

High throughput determination of ammonium and primary amine compounds in environmental and food samples

Fabien Robert-Peillard, Edwin Palacio, Marco Ciulu, Carine Demelas,
Frédéric Théraulaz, Jean Luc Boudenne, Bruno Coulomb

► **To cite this version:**

Fabien Robert-Peillard, Edwin Palacio, Marco Ciulu, Carine Demelas, Frédéric Théraulaz, et al.. High throughput determination of ammonium and primary amine compounds in environmental and food samples. *Microchemical Journal*, Elsevier, 2017, 133, pp.216-221. 10.1016/j.microc.2017.03.048 . hal-01499481

HAL Id: hal-01499481

<https://hal-amu.archives-ouvertes.fr/hal-01499481>

Submitted on 18 Apr 2018

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

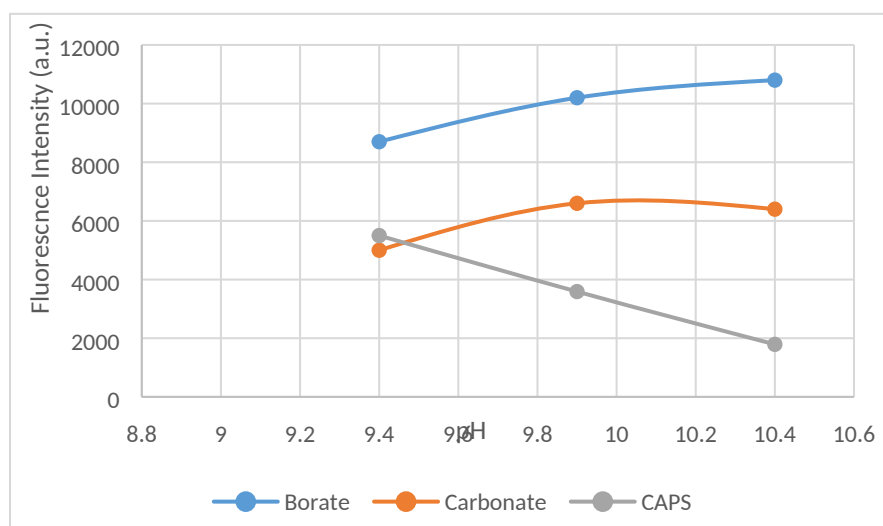
High throughput determination of ammonium and primary amine compounds in environmental and food samples

F. Robert-Peillard¹, E. Palacio Barco², M. Ciulu¹, C. Demelas¹, F. Théraulaz¹, J.-L. Boudenne¹, B. Coulomb¹

¹ Aix Marseille Univ, CNRS, LCE UMR7376, 13331 Marseille, France.

² Laboratory of Environmental Analytical Chemistry. University of the Balearic Islands, E-07122 Palma de Mallorca, Illes Balears, Spain.

SUPPLEMENTARY INFORMATION



Sup. Fig. 1: Influence of buffer. [OPA]: 8 mM; [NAC]: 8 mM; [NH₄⁺]: 100 μM; pH = 10.5

1 **High throughput determination of ammonium and primary amine compounds in**
2 **environmental and food samples**

3
4 Fabien Robert-Peillard^{1*}, Edwin Palacio Barco², Marco Ciulu¹, Carine Demelas¹, Frédéric
5 Théraulaz¹, Jean-Luc Boudenne¹, Bruno Coulomb¹

6
7 ¹ Aix Marseille Univ, CNRS, LCE UMR7376, 13331 Marseille, France.

8 ² Laboratory of Environmental Analytical Chemistry. University of the Balearic Islands, E-
9 07122 Palma de Mallorca, Illes Balears, Spain.

10 *Corresponding author: fabien.robert-peillard@univ-amu.fr

11
12 **Abstract**

13 In this paper, an improved spectrofluorimetric method for the simultaneous and direct
14 determination of ammonium and primary amine compounds is presented. The method is
15 based on the derivatization with *o*-phthaldialdehyde (OPA) / *N*-acetylcysteine (NAC) reagent
16 using high throughput microplates, and OPA/NAC ratio has been optimized in order to
17 suppress interference of ammonium on primary amine determination. Direct measurement of
18 these two parameters is therefore possible with a global procedure time that does not exceed
19 ten minutes. Excellent limits of detection of 1.32 µM and 0.55 µM have been achieved for
20 ammonium and primary amines, respectively. Reagent stability issues have also been
21 addressed and formulation of reagents solution is described for improved reagents shelf life.
22 The proposed protocol was finally applied and validated on real samples such as wine
23 samples, compost extracts and wastewater.

24
25 **Keywords:** ammonium; primary amine; microplate; reagents stability.

26

27 **1. Introduction**

28 Amino compounds are widely distributed in the environment and essentially result from
29 metabolic processes of degradation, hydrolysis and excretion at different levels of the food
30 chains. Soil organic matter thus contains about 30% of its nitrogen pool as amino acids [1],
31 and a significant proportion of NH_4^+ can be released from organic matter by microbial
32 hydrolysis [2]. Anthropogenic activities also contribute to the presence of these compounds
33 and ammonium in the environment, mainly attributed to the incineration of waste [3] and the
34 discharges of waste waters from chemical industry and wastewater treatment plants [3,4].

35 Among the nitrogen pool, primary amino compounds determination is a significant parameter
36 in the food processing or drinking-water treatment industry as it can react with nitrites or
37 nitrates to form nitrosamines which are classified as "probably carcinogenic to human" [5,6].

38 Primary amino compounds (mainly primary amino acids) are also very important regarding
39 the nitrogen management in some specific fields such as wine industry [7]. Indeed, assaying
40 the total primary amino acids concentration is often considered to be the most convenient
41 method to measure assimilable nitrogen, which is critical for wine flavour and style.

42 Regarding ammonium, its determination in environmental samples is highly relevant, due to
43 its important micronutrient function in aquatic systems or as an important index in
44 composting process studies. As example, high concentration in a water body can be an
45 indicator of the environmental impact of human activities, with strong effects on
46 microbiological activities that can potentially lead to eutrophication events [8]. In the
47 composting field, low ammonium concentration coupled to low $\text{N-NH}_4^+/\text{N-NO}_3^-$ ratio has
48 been proposed as indicators of a compost stability [9].

49 Several methods have been proposed for the analysis of ammonium [10], such as
50 spectrophotometry [11,12,13], ion selective electrode [14], fluorimetry [15,16] or ion

51 chromatography [17]. Primary amines/amino acids are generally analyzed individually by
52 liquid chromatography [18,19], but methods have also been developed to measure the total
53 concentrations of these compounds in order to have a rapid and global assay of these
54 important nitrogen compounds. This can be done for example by spectrophotometric
55 measurements after reaction with ninhydrin [20] or by fluorimetry after reaction with *o*-
56 phthaldialdehyde (OPA) and a thiol compound [21].

