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Soil quality — Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates

Qualité du sol — Mesure de l'activité enzymatique dans des échantillons de sol en utilisant des substrats colorimétriques

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

Introduction

Microorganisms are responsible for many key processes in the cycle of elements. Enzymes are responsible for the degradation of organic molecules and their mineralization. The main postulate is the microbial origin of soil enzymes, even if plant root exudates include enzymes. Extracellular enzymes in soil play key roles in the biodegradation of organic macromolecules. The simultaneous monitoring of several enzyme activities important in the biodegradation of organic compounds and mineralization of carbon, nitrogen, phosphorus and sulfur in soil may reveal harmful effects caused by chemicals and other anthropogenic impacts. However, the measurements carried out under selected laboratory conditions using artificial substrates cannot be a substitute for the actual rate of enzymatic processes in soil *in situ*.

Soil quality — Measurement of enzyme activity patterns in soil samples using colorimetric substrates in micro-well plates

1 Scope

This document specifies a method for the measurement of several hydrolase activities (arylamidase, arylsulfatase, *β*-galactosidase, *α*-glucosidase, *β*-glucosidase, N-acetyl-glucosaminidase, acid, alkaline and global phosphatases, urease) simultaneously (or not) in soil samples, using colorimetric substrates. Enzyme activities of soil vary seasonally and depend on soil chemical, physical and biological characteristics. This method can be applied either to detect harmful effects on soil enzyme activities derived from toxic substances or other anthropogenic agents in contaminated soils against a control soil, or to test chemicals.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

3 Terms and definitions, symbols and abbreviated terms

3.1 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at https://www.iso.org/obp

3.2 Symbols and abbreviated terms

ARN	Arylamidase
ARS	Arylsulfatase
E.C.	Enzyme code number by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB)
NAG	N-acetyl-glucosaminidase
PAC	acid phosphatase
PAK	alkaline phosphatase
PHOS	phosphatase

URE urease

β–GAL *β*-galactosidase

α–GLU *α*-glucosidase

β–GLU *β*-glucosidase

4 Principle

This document describes a method for the simultaneous measurement of several enzymes in soil samples (see Table 1). It is based on the use of soil samples solutions and colorimetric substrates, which are incubated during specific times at 25 °C ± 2 °C or 37 °C ± 2 °C in multi-well plates. After the incubation, reactions are stopped, plates are then centrifuged and supernatants transferred into new plates. The intensities of the coloration are measured with absorbance with a 96 wells microplate spectrophotometer UV/visible.

Table 1 — Enzymatic activity measurements with colorimetric method

Enzyme	Abbreviation	N°	Soil cycle	Macromolecule degraded
Arylamidase	ARN	E.C. 3.4.11.2	Nitrogen	
Arylsulfatase	ARS	E.C. 3.1.6.1	Sulfur	Mineralization of organic sulfur
β-Galactosidase	βGAL	E.C. 3.2.1.22	Carbon	Hemicellulose
α-Glucosidase	αGLU	E.C. 3.2.1.20	Carbon	Starch and glycogen
β-Glucosidase	βGLU	E.C. 3.2.1.21	Carbon	Cellulose
N-acetyl-glucosaminidase	NAG	E.C. 3.2.1.52	Carbon	Chitin and other *β*-1,4-linked glucosamine polymers
Phosphatase	PHOS	E.C. 3.1.4.1	Phosphorus	Phosphate esters
Acid phosphatase	PAC	E.C. 3.1.4.1	Phosphorus	Phosphate esters
Alkaline phosphatase	PAK	E.C. 3.1.4.1	Phosphorus	Phosphate esters
Urease	URE	E.C. 3.5.1.5	Nitrogen	Urea

An interlaboratory trial was carried out for the validation of the standard; summary of the international ring test is given in Table 8, and the whole data of the interlaboratory validation are described in Annex B.

5 Reactives

5.1 Buffers and reageants

5.1.1 General

The choice is made to use deionized water as medium to evaluate native soil enzyme activities at soil pH and also to allow the analysis of multiple enzymes using the same soil suspension. The soil (in g)/water (in ml) ratio (4:25) is optimized to maximize reaction, sensitivity and facilitate pipetting technique. The use of the same soil solution for analysing multiple enzymes also makes data more comparable. Arylamidase is measured with Tris buffer 50 mmol/l, pH 7,5 and acid and alkaline phosphatases are involved with the use of Tris-HCl 50 mmol/l at pH 5,5 and Tris base 50 mmol/l at pH 11, respectively.

NOTE The volume can be adapted according to needs.

5.1.2 Tris hydrochloride 50 mmol/l pH 5,5 ± 0,1.

— Tris(hydroxymethyl)aminomethane hydrochloride (CAS N°: 1185-53-1 – Mw:157,6): 7,88 g;

— deionized water: 1 000 ml;

— hydrochloric acid (HCl) (CAS N°7647-01-0) 1 mol/l.

