

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

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 determination of nitrate ion in aqueous compost extracts.

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- 8

## 9 Abstract

In this paper, a new spectrofluorimetric method for the determination of nitrate in aqueous 10 11 compost extracts is presented. The microplate procedure is based on the reduction of nitrate 12 to ammonium by means of Zn powder under acidic conditions and the following derivatization of ammonium with o-phtaladehyde (OPA) and N-acetylcysteine (NAC) to give a fluorescent 13 derivative. Optimization work allowed performing the reduction in 30 min on a small sample 14 15 volume (150 µL) and using a little quantity of metal (20 mg). The use of strong acids is also 16 avoided. Excellent limit of detection was achieved (1.3  $\mu$ M = 0.08 mg.L<sup>-1</sup> NO<sub>3</sub><sup>-1</sup>) along with good 17 precision levels (4.2 and 14.3 % for 50 and 5 µM, respectively) and a satisfactory linear 18 dynamic range (4 - 100 µM). A good agreement between data coming from our method and from ion-exchange chromatography was found on real compost samples, indicating a good 19 20 level of accuracy.

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23 Keywords: Nitrate, microplate, compost extracts, spectrofluorimetric.

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# 1. Introduction

27 Composting represents an useful way to recycle organic wastes into a fertilizing product. The 28 formation of compost occurs by means of an exothermic and aerobic process promoted by 29 micro-organisms who decompose the organic matter. The physical and chemical changes that 30 occur during the compost formation determine the loss of the putrescebility of the biomass 31 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 32 industry has proved to be able to increase the yield of agronomic and horticultural crops and 33 34 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 38 39 management but also to preserve the economic value of a commercial item. During the 40 composting process nitrogen present in the biomass goes through two main mineralization 41 phenomena: ammonification (production of  $NH_4^+$ ) and nitrification (production of  $NO_3^-$ ). Nitrogen-based parameters (i.e. concentration of ion nitrate and ammonium, and NH4<sup>+</sup>/NO3<sup>-</sup> 42 ratio) have proved to be useful to monitor the correct development of the composting process. 43 44 In fact, while ammonium concentration tends to decrease during the compost stabilization, 45 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 46 Union Nitrates Directive [8] (which aims to protect water quality across Europe by preventing 47 nitrates from agricultural sources polluting ground and surface waters) can also benefit from 48 efficient analytical techniques applied to aqueous compost extracts. For these reasons, the 49 50 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 51 extracts. In the past, various methods have been proposed for the quantification of nitrate ion 52 in aqueous solutions including spectrophotometry [9, 10], fluorimetry [11, 12], ion 53 54 chromatography [13] and flow injection analysis [14]. Literature also offers some examples of 55 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 56 57 given by these works for the determination of nitrate, the main problem related to the cited 58 procedures is given by the use of cadmium, which is renowned for his high toxicity, and also 59 by the strong interferences caused by complex samples such as compost extracts, when the measurement is carried out by spectrophotometry. 60

The main goal of our work was to develop a new sensitive method for the determination of 61 62 nitrate by a microplate-based procedure in compost aqueous extracts. In the past, our research group has developed various analytical methods based on the 96-wells technique [17-20]. In 63 64 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 65 basic idea for the assessment of our method was to exploit the previous protocol, reducing first 66 the nitrate to ammonium and then quantifying the latter by means of the method already 67 assessed. An important advantage of this analytical strategy would be the easy determination 68 of both nitrate and ammonium ions with a single set of detection reagents (with or without the 69 reduction step), which would be highly valuable as the  $NH_4^+/NO_3^-$  ratio is one of the principal 70 71 nitrogen-based indicators of compost maturity [7].

72 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 73 74 types of alloys like Devarda's alloy and Arndt's alloy have been reviewed by Fanning [21]. 75 Among all, Zn seems to represent a good candidate for the reduction of nitrate to ammonium. 76 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 77 78 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 79 nitrate in compost water extracts which includes the conversion to ammonium by means of Zn 80 powder, and which is developed as a simple analytical tool that can be used directly in 81 82 composting platforms in a non-specialized environment (no fume hoods for strong acids and 83 non-expert technicians).