57 The numerous advantages of the combination of this OPA-thiol reagent (reaction with
58 ammonium and amines, sensitivity, selectivity, low toxicity and price of reagents...) has led to
59 the development of simultaneous determination methods for ammonium and primary amino
60 compounds. Meseguer Lloret et al.[14] used solution derivatization with OPA-NAC reagent
61 (NAC: N-acetylcysteine), two different excitation and emission fluorescence wavelengths and
62 statistical analysis by multivariate Principal Component Regression in order to separate the
63 responses of ammonium and amine under selected experimental conditions [15]. Darrouzet-
64 Nardi et al. [22] developed a fluorescent assay with OPA and β -mercaptoethanol for analysis
65 of primary amino compounds, by taking into account potential interference of ammonium.
66 The main drawback of this method was the necessity to use a 1-h incubation time in order to
67 reduce interference from ammonium.

68 The aim of the present study is to develop a fast, simple and efficient method for ammonium
69 and primary amino compounds analysis, with no need of statistical analysis, direct
70 measurement from calibration curves and a global procedure time that does not exceed ten
71 minutes. Moreover, this method has been developed as a potential routine analytical method
72 applicable to the large number of samples that an analytical laboratory typically has to deal
73 with (especially for ammonium). Therefore, a high-throughput microplate method based on
74 OPA-NAC reagent was used, with special care on reagents stability which is a key point for
75 routine analysis development (shelf life of at least 3 months without deterioration of analytical

76 performances). The final goal of this study was to conduct a strong validation on complex
77 samples like compost extracts or wastewaters by comparison with a chromatographic
78 reference method, in order to have a robust method for routine analysis of ammonium and
79 primary amino compounds.

80

81 2. Experimental

82 2.1 Reagents and solutions

83 All chemicals were of analytical reagent grade and used without further purification. OPA
84 was obtained from Acros Organics and *N*-acetyl-L-cysteine (NAC) and tris(2-carboxyethyl)
85 phosphine hydrochloride (TCEP) from Sigma-Aldrich. OPA solutions were prepared by
86 dissolving pure compound in appropriate buffer and adjusted at pH=10.5 with sodium
87 hydroxide or hydrochloric acid. CAPS, borate and carbonate buffers were prepared by
88 dissolving *N*-cyclohexyl-3-aminopropanesulfonic acid (Acros Organics), sodium tetraborate
89 decahydrate (Sigma-Aldrich) and anhydrous sodium carbonate (Sigma-Aldrich) respectively
90 in ultrapure water (Millipore, resistivity >18 MΩ cm). Stock solutions of individual amino
91 compounds (10 mM each) were prepared by dissolving appropriate amounts of pure
92 compound (Sigma-Aldrich) in ultrapure water. Stock standard ammonium solution (55.5 mM)
93 was prepared by dissolving appropriate amount of ammonium chloride in deionized water.
94 Working solutions were obtained by diluting stock solutions to proper concentrations.

95

96 2.2 Instruments

97 2.2.1 Microplate

98 Microplate fluorescence measurements were carried out on a microplate reader (Infinite
99 M200, Tecan France SAS, Lyon, France), operated at 30 °C and controlled by i-control™
100 software (Tecan). Detection was performed by top fluorescence reading at $\lambda_{\text{ex}} = 335 \text{ nm}$ and

101 $\lambda_{em} = 455$ nm for total primary amines quantification and at $\lambda_{ex} = 415$ nm and $\lambda_{em} = 485$ nm
102 for ammonium determination. Other parameters were as follows: gain: 80; number of flashes:
103 5; integration time: 20 μ s. Fluorescence intensities were expressed in arbitrary units (a.u.).
104 Polystyrene black 96 V-well microplates (Fisher Scientific, Illkirch, France) were used.

105

106 2.2.2 Ion chromatography analysis of ammonium

107 The ion chromatographic system consisted of an IonPac CS12A 4x250 mm column
108 (ThermoScientific), a CSRS-Ultra 4 mm self-regenerating suppressor, an AS40 auto-sampler,
109 an ED40 electrochemical detector operated in the conductivity mode and a GP40 gradient
110 pump operating at a flow-rate of 1.0 mL/min (Dionex). The injection loop was 50 μ l. Elution
111 was carried out in isocratic mode by 18 mM methanesulfonic acid solution. System control,
112 data collection and data processing was performed with PeakNet 5.1 Chromatography
113 Workstation software (Dionex).

114

115 2.2.3 Ion exchange chromatography for primary amines determination

116 Primary amino acid compounds in selected wines were determined by an external laboratory
117 using an automatic amino acid analyzer (Biochrom 30+, Cambridge, England). Wine samples
118 were initially diluted in a sodium citrate buffer (pH 2.2). All amino acids were
119 spectrophotometrically detected after post-column derivatization with ninhydrin reagent at
120 570 nm. Concentrations of amino acid compounds in unknown samples were determined by
121 comparison with standard peak areas (Sigma-Aldrich amino acid standard kit) and by using
122 norleucine as internal standard. Ion-exchange chromatography analyses on real wine samples
123 were performed on the same day as microplate analyses for validation purposes.

124

125 2.3 Analytical protocol for primary amines and ammonium determination

126 100 μL of sample or standard solution were dispensed into the wells of the microplate, where
127 30 μL of 13 mM OPA in ethanol-0.15 M carbonate buffer pH 10.5 (10:90, v/v) and 20 μL of
128 a solution of 20 mM NAC and 1.5 mM TCEP in 0.1 M HCl were added. The plate was
129 shaken for 10 min and fluorescence intensity was then recorded, with excitation and emission
130 wavelengths set at at $\lambda_{\text{ex}}=335$ nm / $\lambda_{\text{em}}=455$ nm and at $\lambda_{\text{ex}}=415$ nm / $\lambda_{\text{em}}=485$ nm for total
131 primary amines and ammonium determination respectively. Concentrations in unknown
132 samples were determined using the linear calibration curves obtained with standards. All
133 experiments were performed in duplicate.

134

135 3. Results

136 3.1 Optimization of analytical method

137 3.1.1 OPA/NAC concentration and pH

138 Initially derivatization of amino acids by OPA-NAC reagent developed by Aswad [23] was
139 carried out with an OPA/NAC ratio of 1:2. Even if the reaction rate and the fluorescence yield
140 are not dependent on the OPA/NAC ratio [24], it is nevertheless necessary to use an OPA
141 concentration higher than targeted amino acid concentration [25]. In past years OPA/NAC 1:1
142 ratio solution has often been used as pre-column derivatization reagent for HPLC or capillary
143 electrophoresis separation of amino acids or biological amines.