Dissolve 7,88 g of Tris(hydroxymethyl)aminomethane hydrochloride into 800 ml deionized water and adjust to pH 5,5 with hydrochloric acid (1 mol/l). Fill in to 1 000 ml. The storage duration shall not exceed one month at 4 °C ± 2 °C in glass or polypropylene bottle.

5.1.3 Tris base 50 mmol/l pH 11 ± 0,1.

— Tris(hydroxymethyl)aminomethane (CAS N°: 77-86-1 - Mw:121,14): 6,06 g;

— deionized water: 1 000 ml;

— sodium hydroxide (CAS N° 1310-73-2) (1 mol/l).

Dissolve 6,06 g of Tris(hydroxymethyl)aminomethane into 800 ml deionized water and adjust to pH 11 with sodium hydroxide (1 mol/l). Fill in to 1 000 ml. The storage duration shall not exceed one month at 4 °C ± 2 °C.

5.1.4 Tris base 50 mmol/l pH 7,5 ± 0,1.

— Tris(hydroxymethyl)aminomethane (CAS N°: 77-86-1 - Mw:121,14): 6,06 g;

— deionized water: 1 000 ml;

— hydrochloric acid (HCl) (CAS N°7647-01-0) 1 mol/l.

Dissolve 6,06 g of Tris(hydroxymethyl)aminomethane into 800 ml deionized water and adjust to pH 7,5 with hydrochloric acid (1 mol/l). Fill in to 1 000 ml. The storage duration shall not exceed one month at 4 °C ± 2 °C.

5.1.5 Tris base 100 mmol/l pH 12 ± 0,1.

— Tris(hydroxymethyl)aminomethane (CAS N°: 77-86-1 - Mw:121,14): 12,11 g;

— deionized water: 1 000 ml;

— sodium hydroxide (CAS N° 1310-73-2) (5 mol/l).

Dissolve 12,11 g of Tris(hydroxymethyl)aminomethane into 800 ml deionized water and adjust to pH 12 with sodium hydroxide (5 M). Fill in to 1 000 ml. The storage duration shall not exceed one month at 4 °C ± 2 °C.

5.1.6 Calcium chloride dihydrate 0,5 mol/l.

— calcium chloride dihydrate (CAS N°: 10035-04-8 - Mw:147,01): 14,7 g;

— Deionized water: 200 ml.

Dissolve 14,7 g of calcium chloride dihydrate in 200 ml of deionized water. The storage duration shall not exceed one month at 4 °C ± 2 °C.

5.1.7 Salicylate reagent.

— sodium salicylate 270 mmol/l (CAS N°: 54-21-7 - Mw:160,1): 865 mg;

— tri sodium citrate 145 mmol/l (CAS N°: 6132-04-3 - Mw:294,1): 853 mg;

— di sodium tartrate 60 mmol/l (CAS N°: 6106-24-7 - Mw:230,08): 276 mg;

— sodium nitroferricyanide 2 mmol/l (CAS N°: 13755-38-9 - Mw:297,95): 12 mg;

— deionized water: 20 ml.

Salicylate reagent is prepared with the 4 compounds listed above just before analysis; dissolve 865 mg of sodium salicylate, 853 mg of tri sodium citrate, 276 mg of di sodium tartrate and 12 mg of sodium nitroferricyanide in 20 ml of deionized water.

5.1.8 Cyanurate reagent.

— tri sodium citrate 580 mmol/l (CAS N°: 6132-04-3 - Mw:294,1): 3,4 g;

— di sodium tartrate 90 mmol/l (CAS N°: 6106-24-7 - Mw:230,08): 414 mg;

— lithium hydroxide 280 mmol/l (CAS N° : 1310-65-2 - Mw:23,95): 134 mg;

— dichloroisocyanurate 10 mmol/l (CAS N° : 51580-86-0 - Mw:255,98): 51 mg;

— deionized water: 20 ml.

Cyanurate reagent is prepared with the 4 compounds listed above just before analysis; dissolve 3,4 g of tri sodium citrate, 414 mg of di sodium tartrate, 134 mg of lithium hydroxide and 51 mg of dichloroisocyanurate in 20 ml of deionized water.

5.1.9 Ethanol, 96 %.

— Ethanol 96 % (CAS N° 41340-36-7).

5.1.10 Acidified ethanol (0,26 mol/l HCl).

— Hydrochloric acid ACS reagent, 37 % (CAS N°7647-01-0) 4,32 ml;

— Ethanol 96 % (CAS N° 41340-36-7).

Dilute 4,32 mL of concentrated HCl into 200 ml ethanol 96 %. The storage duration shall not exceed one month at 4 °C ± 2 °C.

5.1.11 p-dimethylaminocinnamaldehyde (DMCA) (3,5 mmol/l).