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#### 2. Experimental

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### 87 **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 88 samples (A samples) was exclusively composed by green wastes deriving from activities of 89 gardening and maintenance of green public or private areas. Sampling sites were located in 3 90 private areas (agricultural lands) located in Provence Alpes-Côte d'Azur region (France, GPS 91 coordinates : near Gardanne : 43.466350N, 5.454541E ; near Rousset : 43.468935N, 92 5.634117E, and near Rougiers : 43.411657N, 5.841994E). Ages of the swaths were always 93 94 included between one week and twelve months. It should be noticed that studied swaths can 95 be very heterogeneous in their composition of plant species, size and height. The second type 96 of samples (B samples) consisted in sewage sludge mixed with green wastes (1/3-2/3, v/v). In 97 this case, samplings were performed on an industrial composting platform (Biotechna) located in Ensuès-la-Redonne (Bouches-du-Rhône, France, GPS coordinates 43.376838N, 98 99 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 100 the 2 first months, every two weeks the next month, and every month the three months 101 102 afterwards). For each type of compost, matter was collected in different places of the swath at 103 80 cm depth, in order to get a final composite sample of 2 kg representative of compost.

104 The water extractions were performed in PTFE Teflon tube (triplicates per soil sample) on an 105 orbital shaker (Fisher Scientific Bioblock SM30B) at 125 rpm and at room temperature. Organic 106 matter was extracted through shaking 6 g of fresh compost with 60 mL of ultrapure water 107 (resistivity of 18 M $\Omega$  cm<sup>-1</sup> at 25 °C, and total organic carbon < 20 µg L<sup>-1</sup>) with a soil/water ratio 108 of 1/2 (w/v) for 2 h under N<sub>2</sub>. After extraction step, the tubes were centrifuged at 8000 rpm for 109 15 min (JP SELECTA, Medifriger BL-S), and the supernatants were collected and filtered
110 through 0.45 µm membrane filters (PES membrane, Millipore, France). The resulting solutions
111 were stored in the dark at -18 °C until analysis.

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#### 113 2.2 Reagents and solutions

All chemicals were of analytical reagent grade and used without further purification. Zn powder 114 115 (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 116 (puriss., <212 μM) and AI powder were provided by Sigma-Aldrich (France). O-phthaldehyde 117 (OPA) was obtained from Acros Organics (Belgium) and N-acetyl-L-cysteine (NAC) from 118 Sigma-Aldrich. Buffers solutions were prepared dissolving the proper amount of sodium 119 acetate (Sigma-Aldrich), 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES, Acros 120 organics), anhydrous sodium carbonate (Sigma-Aldrich) and sodium tetraborate decahydrate 121 (Sigma-Aldrich) in ultrapure water (Millipore, USA, resistivity >18 M $\Omega$  cm) and adjusting the pH 122 with sodium hydroxyde or hydrochloridic acid. 123 124 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.

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### 128 2.3 Instruments

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#### 130 2.3.1 Microplate

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

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## 138**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<sup>®</sup> (6.80 version) equipped with a guard column (Dionex AG11-HC), an analytic Dionex AS11-HC (4 × 250 mm) column, a conductimetric detector (Dionex CD-25) and using a 200  $\mu$ L loop injection valve. Analysis were performed in an isocratic mode (22.5 mM NaOH in helium sparged deionized water) at 30 °C, with a flow rate set at 1.5 mL min<sup>-1</sup>.To improve the signal-to-noise ratio of the conductivity measurement, an external flow electrochemical suppressor system (ACRS 500 4 mm) wasadded to the analytical system.

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#### 148 **2.4** Analytical protocol for nitrate determination

Twenty mg of Zn powder (100 mesh) were dispensed into the wells of the microplate, 150 µL 149 of sample or standard solution were introduced and 20 µL of MES buffer (0.1 M, pH 6) were 150 151 added. The plate was shaken for 30 min at 30 °C. Then, 120 µL of the solution were transferred into new wells where 20 µL of a solution of 20 mM NAC and 30 µL of 13 mM OPA in ethanol-152 153 0.15 M carbonate buffer pH 10.5 (10:90, v/v) were subsequently added. The plate was shaken 154 for 10 min and fluorescence intensity was then recorded, with excitation and emission 155 wavelengths set at  $\lambda_{ex}$ =415 nm/ $\lambda_{em}$ =485 nm. Concentrations in unknown samples were determined using the linear calibration curves obtained with standards. Nitrate concentration 156 was calculated after quantifying the ammonium by means of the microplate procedure 157 previously assessed by our research group [17]. 158