144 More recently, this method was adapted for the determination of total primary amine
145 compounds and ammonium in environmental samples by Meseguer Lloret et al. [15] with a
146 fluorescence measurement at two $\lambda_{\text{ex}}/\lambda_{\text{em}}$ couples of wavelengths to separate the response of
147 the primary amines with that of ammonium. However, this method was based on the use of a
148 statistical calibration model to avoid cross interferences of the analytes during fluorescence
149 measurement. Optimization of the concentration of OPA/NAC 1:1 ratio reagent may reduce

150 decrease of the fluorescence response of ammonium adduct at the wavelengths used for amino
151 compounds determination.

152 Fig. 1 displays the evolution of fluorescence intensity of OPA-NAC-ammonium adduct at
153 amino compounds wavelengths as a function of reaction time. The increase in OPA and NAC
154 concentration can reduce ammonium interference during primary amines determination. For a
155 concentration greater than 8 mM of OPA and NAC, a reaction time of 600 seconds allows to
156 fully eliminate the cross-interference of ammonium over amino compounds determination.
157 This reaction time is longer than the one used by Meseguer-Lloret et al. [15] (120 and 300
158 seconds respectively for amines and ammonia) but greatly simplifies the calibration process
159 by avoiding the use of statistical tools. The reaction time also depends on the pH used for the
160 derivatization reaction. Fig. 2 shows the fluorescence intensity of the OPA-NAC-ammonium
161 adduct as a function of pH and reaction time. The reaction time previously set at 600 seconds
162 for the simultaneous measurement of ammonium and primary amines is sufficient to obtain a
163 high and stable fluorescence signal for ammonium derivatization by OPA/NAC 8mM/8mM at
164 pH = 10.5. This reaction time is significantly lower than that proposed by Darrouzet-Nardi et
165 al. (60 minutes) for a OPA/mercaptoethanol (ME) procedure developed in order to limit
166 interference of ammonium on the measurement of primary amines in soils [22].

167

168 3.1.2 Buffer

169 Sodium or potassium tetraborate are certainly the most commonly used buffers for
170 derivatization of ammonia or amines with OPA and NAC or ME in alkaline conditions
171 [14,15,21-25]. However, tetraborate salts have been classified as toxic for reproduction
172 (category 1B) by European regulations since 2008 [26]. Substitution of these products is
173 therefore recommended, especially for analytical procedures that are developed as potential
174 routine methods with high frequency of use for the reagent solutions.

175 In this study, we replaced the borate buffer solution with a carbonate or CAPS buffer which
176 have pKa values compatible with the pH used in the derivatization reaction. Experiments
177 showed that a carbonate buffer could replace the borate buffer but the fluorescence signal
178 obtained decreased by 40%. Nevertheless, despite this significant decrease of fluorescence
179 intensity, the analytical features obtained with the carbonate buffer fit with expected values of
180 ammonium and amines in environmental samples (see 3.3). CAPS, on the other hand, lead to
181 a very significant decrease in the fluorescence signal by 90% (Sup. Fig. 1).

182

183 3.2 Conservation of reagents

184 3.2.1 Reducing agent

185 It is well known that thiol group can easily be oxidized and form disulfide bond. This
186 oxidation reaction will quickly limit NAC reactivity and therefore inhibit derivatization of
187 ammonium or amines by OPA. This usually leads the authors to prepare OPA/NAC solutions
188 daily. However, it may be interesting in an analytical laboratory to keep OPA and NAC
189 solutions for several days, several weeks or even a few months, again especially for analytical
190 methods that are used routinely.

191 Three reducing agents conventionally used in analytical procedures have been studied: 2,2-
192 thiodiethanol (TDE), ascorbic acid and tris(2-carboxyethyl) phosphine hydrochloride (TCEP).
193 Fluorescence intensity of an ammonium standard adduct (100 μ M) was measured over a
194 period of 60 days (Fig. 3). Derivatization was carried out with OPA/NAC solution comprising
195 one of the reducing agent mentioned above at a concentration of 1 mM (TCEP) or 10 mM
196 (ascorbic acid, TDE).

197 We can observe with data of Fig. 3 that ascorbic acid did not enable good preservation of
198 OPA/NAC solutions. TDE and TCEP reduced degradation of OPA/NAC solution with
199 approximately 14% decrease in fluorescence intensity after 30 days in both cases. TCEP was

200 preferred for subsequent experiments because TDE is unpleasant to use due to its malodorous
201 properties. The optimization of the concentration of TCEP was then carried out (fig .4). Data
202 showed that TCEP concentration of 1 mM was sufficient to improve the conservation of
203 OPA/NAC reagent. Higher concentrations lead to an increase in the blank signal. Similar
204 results have been obtained for amino compounds derivatization (glycine as a reference).

205

206 3.2.2 Conservation mode of reagents

207 The optimization of the preservation of reagents was finally optimized by studying the
208 conservation mode of the reagents: OPA and NAC solutions can be stored in a mixture or
209 separately. It is well known that a formulation at low pH can be used to prevent oxidation of
210 *N*-acetylcysteine [27]. We have thus studied the evolution of the slope of calibration curves
211 over time (up to 6 months) either with the two liquid reagents prepared together (condition
212 called 'mixed reagents' on Fig. 5A) or with the two liquid reagents stored separately
213 (condition called 'separated liquid reagents' on Fig. 5B), and also with solid NAC deposited
214 in microplate wells and liquid OPA reagent stored separately (condition called 'solid NAC +
215 liquid OPA' on Fig. 5C). For this last preservation condition of the reagents, NAC was
216 solubilized in acetone and then introduced into microplate wells. Acetone was evaporated at
217 room temperature, then the plate was sealed with a polyethylene film. The OPA reagent was
218 similar to the previous condition (in carbonate buffer at pH 10.5). All the solutions or
219 microplates used in this study were stored in the dark at 4 °C.

220 For each experimental condition, confidence interval (CI) of slope value was determined at
221 initial time (0 day) from the standard deviation of the b-slope value of the calibration curve
222 $s(b)$, depending on the residual standard deviation of the regression ($n=5$, $P=0.05$) [28].
223 Calculated values of $(b \pm UM)$, with UM the uncertainty of measurement, for separated liquid
224 reagents, mixed reagents and solid NAC/liquid OPA were 58.1 ± 8.1 , 54.6 ± 1.6 and 49.1 ± 9.2 ,

225 respectively. On figure 5, CI limits are noticed by dotted lines. We can observe that the slope
226 remained inside CI for separated liquid reagents up to 4 months, on contrary to mixed
227 reagents only after 7 days. For solid NAC/liquid OPA condition, a great variability of slope
228 value during experimental period was noticed and one data was outside CI after only 4 weeks.
229 These results prompted us to use separated liquid reagents condition.

230

231 3.3 Analytical features

232 3.3.1 Screening of primary amines

233 Screening of some biogenic amines and primary amino acids (25 μ M) that could be likely
234 present in environmental or food samples was performed using OPA/NAC optimized
235 analytical protocol (Fig. 6). The fluorescence intensity of the different adducts was
236 standardized over that of the glycine adduct (reference 100).