— DMCA (CAS N°: 6203-18-5 - Mw:175,23): 0,12 g;

— Ethanol 96 % (CAS N° 41340-36-7).

Dissolve 0,12 g of DMCA into 200 ml ethanol 96 %. The storage duration shall not exceed one week at –20 °C ± 2 °C.

Table 2 — Buffer utilization for enzymatic activity measurement

	ARS; α-GLU; β-GLU; β-GAL; NAG; PHOS;	ARN	URE	PAC	PAK
Soil solution	deionized water	Tris base 50 mmol/l, pH 7,5	deionized water	Tris HCl 50 mmol/l, pH 5,5	Trisbase 50 mmol/l, pH 11
Stop/ revelation	Tris 100 mmol/l pH12	Ethanol 96 % Acidified ethanol	salicylate reagent	Tris 100 mmol/l pH 12	
	$CaCl_2$ 0,5 mol/l	DMCA	cyanurate reagent	$CaCl_2$ 0,5 mol/l	

5.2 Substrates and standards

5.2.1 Preparation of standard solutions

5.2.1.1 Para-nitrophenol (CAS N°: 100-02-7 - PNP) at 3,6 mmol/l.

— para-nitrophenol (PNP) (Mw:139,11): 10 mg;

— deionized water: 20 ml.

PNP as a powder should be stored at −20 °C ± 2 °C and protected from light. Weigh PNP carefully and dissolve it into deionized water. Working concentration is 0,36 mM (i.e. dilution of the concentrated solution 1/10). The storage of the concentrated and working concentrations shall not exceed two years at −20 °C ± 2 °C. Solutions could be aliquoted for one use or maximum 3 freeze/defreeze cycles.

NOTE Paranitrophenol can cause damage to organs through prolonged or repeated exposure if swallowed (H373) and harmful if swallowed, in contact with skin or if inhaled (H302, H312, and H332). Appropriated ventilation and protections need to be used.

5.2.1.2 Ammonium chloride (NH_4Cl) at 62 mmol/l.

— ammonium chloride (CAS N°: 12125-02-9 - Mw:53,49): 66,4 mg;

— deionized water: 20 ml.

Ammonium chloride as a powder can be stored at room temperature and protected from light. Weigh ammonium chloride carefully and dissolve it into water. The concentrated solution should be stored at −20 °C ± 2 °C for two years. The storage of the concentrated solution shall not exceed two years at −20 °C ± 2 °C. Working concentration is 0,62 mmol/l (dilution 1/100). The storage of the working concentration shall not exceed two years at −20 °C ± 2 °C.

5.2.1.3 *β*-naphthylamine, 1 mmol/l.

— *β*-naphthylamine (CAS N°: 91-59-8 - Mw:143,19): 35,8 mg;

— ethanol 96 %: 20 ml;

— deionized water.

Dissolve 35,8 mg of *β*-naphthylamine into 50 ml ethanol 96 % (0,071 %). This 5 mmol/l stock solution shall be stored at −20 °C for one year. Working concentration is 1 mmol/l (dilution 1/5 in water) and shall be stored at −20 °C ± 2 °C for one year.

NOTE β-Naphtylamine has acute toxicity (oral, dermal, inhalation), category 4, respiratory sensitization, category 1, and hazardous to the aquatic environment. Eyeshields[1)], full-face particle respirator type N100 (US)[2)], appropriated ventilation, specific gloves[3)], glasses and protective screen need to be used.

5.2.2 Preparation of substrate solutions

Commercially available colorimetric substrates are delivered as powders that should be stored, according to the specifications, frozen at −20 °C ± 2 °C or cooled at 4 °C ± 2 °C or stored at room

1) https://www.sigmaaldrich.com/labware/labware-products.html?TablePage=20009868

2) http://www.sigmaaldrich.com/catalog/search?interface=Substance&term=msaadvantageseries1000fullfacerespirator1234598765+OR+msaultratwinfullfacerespirator1234598765&focus=product&mode=boolean

3) http://www.sigmaaldrich.com/labware/labware-products.html?TablePage=9577418

temperature (RT). All the substrates should be prepared in advance according to the requirements of Table 3.

NOTE The volume needs to be sufficient for reliable weighing and measurement of substrates. It depends also on the number of plates needed. Examples are given in Table 3.

