricultural industry has proved to be able to increase the yield of agronomic and horticultural crops and to restore soil conditions after tree harvesting [4]. Moreover, it represents a valid alternative to the traditional incineration and to landfills. In fact, thanks to the water loss and to the mineralization of organic matter, the global volume and mass of the initial waste products can be reduced up to 50% [5,6].

The monitoring of the composting process is essential not only to pursue a correct waste management but also to preserve the economic value of a commercial item. During the composting process nitrogen present in the biomass goes through two main mineralization phenomena: ammonification (production of  $\text{NH}_4^+$ ) and nitrification (production of  $\text{NO}_3^-$ ). Nitrogen-based parameters (i.e. concentration of ion nitrate and ammonium, and  $\text{NH}_4^+/\text{NO}_3^-$  ratio) have proved to be useful to

monitor the correct development of the composting process. In fact, while ammonium concentration tends to decrease during the compost stabilization, formation of nitrate is facilitated in mature compost thanks to the lower respiration rates [7]. Monitoring of nitrate in various environmental samples in the frame of the 1991 European Union Nitrates Directive [8] (which aims to protect water quality across Europe by preventing nitrates from agricultural sources polluting ground and surface waters) can also benefit from efficient analytical techniques applied to aqueous compost extracts. For these reasons, the attention of researchers and enterprises has been focused on the assessment of reliable analytical protocols aimed to quantify these chemical species in compost and compost extracts. In the past, various methods have been proposed for the quantification of nitrate ion in aqueous solutions including spectrophotometry [9,10], fluorimetry [11,12], ion chromatography [13] and flow injection analysis [14]. Literature also offers some examples of microplate-based procedures where nitrate is first reduced to nitrite which is later spectrophotometrically revealed after derivatization [15,16]. In spite of the strong contribution given by these works for the determination of nitrate, the main problem related to the cited procedures is given by the use of cadmium, which is renowned for his high toxicity, and also by the strong interferences caused by complex samples such as compost extracts, when the measurement is carried out by spectrophotometry.

The main goal of our work was to develop a new sensitive method for the determination of nitrate by a microplate-based procedure in compost aqueous extracts. In the past, our research group has

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developed various analytical methods based on the 96-wells technique [17–20]. In particular, a fluorescence-based microplate procedure for the quantification of ammonium and primary amines in compost water extracts and other matrices was recently assessed [17]. The basic idea for the assessment of our method was to exploit the previous protocol, reducing first the nitrate to ammonium and then quantifying the latter by means of the method already assessed. An important advantage of this analytical strategy would be the easy determination of both nitrate and ammonium ions with a single set of detection reagents (with or without the reduction step), which would be highly valuable as the  $\text{NH}_4^+/\text{NO}_3^-$  ratio is one of the principal nitrogen-based indicators of compost maturity [7].

In the last years, metals have been employed for the reduction of nitrate to nitrite or ammonia in aqueous solutions. Reducing properties of cadmium, aluminum, zinc, iron and also of some types of alloys like Devarda's alloy and Arndt's alloy have been reviewed by Fanning [21]. Among all, Zn seems to represent a good candidate for the reduction of nitrate to ammonium. Indeed, besides being less toxic than other metals (i.e. cadmium), it has been already successfully used under mild reduction conditions for the quantification of nitrate in water solutions and other matrices [22–24], but never for compost extracts so far. For this reason, we present in this work a new fluorescence-based microplate method for the quantification of nitrate in compost water extracts which includes the conversion to ammonium by means of Zn powder, and which is developed as a simple analytical tool that can be used directly in composting platforms in a non-specialized environment (no fume hoods for strong acids and non-expert technicians).

## 2. Experimental

### 2.1. Samples and water extraction of organic matter

Two type of compost samples were selected for this study. More specifically, the first type of samples (A samples) was exclusively composed by green wastes deriving from activities of gardening and maintenance of green public or private areas. Sampling sites were located in 3 private areas (agricultural lands) located in Provence Alpes-Côte d'Azur region (France, GPS coordinates: near Gardanne: 43.466350N, 5.454541E; near Rousset: 43.468935N, 5.634117E, and near Rougiers: 43.411657N, 5.841994E). Ages of the swaths were always included between one week and twelve months. It should be noticed that studied swaths can be very heterogeneous in their composition of plant species, size and height. The second type of samples (B samples) consisted in sewage sludge mixed with green wastes (1/3–2/3, v/v). In this case, samplings were performed on an industrial composting platform (Biotechna) located in Ensues-la-Redonne (Bouches-du-Rhône, France, GPS coordinates 43.376838N, 5.190849E). A single swath was studied in a period of time included between 1 week and 6 months, with a various frequency of sampling (samplings were performed every week during the 2 first months, every two weeks the next month, and every month the three months afterwards). For each type of compost, matter was collected in different places of the swath at 80 cm depth, in order to get a final composite sample of 2 kg representative of compost.