237 A great diversity of amines and primary amino acids can therefore be detected by the
238 developed method, although their responses are not all similar. Secondary amines (proline)
239 and primary amines involved in a conjugated system (creatine, urea, creatinine) do not react.

240 The method allows for example the control of amino compounds in raw waters at the inlet of
241 treatment plants. Brosillon et al. [6] found that alanine, valine and tyrosine were the main
242 amino acids at the origin of disinfection by-products in drinking water during chlorination.
243 These 3 amino acids exhibit responses of 103, 82 and 78% compared to glycine. Although
244 individual concentrations of amino acids in raw waters range from 0.2 to 0.9 nM depending
245 on the season, the method is sensitive enough to quantify the total amount of amino
246 compounds. Likewise, the most frequently measured primary amino acids in wine samples
247 (alanine, glutamic acid, arginine) all lead to responses comparable with that of glycine, and a
248 global measurement based on a glycine standard is therefore relevant for this study.

249

250 3.3.2 Cross-interference and other interferences

251 In this section, a compound was considered as interferent when its presence resulted in more
252 than 5% modification of the pure ammonium (100 μM) or glycine (as reference amino acid;
253 100 μM) response.

254 The cross-interference of amines over ammonium ($\lambda_{\text{ex}}=415 \text{ nm} / \lambda_{\text{em}}=485 \text{ nm}$) and inversely
255 ammonium over amines ($\lambda_{\text{ex}}=335 \text{ nm} / \lambda_{\text{em}}=455 \text{ nm}$) was evaluated. The results showed that
256 ammonium could be quantified in the presence of a 1000-fold excess of glycine and that
257 primary amines could be quantified in the presence of a 100-fold excess of ammonium.

258 The interference of various metal cations has also been studied. The presence of Fe^{3+} or Cu^{2+}
259 at 15 μM resulted in an interference of more than 5%. The addition of 50 mM EDTA to the
260 reaction mixture reduced this interference. Ammonium could thus be quantified in the
261 presence of 750 μM of Fe^{3+} or Cu^{2+} and the amines could be quantified in the presence of 180
262 μM of Fe^{3+} and 450 μM of Cu^{2+} . The other metallic elements tested (Al^{3+} , Cd^{2+} , Co^{2+} , Ni^{2+} ,
263 Pb^{2+} , Zn^{2+}) did not interfere up to 1 mM.

264

265 3.3.3 Performance and validation of the analytical method

266 The developed method was characterized and validated according to the AFNOR procedure
267 XP T 90–210 [28]. Regarding analytical features, calibration curves for ammoniacal nitrogen
268 and amine compounds were constructed with several standards (respectively, $n=6$ and $n=5$)
269 with triplicate measurements for each, and have been obtained for each compound by linear
270 regression of the fluorescence intensity against the concentrations of standards. The
271 calibration range lies between the limit of quantification and a maximum concentration
272 depending on the concentration range allowing to keep linearity between instrumental signal
273 and standard concentrations. The limit of detection (LOD) of the analytical procedure is
274 defined as the lowest amount of analyte in a sample that can be detected and considered as

275 different from the blank value but not necessarily quantified as an exact value, whereas the
276 limit of quantification (LOQ) is the lowest amount of analyte in a sample which can be
277 quantitatively determined with the analytical procedure with a defined variability. The LOD
278 and LOQ were evaluated from the residual standard deviation of the regression (linearity
279 study method) as $LOD = 3.s(a)/b$ and $LOQ = 10.s(a)/b$, where $s(a)$ is the standard deviation of
280 the a-intercept and b is the slope of the calibration curve.

281 The accuracy of an analytical procedure is defined as the closeness of agreement between the
282 conventional “true” value (obtained by the reference analytical procedure) and the measured
283 value. The accuracy of our microplate procedure with fluorescence detection was assessed by
284 analyzing a great number of samples from various origins. The calculation of the existing
285 difference (d) between the measured value and the value issue from cationic chromatography,
286 for each sample, and the calculation of the absolute value of their mean (\bar{d}) and the
287 standard deviation (s) of all calculated data, is used to evaluate the good accuracy by check of
288 the normal distribution of these data around zero ($w = \bar{d}/s$; $P = 0.01$) [28].

289 The repeatability, i.e. the precision, of analytical procedures was assessed by calculating the
290 standard deviation of repeatability taking into account each replicate measurement ($n=3$) for
291 each sample. A comparison of variance of repeatability obtained with the two analytical
292 methods on the same multiple samples as previously is realized in order to determine their
293 potential significative difference or not (Fisher-test, $P = 0.01$) [28].

294

295 3.3.3.1 Ammonium

296 The calibration curve ($y = 57.076x + 623.96$) was linear up to 100 μM with a correlation
297 coefficient of 0.997 ($n=6$). A LOD of 1.32 μM and a LOQ of 4.41 μM have been obtained.
298 The working range of this analytical method is then 4.4-100 μM . The relative standard
299 deviation evaluated from a sample containing 50 μM ($n=6$) was 1.58%.

300 Validation of the analytical method requires satisfactory results for the accuracy and the
301 repeatability. No significant differences (Fisher-test, $n=54$, $P=0.01$) were noticed between
302 variance of repeatability of our new analytical method and cationic chromatography, and the
303 closeness of agreement of results proved a good accuracy ($w=0.16 < 3$; $P=0.01$). Figure 7
304 shows regression line obtained by comparison of results of 54 samples (surface water,
305 wastewater, water extracts of compost). Slope of regression line is 0.96 ± 0.05 and intercept
306 were 1.13 ± 1.88 . Result was satisfactory for the intercept and slope as confidence intervals of
307 their value included 0 and 1 respectively, in accordance with previous statistical test for
308 accuracy. The analytical range and the low limit of quantification allow the determination of
309 ammonium in various types of samples as natural waters, raw or treated wastewaters, aqueous
310 extracts of soils or wastes.

311

312 3.3.3.2 Primary amines

313 For primary amines, the calibration curve ($y=153.94x+374.89$) was linear up to $100\ \mu\text{M}$ with
314 a correlation coefficient of 0.995 ($n=5$). A LOD of $0.55\ \mu\text{M}$ and a LOQ of $1.84\ \mu\text{M}$ have been
315 obtained. The working range of this analytical method is then $0.55\text{-}100\ \mu\text{M}$. The relative
316 standard deviation evaluated from a sample containing $50\ \mu\text{M}$ ($n=6$) was 1.25%.