159

#### 160 **3. Results**

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#### 162 **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 163 the subsequent determination of the latter by fluorescence (Figure 1). One of the main 164 165 challenges of this work was to find a metal for the effective reduction of nitrates under mild conditions (moderate temperature to apply the developed protocol in polystyrene microplates 166 167 which don't withstand high temperatures, and also because the most affordable microplate 168 readers don't enable heating above 40°C), but which does not interfere with the second step 169 of the protocol (derivatization of ammonium with OPA/NAC and formation of isoindol adduct). 170 Alloys like Devarda's alloy generally provide efficient reduction of nitrates, but only under quite harsh reaction conditions (i.e. boiling water). Several tests were performed in order to reduce 171 172 the nitrate to ammonium with various metals. Experiments conducted with aluminum (foil ad powder) and copper (granules) at various pH conditions did not provide any significant result 173 proving to be not efficient for the reduction of the analyte. On the other hand, tests performed 174 175 with iron (thread and powder) revealed that this metal is able to convert nitrate to ammonium 176 in acidic conditions (encouraging results were obtained at pH 5 and 6). Unfortunately, the corrosion products formed during the reduction phase constituted a strong interference for the 177 revelation of ammonium preventing a precise and accurate determination. Attempts to 178 179 suppress the interference by adding EDTA at various concentration levels (0.1-1 M) were 180 unsuccessful. Finally, Zinc proved to be promising both for reduction of nitrate and ammonium derivatization under mild conditions in our first experiments, so we decided to optimize ourmethod with this metal.

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#### 184 **3.2 Optimization of the analytical method with Zn**

185 Reduction of nitrate with Zn was performed in presence of various selected buffers in order to 186 identify the best pH conditions for the conversion of nitrate to ammonium and for the 187 derivatization. These first experiments were performed by means of Zn granules. A quantity of metal granules large enough to entirely cover the bottom of the microplate wells was cut and 188 introduced. 100 µL of a water solution of NaNO<sub>3</sub> 100 µM were introduced in the wells containing 189 190 the metal along with 20 µL of : i) acetate buffer (0.1 M, pH 5), ii) MES buffer (0.1 M, pH 6), iii) 191 HEPES buffer (0.1 M, pH 7), iv) carbonate buffer (150 mM, pH 10.5), v) borate buffer (100 mM, pH 11.5). Also HCl 2.5 M and H<sub>2</sub>SO<sub>4</sub> 2.5 M were tested for the reduction. In this case, 192 193 NaOH 0.1 M was added to the wells until basic pH, before derivatization with NAC and OPA. The suspensions were shaken for 1 h at 30 °C before addition of OPA and NAC reagents. 194 Experiments were also conducted on a solution of NH<sub>4</sub>Cl 100 µM in order to detect possible 195 196 interferences of the reducing conditions on the ammonium derivatization. As shown in Figure 2, best results were obtained at pH 5 and pH 6. In fact, in both cases conversion of nitrate to 197 198 ammonium occurred and high values of fluorescence for ammonium were detected, resulting in good sensitivity under these conditions. According to data obtained, reduction of nitrate is 199 200 much less efficient at pH 7 or when basic carbonate and borate buffers are employed, also 201 probably because of the evaporation of NH<sub>3</sub> which is predominant at neutral/basic pH. Regarding the acidic conditions selected for the study, it appears that in the case of HCI 2.5 M 202 203 there is no substantial difference between the blank values and those of nitrate and 204 ammonium, while in the case of  $H_2SO_4$  data related to ammonium derivatization are very low. 205 The MES buffer (0.1 M, pH 6) was selected for the following experiments, given the higher 206 value provided by the test on the ammonium solution revealing a higher sensitivity for this pH level. The subsequent experiments were mainly aimed to optimise the conditions for the 207 208 enhancement of the conversion of nitrate to ammonium.

A kinetic test was carried out in order to select the best time condition for the reduction of 209 210 nitrate with zinc powder (more efficient than zinc granules). As shown in Figure 3, after 30 min about 90% of the nitrate is reduced to ammonium indicating that this could represent a good 211 212 time condition for the proposed procedure. The influence of Zn powder quantity on the reduction rate at the selected conditions (30 °C, 30 min) was also studied. Calibration curves 213 obtained with 10, 20 and 30 mg of Zn are shown in Figure 4. Tests performed with 10 mg of 214 powder showed that there is a loss of linearity at concentrations higher than 50 µM indicating 215 also that, in this case, contact surface is not large enough to provide efficient conversion of 216 217 nitrate to ammonium. At the same time, experiments carried out with 30 mg of powder, showed a worsening of the R<sup>2</sup> value probably due to an interference caused by the excess of solid
particles in the suspension. For this reason, 20 mg was selected as the best compromise for
the procedure.