Table 3 — Colorimetric substrates available commercially for enzymatic activity measurements and their preparation for measures

Enzyme	Substrate[a] (Storage temperature)	Molar mass g/mol	Concentration mol/l	Examples of preparation	Storage of solutions Temperature, duration
Arylamidase	L-leucine β-naphthylamide hydrochloride (–20 °C ± 2 °C) CAS N°: 893–36–7	292,8	0,008	Dissolve 0,2342 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year
Arylsulfatase	Potassium 4-nitrophenyl sulfate (–20 °C ± 2 °C) CAS N°: 6217–68–1	257,26	0,025	Dissolve 0,322 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year
β-Galactosidase	p-nitrophenyl β-D-galactopyranoside (–20 °C ± 2 °C) CAS N°: 3150–24–1	301,25	0,05	Dissolve 0,753 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year
α-Glucosidase	4-nitrophenyl α-D-glucopyranoside (–20 °C ± 2 °C) CAS N°: 3767–28–0	301,25	0,025	Dissolve 0,375 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year
β-Glucosidase	4-nitrophenyl β-D-glucopyranoside (–20 °C ± 2 °C) CAS N°: 2492–87–7	301,25	0,05	Dissolve 0,753 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year

[a] Sigma-Aldrich is an example of producer of colorimetric substrates. This information is given for the convenience of users of this international standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

NOTE All Substrates need to be manipulated with appropriated ventilation, specific gloves, and protective screen.

Table 3 *(continued)*

Enzyme	Substrate[a] (Storage temperature)	Molar mass g/mol	Concentration mol/l	Examples of preparation	Storage of solutions Temperature, duration
N-acetyl-glucosaminidase	para-nitrophenyl N-acethyl β-D glucopyranoside (–20 °C ± 2 °C) CAS N°: 3459–18–5	342,31	0,01	Dissolve 0,171 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year
Phosphatase Acid phosphatase and Alkaline phosphatase	4-nitro-phenylphosphate disodium salt hexahydrate (+4 °C ± 2 °C) CAS N°: 333338–18–4	371,12	0,05	Dissolve 0,928 g in 50 ml of deionized water	–20 °C ± 2 °C 1 year
Urease	Urea > 98 % (RT) CAS N°: 57–13–6	60,06	0,4	Dissolve 0,480 5 g in 20 ml of deionized water	+4 °C 1 week

[a] Sigma-Aldrich is an example of producer of colorimetric substrates. This information is given for the convenience of users of this international standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead to the same results.

NOTE All Substrates need to be manipulated with appropriated ventilation, specific gloves, and protective screen.

6 Apparatus and materials

In addition to usual laboratory equipment the following materials are required:

6.1 Sieves, with grid size 2 mm, up to 5 mm according to soil texture and humidity.

NOTE Other grid sizes can be used depending on the soil texture.

6.2 Balance (±0,001 g).

6.3 Orbital shaker.

6.4 Multi-well plates, 96 wells, polystyrene, with flat bottoms but without treatment, with lids.

6.5 Automatic dispenser for water (optional).

NOTE Compared with manual pipetting, an automatic dispenser decreases the uncertainty of volumes dispensed significantly.

6.6 Multichannel micropipettes (50 µL and 200 µL).

6.7 Incubators, set at 25 °C ± 2 °C and 37 °C ± 2 °C.

6.8 Plate centrifuge, with temperature regulation at 20 °C and acceleration of 1 500 g.

6.9 Plate reading spectrophotometer, with reading at 405 nm ± 5 nm, 540 nm ± 5 nm and 650 nm ± 5 nm (monochromator or BP ± 10 nm).

7 Procedure

7.1 Establishment of calibration curves

7.1.1 General

Calibration curves require several concentrations of para-nitro phenol (PNP), ammonium chloride (NH_4Cl) or *β*-naphthylamine, at least in duplicates, preferably in triplicates; all the volumes are given per well. Homogenizations are realized with a micropipette and 2 or 3 aspirations/ejections. Exposure to light shall be limited during preparation of standard curves. Examples of calibration curves are given in Annex B.

7.1.2 Solution of PNP

The stock solution of PNP with a concentration of 0,36 mmol/l is used to establish the calibration curve. Distribute the volumes needed into multi-well plate wells for concentrations 0 nmol/ml, 14 nmol/ml, 29 nmol/ml, 72 nmol/ml, 140 nmol/ml, 220 nmol/ml, 290 nmol/ml and 360 nmol/ml in replicate (Table 4).

Table 4 — Preparation of calibration curve of para-nitrophenol in 96 wells microplate

[PNP] nmol/ml	0	14	29	72	140	220	290	360
PNP (µl)	0	5	10	25	50	75	100	125
Water (µl)	125	120	115	100	75	50	25	0

To reveal yellow coloration of PNP, 25 µl of water, 25 µl of calcium chloride and 100 µl of basic Tris base 100 mmol/l pH 12 are added. After homogenization, 200 µl are transferred in new plates and reading of absorbance is realized with a microplate spectrophotometer at 405 nm.

7.1.3 Solution of *β*-naphthylamine

The working solution of β-naphthylamine with a concentration of 1 mmol/l, is used to produce the standard curve. Distribute the volumes needed into multi-well plate wells for concentrations 0 nmol/ml, 10 nmol/ml, 20 nmol/ml, 50 nmol/ml, 100 nmol/ml, 200 nmol/ml (Table 5).