317 As for ammoniacal nitrogen, the accuracy and the repeatability for amine compounds gave
318 satisfactory results. No significant difference (Fisher-test, $n=13$, $P=0.01$) was noticed between
319 variance of repeatability of the developed analytical method and cationic chromatography,
320 and the closeness of agreement of results proved a good accuracy ($w=0.22 < 3$; $P=0.01$).
321 Figure 8 shows regression line obtained by comparison of results of 13 samples (various
322 samples of wines). Slope of regression line is 0.94 ± 0.23 and intercept were 178.7 ± 535.2 .
323 Confidence intervals for the intercept and slope were relatively wide, essentially due to higher
324 residual standard deviation of the regression, including however 0 and 1 respectively, equally

325 in accordance with previous statistical test for accuracy. The analytical range and the low
326 limit of quantification allow the determination of primary amines in various types of samples
327 as natural waters or food samples for example.

328

329 **4. Conclusion**

330 In this study, we presented the development and validation of a method for the determination
331 of ammonium and primary amine compounds in various environmental and food samples.
332 Optimization of reagents ratio and pH buffer enables fast and stable responses with no cross
333 interferences between these two structurally close analytes. Reagent storage conditions were
334 also evaluated in order to improve reagents shelf life, and we showed that separated storage of
335 OPA and NAC solutions was the best option. Regarding analytical features, excellent limits of
336 detection of 1.32 μM and 0.55 μM have been achieved for ammonium and primary amines,
337 respectively, with RSD below 2%. Validation on various real samples by comparison with
338 reference methods resulted in very good accuracies, proving the efficiency of this
339 methodology for routine analysis of these nitrogen compounds.

340

341 **Acknowledgment**

342 This work was financially supported by the French Research Agency (ANR) through the
343 programme “EMERGENCE” [ANR-10-EMMA-038] and by the French Environment and
344 Energy Management Agency (ADEME) through the programme “DOSTE” [1506C0034].

345

346 **References**

347 [1] F.J. Stevenson, Humus chemistry: Genesis, composition, reactions, second ed., John
348 Wiley & Sons, 1994.

- 349 [2] D. Barraclough, The direct or MIT route for nitrogen immobilization: A ¹⁵N mirror image
350 study with leucine and glycine, *Soil Biol. Biochem.* 29 (1997) 101-108.
- 351 [3] G. Seeber, M.R. Buchmeiser, G.K. Bonn, T. Bertsch, Determination of airborne, volatile
352 amines from polyurethane foams by sorption onto a high-capacity cation-exchange resin
353 based on poly(succinic acid), *J. Chromatogr. A.* 809 (1998) 121-129.
- 354 [4] G. Skarping, M. Dalene, P. Lind, Determination of toluenediamine isomers by capillary
355 gas chromatography and chemical ionization mass spectrometry with special reference to the
356 biological monitoring of 2,4- and 2,6-toluene diisocyanate, *J. Chromatogr. A.* 663 (1994) 199-
357 210.
- 358 [5] L. Hureiki, J.P. Croué, B. Legube, Chlorination studies of free and combined amino acids,
359 *Water Res.* 28 (1994) 2521-2531.
- 360 [6] S. Brosillon, M. Lemasle, E. Renault, D. Tozza, V. Heim, A. Laplanche, Chlorination
361 studies of free and combined amino acids, *Chemosphere* 77 (2009) 1035-1042.
- 362 [7] M. Ugliano, P.A. Henschke, M.J. Herderich, I.S. Pretorius, Nitrogen management is
363 critical for wine flavour and style, *Wine Ind. J.* 22 (2007) 24-30.
- 364 [8] M. Zamparas, I. Zacharias, Nitrogen management is critical for wine flavour and style, *Sc.*
365 *Tot. Env.* 496 (2014) 551-562.
- 366 [9] M.P. Bernal, A.F. Navarro, M.A. Sanchez-Monedero, A. Roig, J. Cegarra, Influence of
367 sewage sludge compost stability and maturity on carbon and nitrogen mineralization in soil,
368 *Soil Biol. Biochem.* 30 (1998) 305-313.
- 369 [10] C. Molins-Lagua, S. Meseguer-Lloret, Y. Moliner-Martinez, P. Campins-Falco,
370 Influence of sewage sludge compost stability and maturity on carbon and nitrogen
371 mineralization in soil, *Trends Anal. Chem.* 25 (2006) 282-290.

372 [11] M. S. F. Marina, B. Freire dos Reis, H. Bergamin, N. Baccan, Flow-injection
373 determination of low levels of ammonium ions in natural waters employing preconcentration
374 with a cation-exchange resin, *Anal. Chim. Acta* 261 (1992) 339-343.

375 [12] M.T. Oms, A. Cerda, A. Caldera, V. Cerda, R. Forteza, Gas diffusion techniques coupled
376 sequential injection analysis for selective determination of ammonium, *Anal. Chim. Acta* 318
377 (1996) 251-260.

378 [13] H. Jeong, J. Park, H. Kim, Determination of NH_4^+ in environmental water with
379 interfering substances using the modified Nessler method, *J. Chem.* 2013 (2013) 1-9.

380 [14] S. Meseguer-Lloret, C. Molins-Legua, P. Campins-Falco, Ammonium determination in
381 water samples by using OPA-NAC reagent: a comparative study with Nessler and ammonium
382 selective electrode methods, *Intern. J. Environ. Anal. Chem.* 82 (2002) 475-489.

383 [15] S. Meseguer-Lloret, J. Verdu Andres, C. Molins-Legua, P. Campins-Falco,
384 Determination of ammonia and primary amine compounds and Kjeldahl nitrogen in water
385 samples with a modified Roth's fluorimetric method, *Talanta* 65 (2005) 869-875.

386 [16] G. Cao, Y. Su, Y. Zhuang, J. Lu, A new fluorescence method for determination of
387 ammonium nitrogen in aquatic environment using derivatization with benzyl chloride, *J. Braz.*
388 *Chem. Soc.* 27 (2016) 950-957.

389 [17] H. Shen, T.J. Cardwell, R.W. Cattrall, The application of a chemical sensor array
390 detector in ion chromatography for the determination of Na^+ , NH_4^+ , K^+ , Mg^{2+} and Ca^{2+} in
391 water samples, *Analyst* 183 (1998) 2181-2184.

392 [18] P. Krumpochova, B. Bruyneel, D. Molenaar, A. Koukou, M. Wuhler, W.M.A. Niessen,
393 M. Giera, Amino acid analysis using chromatography–mass spectrometry: an inter platform
394 comparison study, *J. Pharm. Biomed. Analysis* 114 (2015) 398-407.

395 [19] P.G. Rigas, Post-column labeling techniques in amino acid analysis by liquid
396 chromatography, *Anal. Bioanal. Chem.* 405 (2013) 7957-7992.

397 [20] S. Moore, W.H. Stein, A modified ninhydrin reagent for the photometric determination
398 of amino acids and related compounds, *J. Biol. Chem.* 211 (1954) 907-913.