- Finally, influence of an increase of the reaction temperature was also assessed (up to 40 °C, the maximum heating capacity of most microplate readers/shakers), with no significant
- 223 improvements, and 30 °C was therefore selected for the optimized protocol.
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### 225 3.3 Interferences

226 The interference of various major anions and cations that could be found in compost extract 227 samples was investigated. More specifically, K<sub>2</sub>SO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>CO<sub>3</sub>, MgCl<sub>2</sub>, NaCl, NaBr and 228 CaCl<sub>2</sub> were selected for the study. Other less common redox active anions (low redox potential/high reducing ability) such as HPO<sub>3</sub><sup>2-</sup>, SO<sub>3</sub><sup>2-</sup> and C<sub>2</sub>O<sub>4</sub><sup>2-</sup> have also been included in 229 these interferences tests. The analytical protocol was applied to pure solutions of the cited 230 salts or anions at a concentration of 2 mM, giving a response not significantly different from 231 the blank. Moreover, tests were also performed on mixtures of NaNO<sub>3</sub> 100  $\mu$ M and selected 232 interfering compounds in order to compare results with those given by the pure NaNO<sub>3</sub>. Results 233 234 showed that the salts tested did not interfere up to at least 2 mM.

- Experiments conducted on a standard solution of  $NaNO_2 100 \mu M$  revealed that nitrite shows the same behavior as nitrate, being reduced to ammonium by Zn at the same pH conditions. Anyway, analysis performed by ion-exchange chromatography on the samples confirmed the already expected absence of nitrite in the aqueous compost extracts. Interferences from nitrite should therefore be negligible in this type of environmental samples, but has to be considered (separate quantification by Griess reagent) if the method is applied to other samples with significant nitrite concentrations.
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#### 243 3.4 Analytical features

244 The assessed procedure was validated in terms of sensitivity, linearity, precision and accuracy.

245 The limit of detection (LOD) was calculated from the residual standard deviation of the

- regression (linearity study method). More specifically, LOD was evaluated as follows:
- 247 LOD=3s/b
- where s is the standard deviation (SD) of the a-intercept and b the slope of the calibration curve. LOD is 1.30  $\mu$ M (0.08 mg L<sup>-1</sup>). It is interesting to note that detection limit is almost an order of magnitude smaller than the LOD estimated by Murray et al. [22], for their procedure using zinc reduction and a spectrophotometric method (0.5 mg L<sup>-1</sup>).
- 252 The calibration curve (I=11.10C+316.2; I = fluorescence intensity (arbitrary units), C = nitrate
- concentration ( $\mu$ M)) was linear up to 100  $\mu$ M with a correlation coefficient of 0.9941, resulting
- in a large enough linear dynamic range for our analytical purposes (4.5-100  $\mu$ M). Relative

- standard deviations were assessed on a 5  $\mu$ M (RSD = 14.3%) and on a 50  $\mu$ M (RSD=4.2%) nitrate standards (n=10 replicates) indicating a good repeatability for both concentration levels.
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#### 258 **3.5 Comparison with other microplate methods for nitrate determination**

259 The microplate format is an ideal tool for routine analysis when a large number of samples has 260 to be analyzed in a short time (typical case for ammonium or nitrate determination in aqueous 261 samples). All methods adapted in microplate are based on the reduction of nitrate to nitrite and subsequent spectrophotometric measurements after reaction with Griess reagent. Reduction 262 263 of nitrate to nitrite has been performed by various methods: copperised cadmium granules [25] 264 or pins [15], vanadium (III) [26] or enzymatic reduction [27]. Global reaction times are a bit 265 longer with these methods (60-90 min at room temperature) compared to our method (40 min). The main advantage of these spectrophotometric techniques is the low detection limits (12-20 266 µg L<sup>-1</sup>) which are 4 to 6 times lower than our spectrofluorimetric method. However, these 267 methods also have limitations for compost analysis: i) the main reduction methods still use 268 cadmium which is highly toxic; ii) complex and colored samples such as compost extracts will 269 270 display strong matrix effects using spectrophotometric techniques [28], making fluorimetric measurements highly desirable; iii) determination of nitrate and ammonium  $(NH_4^+/NO_3^-)$  ratio is 271 one of the principal nitrogen-based indicators of compost maturity) requires two sets of 272 detection reagents if the Griess reagent is used, while our methodology easily provides 273 274 quantification for both analytes (with or without reduction step).

275 Similarly, nitrate determination using nitration of resorcinol and spectrophotometric 276 measurement is not suitable for this type of analysis, as it uses pure sulfuric acid (not 277 compatible with microplates) and displays strong interferences due to sample color (own 278 experiments, data not shown).