Table 5 — Preparation of calibration curve of *β*-naphthylamine in 96 wells microplate

[*β*-Naphthylamine] nmol/ml	0	10	20	50	100	200
β-Naphthylamine (µl)	0	1	2	5	10	20
Tris buffer 50 mmol/l pH 7,5 (µl)	50	49	48	45	40	30
Ethanol 96 % (µl)	50	50	50	50	50	50

To reveal coloration of *β*-naphthylamine, 100 µl acidified ethanol and 100 µl of p-dimethylaminocinnamaldehyde (DMCA) are added and mixed. After 20 min incubation in the dark at room temperature, the absorbance is read with a microplates spectrophotometer UV/visible at 540 nm.

7.1.4 Solution of ammonium chloride

The stock solution of ammonium chloride with a concentration of 62 mmol/l is diluted 1/100 and working solution with 0,62 mmol/L (620 nmol/ml) is used to produce the standard curve. Distribute the volumes needed into multi-well plate wells for concentrations 0 nmol/ml, 6 nmol/ml, 16 nmol/ml, 31 nmol/ml, 62 nmol/ml, 155 nmol/ml, 233 nmol/ml and 310 nmol/ml in replicate (Table 6).

Table 6 — Preparation of calibration curve of ammonium chloride in 96 wells microplate

[NH_4Cl] nmol/ml	0	6	16	31	62	155	233	310
NH_4Cl (µl)	0	2	5	10	20	50	75	100
Water (µl)	200	198	195	190	180	150	125	100

To quantify NH_4Cl concentration, 40 µl of the salicylate reagent are added to each well. After a 3 min reaction period, 40 µl of cyanurate reagent are dispensed into each well, and each well homogenized then incubated in dark for 30 min (absorbance is stable for two hours). After incubation, plates are homogenized with a micropipette (2 or 3 aspirations/ejections) and 200 µl are transferred in new plates and the reading of absorbances is realized with a microplates spectrophotometer UV/visible at 650 nm.

7.2 Sampling

7.2.1 Sample preparation

7.2.1.1 Homogenization

Take and handle soil samples shall be as specified in ISO 18400-206. A composite sample taken from the field, homogenized and sieved through 2 mm mesh (up to 5 mm according to soil texture and humidity), has been observed to yield reasonably low uncertainty of measurement for soil samples.

To improve the link between *in situ* activities, samples should be stored at 15 °C ± 2 °C until four days. But, if it is not possible, weakest modifications will occur with storage at −80 °C ± 5 °C. The storage at −20 °C ± 2 °C before or after sieving is not suitable.

The sieved soil is homogenized, and triplicate of exactly 4 g are weighed and deposited into flat bottom flask (30 to 60 ml). Twenty-five ml of deionized water and cross shaped stirring bars are added. Containers are closed and homogenized for 10 min on orbital agitator (250 min^{-1}).

7.2.1.2 Preparation of sample plates

Cross shaped stir bars cross are added in flat bottom flask and soil suspension shall stay under stirring during pipetting. Use multichannel micropipettes (one to three channels) to distribute each soil suspension in microplates, in four replicate wells, with a specific volume according to Table 7 (part A), and place the lid on each plate. Three wells are analytical points and one well is the blank (to reveal chemical interactions with soil compounds). Prepare a same number of plates as enzyme tested (one plate per one enzyme).

NOTE 1 An example of plate schedule is described in Figure 1.

NOTE 2 There is a possibility that humic soils necessitate the use of enlarged cones for the pipetting.

S1a	S1b	S1c	S2a	S2b	S2c	S3a	S3b	S3c	S4a	S4b	S4c
S1a	S1b	S1c	S2a	S2b	S2c	S3a	S3b	S3c	S4a	S4b	S4c
S1a	S1b	S1c	S2a	S2b	S2c	S3a	S3b	S3c	S4a	S4b	S4c
S1at	S1bt	S1cT	S2aT	S2bT	S2cT	S3aT	S3bT	S3cT	S4aT	S4bT	S4cT
S4a	S4b	S4c	S5a	S6b	S6c	S7a	S7b	S7c	S8a	S8b	S8c
S4a	S4b	S4c	S5a	S6b	S6c	S7a	S7b	S7c	S8a	S8b	S8c
S4a	S4b	S4c	S5a	S6b	S6c	S7a	S7b	S7c	S8a	S8b	S8c
S4at	S4bt	S4cT	S5aT	S6aT	S6cT	S7aT	S7bT	S7cT	S8aT	S8bT	S8cT

Key

S1 sample number

a, b, c triplicate of sample

T control well for each soil

Figure 1 — Example of sample organization in plates for each enzyme

7.2.2 Addition of substrate

Simple organization would occur if one plate is used for one enzyme. When all samples are distributed in plates, use multichannel micropipettes to distribute each substrate, in three replicates and homogenized with 2 or 3 aspirations/ejections, and incubate the multi-well plates (Table 7 part A). After incubation, reactions are stopped, plates are centrifuged 5 min at 1 500 g and the supernatants are transferred in new plates. Then, absorbance is measured on a spectrophotometer for microplates. Table 7 part B summarizes protocols for each enzyme measurement.