399 [21] D.L. Jones, A.G. Owen, J.F. Farrar, Simple method to enable the high resolution
400 determination of total free amino acids in soil solutions and soil extracts, *Soil Biol. Biochem.*
401 34 (2002) 1893-1902.

402 [22] A. Darrouzet-Nardi, M.P. Ladd, M.N. Weintraub, Fluorescent microplate analysis of
403 amino acids and other primary amines in soils, *Soil Biol. Biochem.* 57 (2013) 78-82.

404 [23] D.W. Aswad, Determination of D- and L-Aspartate in amino acid mixtures by High-
405 Performance Liquid Chromatography after derivatization with a chiral adduct of *o*-
406 Phthaldialdehyde, *Anal. Biochem.* 137 (1984) 405-409.

407 [24] M. Zhao, J. L. Bada, Determination of α -dialkylamino acids and their enantiomers in
408 geological samples by high-performance liquid chromatography after derivatization with a
409 chiral adduct of *o*-phthaldialdehyde, *J. Chromatogr. A* 690 (1995) 55-63.

410 [25] V. J. K. Svedas, I. J. Galaev, I. L. Borisov, I. V. Berezin, The interaction of amino acids
411 with *o*-Phthaldialdehyde: a kinetic study and spectrophotometric assay of the reaction
412 product, *Anal. Biochem.* 101 (1980) 188- 195.

413 [26] Regulation (EC) No 1272/2008 of the European Parliament and of the council of 16
414 December 2008 on classification, labelling and packaging of substances and mixtures,
415 amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation
416 (EC) No 1907/2006.

417 [27] R. Pearlman, J. Y. Wang, Formulation, characterization, and stability of protein drugs:
418 case histories, *Pharmaceutical Biotechnology Vol. 9*, Plenum Press, New-York, 1996.

419 [28] AFNOR, XP T 90-210, Protocole d'évaluation d'une méthode alternative d'analyse
420 physico-chimique quantitative par rapport à une méthode de référence, 1999.

421

422

423

424 **Figure captions**

425 Fig. 1: Fluorescence intensity of OPA-NAC-ammonium adduct at $\lambda_{\text{ex}}=335$ nm / $\lambda_{\text{em}}=455$ nm
426 as a function of reaction time and OPA/NAC concentration. $[\text{NH}_4^+]$: 50 μM ; borate buffer
427 pH=10.5.

428 Fig. 2: Fluorescence intensity of OPA-NAC-ammonium adduct at $\lambda_{\text{ex}}=415$ nm / $\lambda_{\text{em}}=485$ nm
429 as a function of pH and reaction time. [OPA]: 8 mM; [NAC]: 8 mM; $[\text{NH}_4^+]$: 100 μM ; borate
430 buffer.

431 Fig. 3: Fluorescence intensity ($\lambda_{\text{ex}}=415$ nm / $\lambda_{\text{em}}=485$ nm) of an ammonium standard adduct
432 (100 μM) over a period of 60 days depending on the reducing agent used. [OPA]: 8 mM;
433 [NAC]: 8 mM; carbonate buffer pH=10.5.

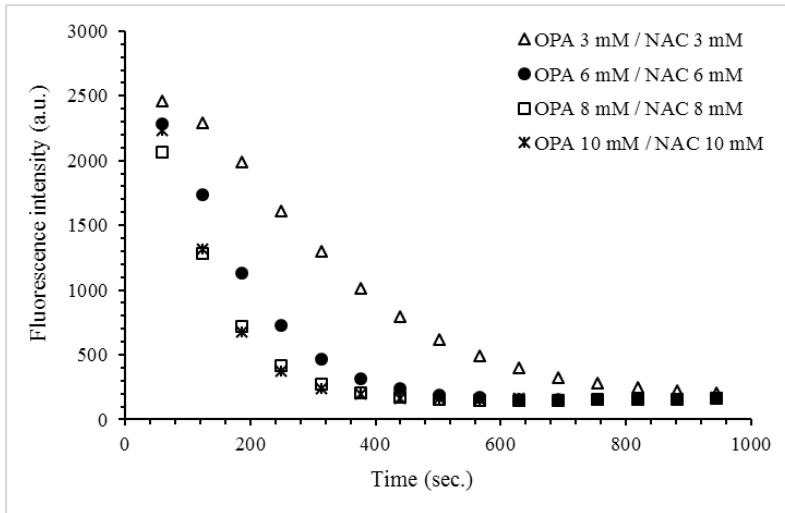
434 Fig. 4: Evolution of fluorescence intensity ($\lambda_{\text{ex}}=415$ nm / $\lambda_{\text{em}}=485$ nm) of an ammonium
435 standard adduct (100 μM) over a period of 70 days as a function of TCEP concentration.
436 [OPA]: 8 mM; [NAC]: 8 mM; carbonate buffer pH=10.5.

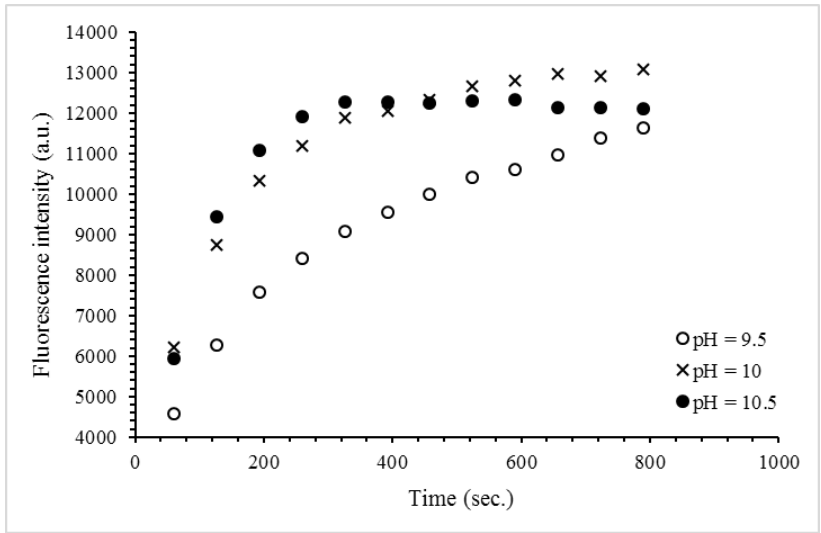
437 Fig. 5: Evolution of the slope of calibration curves over time depending on conservation mode
438 of reagents. A: OPA and NAC mixed reagent; B: OPA and NAC separated liquid reagents; C:
439 solid NAC and liquid OPA. Confidence intervals (CI) are represented in dotted lines. [OPA]:
440 8 mM; [NAC]: 8 mM; [TCEP]: 1 mM; carbonate buffer pH=10.5.