- 279 Overall, our fluorimetric method seems more adapated to compost extract analysis in the 280 microplate format than methods based on spectrophotometry.
- 281

#### 282 **3.6** Quantification of nitrate ion and method validation in aqueous compost extracts

Nitrate was quantified by means of the assessed procedure in twenty-five aqueous extracts of 283 284 compost of various sources, using the external calibration method. Sample dilution represents a critical point of the whole analytical protocol because the highly-colored matrix can heavily 285 286 disturb spectroscopic measurements. Moreover, dilution is required in order to match the defined calibration range both for ammonium and nitrate. A comparison between the slope of 287 calibration curves obtained by means of external calibration and multiple standard addition 288 289 methods for some selected samples was exploited in order to select a dilution ratio able to 290 minimize the interferences given by matrix, but still able to detect nitrates and ammonium. A 5-fold dilution was adopted for A samples and a 50-fold one for B samples. Nitrate concentration was quantified in a range included between  $2.0\pm0.1$  and  $90\pm10$  mg L<sup>-1</sup>.

293 These twenty-five samples were also chosen for comparison purposes between ion-exchange 294 chromatography and microplate measurements with the developed procedure. As shown by Figure 5, a good correlation ( $R^2$ =0.9127) was found between the two data sets, and a very 295 weak intercept value with regard to the studied concentration range sets. The slope reveals a 296 297 slight underestimation of data obtained by the microplate method, probably due to residual matrix effects of these compost samples. A higher dilution rate would have improved these 298 299 results, but was not possible because most samples would have too low concentrations for 300 proper nitrate or ammonium quantification. Results are still satisfactory for a field test tool, 301 which is aimed to assess the evolution of nitrate concentration during the composting process and which mainly requires a good correlation between the measurements and the actual 302 303 concentrations in the compost samples.

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#### 305 **4. Conclusions**

In this study, we presented the assessment and validation of a new analytical method using 306 307 the microplate format for the determination of nitrate ion in aqueous compost extracts. Optimization work allowed to obtain fast and stable responses by employing a small quantity 308 of a non-toxic metal (zinc) for the reduction. Nitrate reduction with zinc (mainly studied for the 309 310 reduction of nitrate to nitrite, much less to the final ammonium reduction), was coupled with a 311 fluorimetric detection method, offering several advantages such as lower detection limits (an order of magnitude lower than the spectrophotometric method described with zinc reduction 312 313 [22], especially using the microplate format) and less interferences due to complex and colored 314 samples such as compost extracts. It also potentially enables the easy determination of both 315 nitrate and ammonium ions with a single set of detection reagents (with or without the reduction step), which is highly valuable as the  $NH_4^+/NO_3^-$  ratio is one of the principal nitrogen-based 316 indicators of compost maturity. Besides, no strong acids are used in this method, on the 317 318 contrary of other frequently used analytical methods for nitrate [10,12]. Preventing the use of strong acids or toxic metals and reagents is highly desirable for this type of analytical method 319 320 which is meant to be used directly on composting platforms by non-expert technicians. Only a 321 small volume of sample is required for the analysis and the whole procedure is completed in 322 about 40 min with the possibility to analyze several extracts at the same time thanks to the microplate instrumentation. Validation showed that the proposed procedure is sensitive, 323 precise and with a good level of accuracy. For all these reasons, the method is a good 324 candidate for routine analysis of nitrate in aqueous compost extracts, either in a traditional 325 326 laboratory or directly in composting platforms. A simple on-site compost extraction with a

327	modified coffee machine is currently under evaluation in our laboratory in order to reach a full
328	on-site analytical procedure.
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## 442 Figure captions

443 Figure 1. Proposed protocol for nitrate determination; Figure 2. Influence of the pH of the 444 reduction step on the fluorescence intensity response of standard solutions after reduction and 445 derivatization with OPA/NAC (zinc granules, 1h reduction time, n=2). Figure 3. Influence of the time of the reduction step on the fluorescence intensity response of standard solutions after 446 reduction and derivatization with OPA/NAC (20 mg zinc powder, n=2). Figure 4. Calibration 447 curves (0-100 µM) depending on the quantity of zinc powder used for the reduction step (30 448 449 min reduction time, n=2). Figure 5. Comparison between microplate method and ion-exchange chromatography (n=2). 450

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Step 1: reduction

$$NO_2^- \xrightarrow{Zn} NH_4^+ NH_3$$
 (function of pH)



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