The water extractions were performed in PTFE Teflon tube (triplicates per soil sample) on an orbital shaker (Fisher Scientific Bioblock SM30B) at 125 rpm and at room temperature. Organic matter was extracted through shaking 6 g of fresh compost with 60 mL of ultrapure water (resistivity of  $18 \text{ M}\Omega \text{ cm}^{-1}$  at  $25^\circ\text{C}$ , and total organic carbon  $<20 \mu\text{g L}^{-1}$ ) with a soil/water ratio of 1/2 (w/v) for 2 h under  $\text{N}_2$ . After extraction step, the tubes were centrifuged at 8000 rpm for 15 min (JP SELECTA, Medifriger BL-S), and the supernatants were collected and filtered through  $0.45 \mu\text{m}$  membrane filters (PES membrane, Millipore, France). The resulting solutions were stored in the dark at  $-18^\circ\text{C}$  until analysis.

### 2.2. Reagents and solutions

All chemicals were of analytical reagent grade and used without further purification. Zn powder (100 mesh) and Zn granules were purchased from Alfa Aesar (Germany) and Prolabo (France), respectively. Copper granules were purchased by Fluka (France) while Fe powder (puriss.,  $<212 \mu\text{M}$ ) and Al powder were provided by Sigma-Aldrich (France). *o*-Phthalaldehyde (OPA) was obtained from Acros Organics (Belgium) and *N*-acetyl-L-cysteine (NAC) from Sigma-Aldrich. Buffers solutions were prepared dissolving the proper amount of sodium acetate (Sigma-Aldrich), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Acros organics), anhydrous sodium carbonate (Sigma-Aldrich) and sodium tetraborate decahydrate (Sigma-Aldrich) in ultrapure water (Millipore, USA, resistivity  $>18 \text{ M}\Omega \text{ cm}$ ) and adjusting the pH with sodium hydroxide or hydrochloric acid.

Stock standard nitrate solution (0.1 M) was prepared by dissolving appropriate amount of sodium nitrate (Sigma-Aldrich) in ultrapure water. Working solutions were obtained by diluting stock solutions to proper concentrations.

### 2.3. Instruments

#### 2.3.1. Microplate

Microplate fluorescence measurements were carried out on a microplate reader (Infinite M200, Tecan France SAS, France), operating at  $30^\circ\text{C}$  and controlled by i-control™ software (Tecan). Detection was performed by top fluorescence reading at  $\lambda_{\text{ex}} = 415 \text{ nm}$  and  $\lambda_{\text{em}} = 485 \text{ nm}$ . Other parameters were as follows: gain: 80; number of flashes: 5; integration time:  $20 \mu\text{s}$ . Fluorescence intensities were expressed in arbitrary units (a.u.). Polystyrene black 96 V-well microplates (Fisher Scientific, France) were used.

#### 2.3.2. Ion chromatography analysis of nitrate

Nitrate analysis was carried out by ion chromatography (EN ISO 10304-2:1996) on a ICS-3000 HPLC system (Dionex, USA), driven by Chromeleon® (6.80 version) equipped with a guard column (Dionex AG11-HC), an analytic Dionex AS11-HC ( $4 \times 250 \text{ mm}$ ) column, a conductimetric detector (Dionex CD-25) and using a  $200 \mu\text{L}$  loop injection valve. Analysis were performed in an isocratic mode ( $22.5 \text{ mM NaOH}$  in helium sparged deionized water) at  $30^\circ\text{C}$ , with a flow rate set at  $1.5 \text{ mL min}^{-1}$ . To improve the signal-to-noise ratio of the conductivity measurement, an external flow electrochemical suppressor system (ACRS 500 4 mm) was added to the analytical system.

### 2.4. Analytical protocol for nitrate determination

Twenty milligrams of Zn powder (100 mesh) were dispensed into the wells of the microplate,  $150 \mu\text{L}$  of sample or standard solution were introduced and  $20 \mu\text{L}$  of MES buffer (0.1 M, pH 6) were added. The plate was shaken for 30 min at  $30^\circ\text{C}$ . Then,  $120 \mu\text{L}$  of the solution were transferred into new wells where  $20 \mu\text{L}$  of a solution of 20 mM NAC and  $30 \mu\text{L}$  of 13 mM OPA in ethanol-0.15 M carbonate buffer pH 10.5 (10:90, v/v) were subsequently added. The plate was shaken for 10 min and fluorescence intensity was then recorded, with excitation and emission wavelengths set at  $\lambda_{\text{ex}} = 415 \text{ nm}/\lambda_{\text{em}} = 485 \text{ nm}$ . Concentrations in unknown samples were determined using the linear calibration curves obtained with standards. Nitrate concentration was calculated after quantifying the ammonium by means of the microplate procedure previously assessed by our research group [17].

## 3. Results

### 3.1. Choice of the metal system for the reduction of nitrates

The proposed analytical procedure is based on the reduction of nitrate ion to ammonium and the subsequent determination of the latter

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