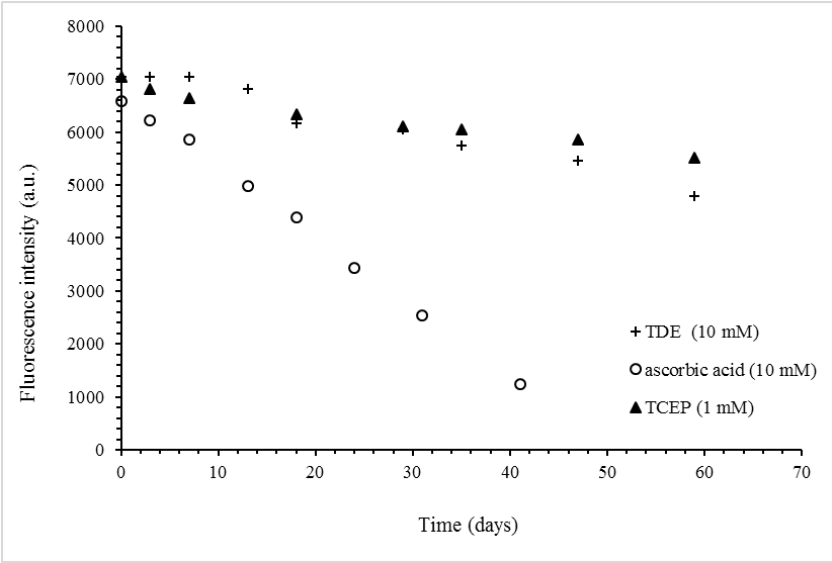
441 Fig. 6: Screening of biogenic amines and primary amino acids (25 μM). [OPA]: 8 mM;
442 [NAC]: 8 mM; [TCEP]: 1 mM; carbonate buffer pH=10.5.

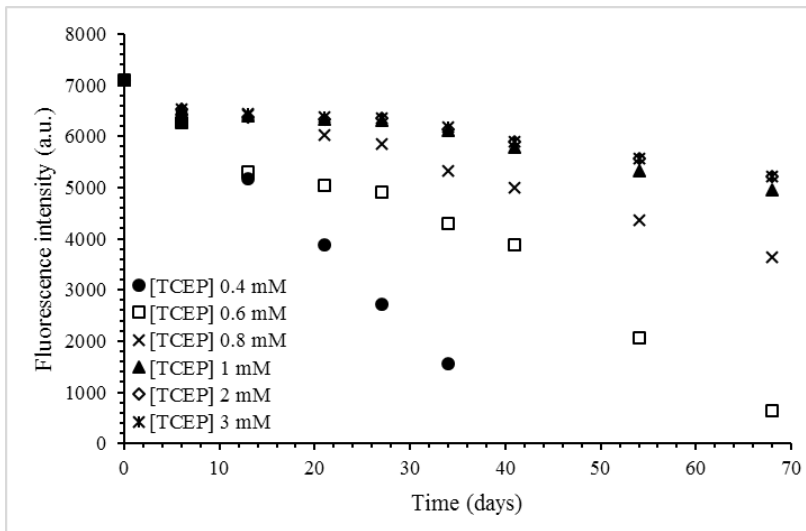
443 Fig. 7 : Regression line for comparison of analytical results between Microplate Assay and
444 Ion Chromatography for ammonium determination.

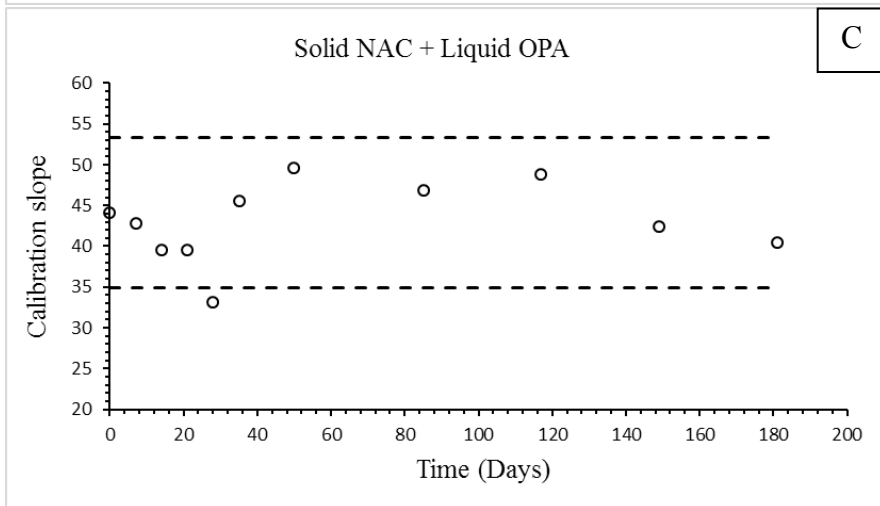
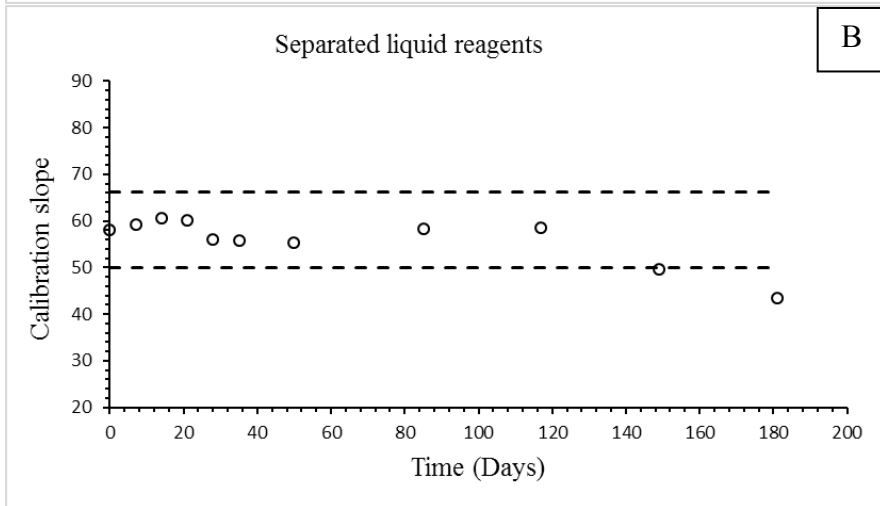
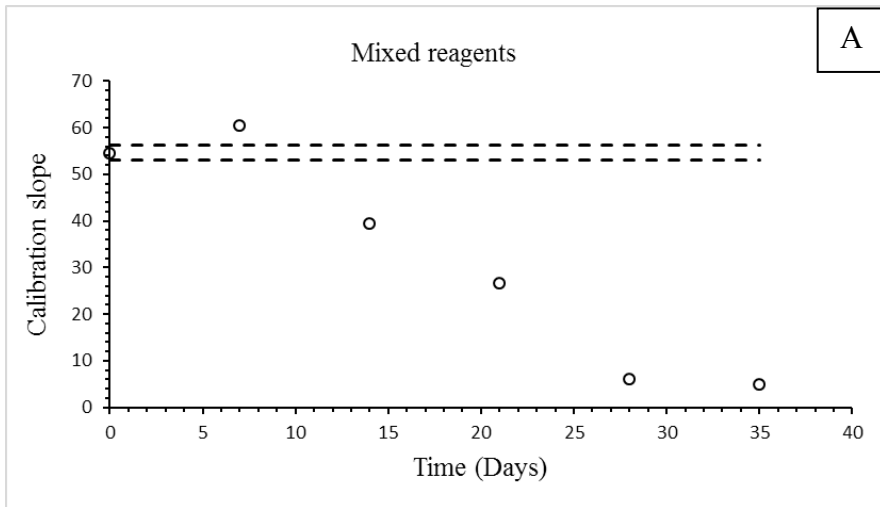
445 Fig. 8 : Regression line for comparison of analytical results between Microplate Assay and
446 Ionic Exchange Chromatography for primary amines determination.