Table 7 — Specific experimental details for enzyme activity measurements

A INCUBATION	**ARN**	**ARS**	***β*-GAL**	***α*-GLU**	***β*-GLU**	**NAG**	**PHOS PAC PAK**	**URE**
Soil solution (µl)	125							50
Substrate (µl)	25							40
Deionized water (µl)	0							150 (+40 µl in control wells)
Incubation time	2H	4H	2H	1H	1H	1H	0H30	3H
Incubation temperature	37 °C							25 °C
B STOP REACTION								

Table 7 *(continued)*

A INCUBATION	ARN	ARS	β-GAL	α-GLU	β-GLU	NAG	PHOS PAC PAK	URE
Stop reaction	In each well: 150 µl Ethanol 96 % homogenization	In each well: 25 µl $CaCl_2$ 100 µl Trizma pH 12 homogenization						In each well: 40 µl salicylate reagent 40 µl cyanurate reagent homogenization
Substrate	25 µl in control wells	25 µl in control wells						
Incubation								30 min, RT, in dark
Centrifugation	5 min, 1 500g							
Transfer	100 µl	200 µl						
Revelation	100 µl acidified ethanol 100 µl DMCA 20 min, RT, in dark							
Reading	λ = 540 nm	λ = 405 nm						λ = 650 nm

NOTE 1 Incubation temperature affects reaction rates and optimum depends on the enzymes. For specific purposes, a different temperature as described in this document can be used, e.g. *in situ* temperature.

NOTE 2 For urease activity, it is necessary to quantify free ammonium brought by urea solution and subtract it from ammonium quantified after soil incubation to obtain specific ammonium released by urease. Urea control is prepared with 40 µl urea, 200 µl of deionized water, 40 µl salicylate reagent and 40 µl cyanurate reagent. After 30 min of incubation, 200 µl are transferred into new wells and absorbances are reading at 650 nm.

After incubation, the reactions are stopped according to enzyme specific protocols (Table 7).

7.2.3 Absorbance measurements

7.2.3.1 Measurements of enzyme activities with PNP as reaction product (ARS, β-GAL, α-GLU, β-GLU, NAG, PHOS, PAC, PAK)

The addition of 25 µl of calcium chloride and 100 µl of basic Tris buffer pH 12 stops the reaction, and it is the key action to eliminate colloids which introduce turbidity in solutions and increase pH for PNP quantification. Add 25 µl of substrate in control wells and homogenise with 2 or 3 aspirations/rejections. Furthermore, plates are centrifuged five minutes at 1 500 g and 20 °C, and 200 µl of the supernatant transferred in a new plate. The reading of absorbance is realized with a microplates spectrophotometer UV/visible at λ = 405 nm.

7.2.3.2 Measurements of arylamidase activities (ARN)

After incubation, add 150 µl ethanol 96 % in each well (including controls) and 25 µl substrate solution in control wells. The plate is centrifuged 5 min at 1500 g and 100 µl of supernatant are transferred into a new plate. To reveal the quantity of naphthylamine produced, 100 µl of acidified ethanol and 100 µl of DMCA were added in all wells and homogenized with a micropipette (2 aspirations/ejections). After 20 min, reading of absorbance is realized with a microplates spectrophotometer UV/visible at 540 nm.

7.2.4 Measurements of urease activities

After incubation of 3 h, 40 µl of the salicylate reagent are added to each well, including controls. After a 3 min reaction period, 40 µl of cyanurate reagent is dispensed into each well including controls. Colorimetric reaction is achieved in 30 min and stable for two hours. Furthermore, plates are centrifuged five minutes at 1 500 g and 20 °C, and 200 µl of the supernatant are transferred in a new plate. The reading of absorbance is performed with a microplate spectrophotometer UV/visible at λ = 650 nm.

8 Calculation of results

The standard curve is plotted for PNP or β-naphthylamine and ammonium chloride nanomolar concentration (nmol/ml) versus absorbance. The PNP or β-naphthylamine or ammonium chloride concentration of blank (C_b) and sample (C_s) are read from the standard curve.

Examples of calibration curves are given in Annex B.

The result is calculated by subtracting measurement of blank from triplicate of sample and multiplying the difference with dilution factor (D) and soil volume (V_{ss}), and dividing with reaction time and dry mass of sample (W_{ds}).

$$A = \frac{(C_s - C_b) \times D \times V_{ss}}{RT \times W_{ds}} \quad (1)$$

where

A *is* the enzymatic activity in mU/g of dry sample (nmol/min/g of dry sample);

C_s is the concentration of product formed in sample (nmol/ml);

C_b is the concentration of product formed in blank (nmol/ml);

D is the dilution of sample in microplate;

V_{ss} is the volume of sample solution (ml);

RT is the reaction time (min);

W_{ds} is the mass of dry sample (g).

9 Expression of results

Results are expressed as milliunit for one gram of dry soil corresponding to nmole of PNP, β-naphthylamine or ammonium chloride released per minute and g soil dry mass of sample. The measurements necessary for each expression, percentage of dry matter determination shall be carried out.

NOTE The more practical and commonly unit used for enzymatic activity is Units (U) = 1 µmol min^{-1}. For soil, activity expressed as milliunit for one gram of dry soil (mU/g DS).

Soil characteristics vary widely due to geography, climate and land use. The interpretation of results cannot, presently, be based on set limit values for each enzyme activity. The experimental design shall facilitate comparisons with a control soil or between samples from relevant sites.

10 Validity criteria

a) The standard curve is valid if the following criteria are met (according to the results of the ring test summarized in Annex B):

— $r^2 \geq 0{,}990$;

— the absorbance for PNP standard curve range is within [0,04 - 1,8] ± 10 %;

— the absorbance for β-naphtylamine standard curve range is within [0,06 - 2,3] ± 10 %;

— the absorbance for ammonium standard curve range is within [0,05 - 1,6] ± 10 %.

b) For the samples:

— Triplicates % CV shall be ≤ 15 %;

— Urea blank absorbance shall be ≤ 0,2;

— For urease measurements, sample blank shall be ≤ 1;

— Differences of absorbance for sample assay and blank shall be ≥ 0,02 to 0,05 according to spectrophotometer sensibility and repeatability.

11 Interlaboratory validation

Summary of the international ring test is given in Table 8, and the whole data of the interlaboratory validation are described in Annex B.

Table 8 — synthetic data of interlaboratory study

	α-GLU	β-GLU	PHOS	PAC	PAK	ARS	β-GAL	NAG	URE	ARN
CV_r	4,0 to 27,7 %	2,4 to 15,2 %	4,2 to 9,7 %	4,3 to 13,0 %	3,8 to 14,1 %	2,7 to 10,4 %	3,9 to 18,4 %	5,4 to 23,3 %	5,5 to 16,2 %	4,3 to 13,1 %
CV_R	9,7 to 17,9 %	9,5 to 24,0 %	27,4 to 36,5 %	12,6 to 23,0 %	5,7 to 23,8 %	15,5 to 30,5 %	21.7 to 32,6 %	9,8 to 19,6 %	14,0 to 35,9 %	15,9 to 31,9 %

(Abbreviations: CVr: range of variability of the mean intralaboratory coefficients of variation for all laboratories (calculated for each activity considering all soils and a single laboratory). CV_R: range of variability of the mean interlaboratory coefficients of variation for all soils (calculated for each activity considering all laboratories and a single soil)

12 Test report

The test report shall include following information:

a) a reference to this document, i.e. ISO 20130:2018;

b) adequate identification of the sample;

c) details of storage temperature and duration;

d) type of soil, soil physical and chemical characterization, water content, soil temperature at sampling time (pH value of the soil sample);

e) incubation conditions applied;

f) test results;

g) any detail not specified in this document or which are optional, as well as any effect which may have affected the results.

Annex A
(informative)

Validation of the method and intralaboratory tests for evaluating soil enzymatic activities with colorimetric method

A.1 Aim

Evaluate validity of the method described in this document to assess the soil enzymatic activities in microplates with colorimetric methods. The validation is performed with comparison tests for specificity, sensibility, linearity, specific operating parameters and the robustness with the operator effect.

A.2 Background

The intralaboratory test is realized in ECOSYS unit, Plateforme Biochem-Env, INRA, France.

A.3 Test materials and methodology

Effect of sieving were tested on PHOS, GLU, ARS, GAL, NAG, URE and ARN activities, on 4 soils (1 sandy soil, 1 clay loamy soil, 2 silt loam soils).

Effect of pH reaction: 2 soils were tested for each enzyme activity and prepared in water or in Tris buffer 50 mmol/l, pH[5-9].

Incubation time effect: For each enzyme, 4 incubations times are chosen to evaluate the robustness of the method according to Table A.1.

Table A.1 — Range of incubation time tested on enzymatic activities

Enzymes	ARN	ARS	β-GAL	α-GLU	β-GLU	NAG	PHOS-PAC-PAK	URE
Incubation times (min)	30–60–120–180	60–120–180–240	60–120–180–240	30–60–90–120	30–60–90–120	30–60–90–120	15–30–45–60	60–120–180–240

Operator effect: 3 soils were analysed by four different persons for each enzyme to evaluate operator effect.