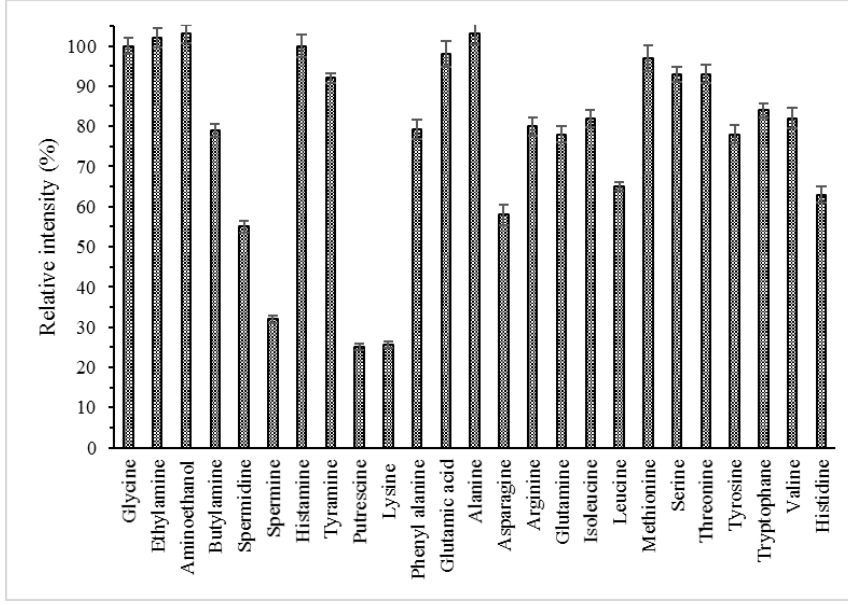


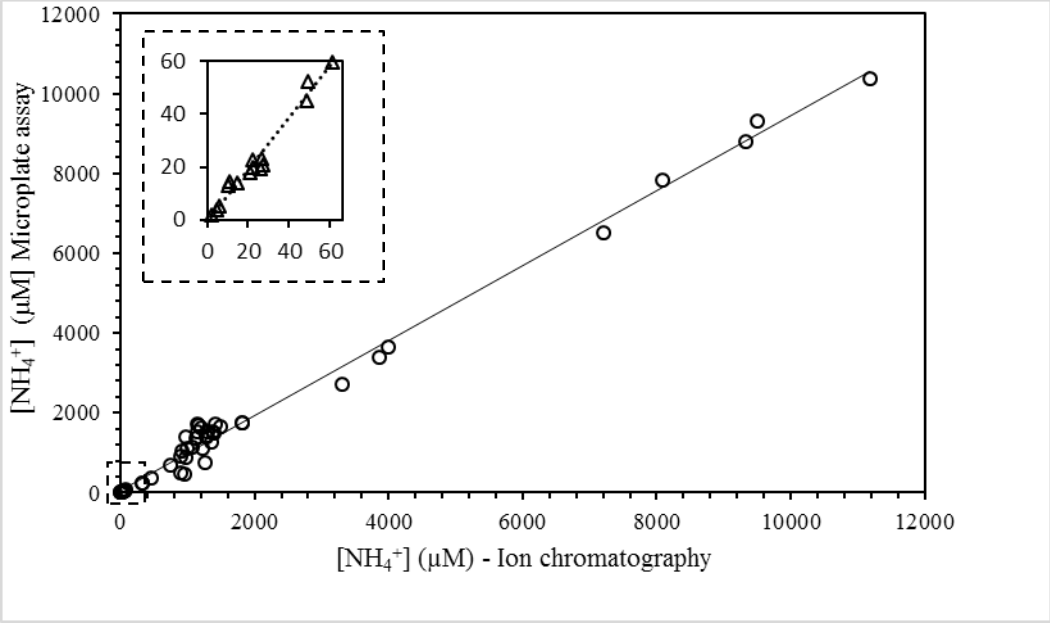


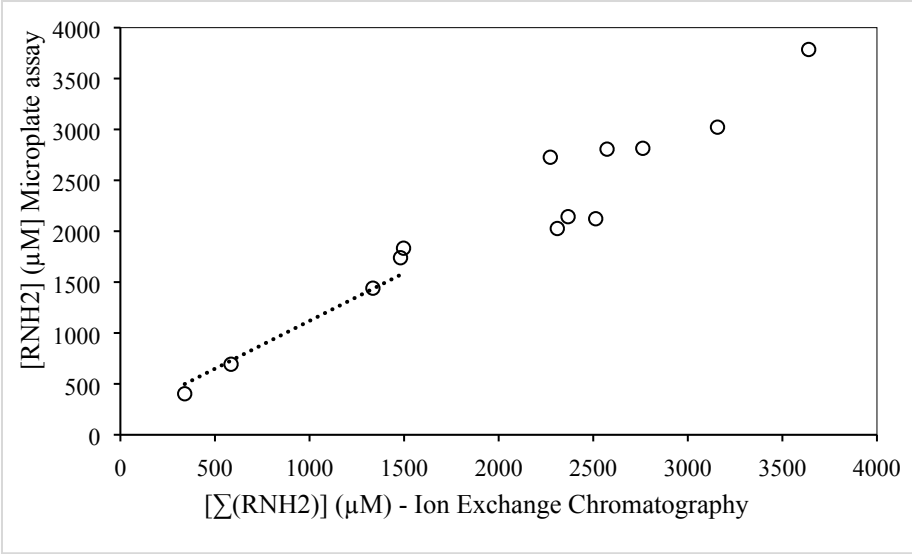












HIGHLIGHTS

- An improved spectrofluorimetric method for the simultaneous and direct determination of ammonium and primary amine compounds is presented.
- Reagents ratio has been optimized in order to suppress known cross-interferences.
- Optimization of reagents formulation led to high reagents stability for routine analysis.
- The method was validated on real samples (wine samples, compost extracts or wastewater).