Specificity for each enzyme is testing on a clay loamy soil with or without autoclaving.

Validation parameters: linearity of product detection: Standard curve are realized with PNP (0 µmol/l to 600 µmol/l), ammonium chloride (0 µmol/l to 600 µmol/l) and β-naphtylamine (0 µmol/l to 300 µmol/l).

Linearity of soil response: soil solutions with 0,5 g to 12 g of soils and 25 ml of distilled water were used for enzymatic activities measurements.

Limits of quantification and precision: dispersion parameters are determined with accuracy profile, which combined ISO 17025 and estimation of the uncertainty of measurements. The methods based on the use of the total error derived from performance criteria such trueness and precision [4, 12]. Four soils (sandy, loamy, clay and humic soils) were analysed in triplicate, with 3 repetitions (repeatability) and 6 series (intermediate precision).

A.4 Data analysis

Robustess of the method

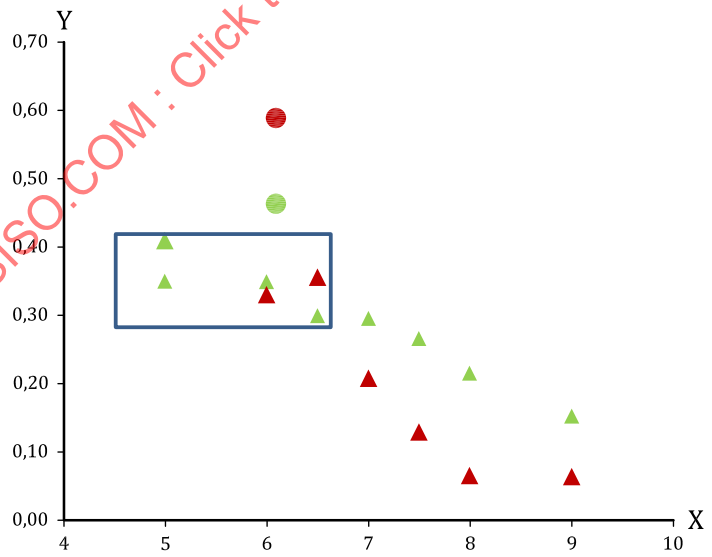
Operator effect: there is no operator effect, whatever enzyme considered.

Effect of sieving: no significate difference is noticed for all enzyme and all soils, except for NAG in two soils (some examples in Table A.2).

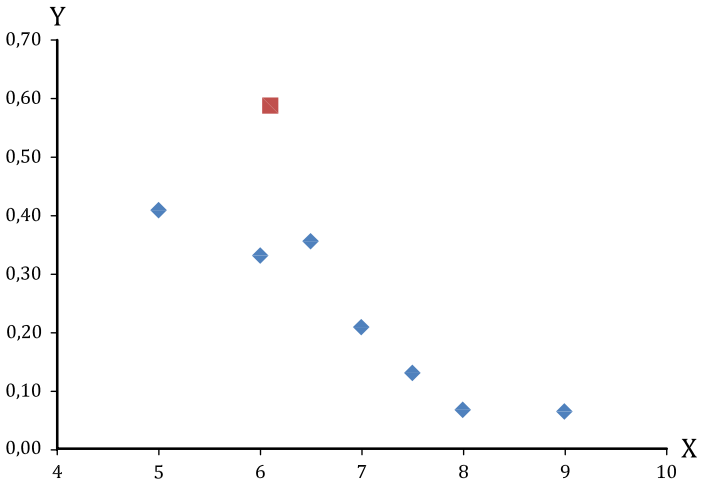
Table A.2 — Effect of sieving on enzymatic activities

SOILS	SIEVING	ENZYMATIC ACTIVITIES (n = 3, mU/g of dry soil)				
		PHOS	β-GLU	ARS	β-GAL	NAG
sandy soil	5 mm	11,20 +/– 0,53	7,67 +/– 0,50	1,08 +/– 0,04	1,10 +/– 0,02	0,82 +/– 0,16
	2 mm	10,47 +/– 1,22	7,36 +/– 0,12	1,22 +/– 0,15	1,13 +/– 0,17	0,93 +/– 0,39
clay loamy soil	5 mm	20,09 +/– 0,56	11,14 +/– 0,17	3,52 +/– 0,05	1,39 +/– 0,09	1,84 +/– 0,08
	2 mm	21,82 +/– 1,37	11,70 +/– 0,12	3,69 +/– 0,07	1,55 +/– 0,09	1,49 +/– 0,03
silt loamy soil	5 mm	18,87 +/– 0,16	7,38 +/– 0,07	4,47 +/– 0,30	0,95 +/– 0,07	1,31 +/– 0,37
	2 mm	17,34 +/– 0,28	7,61 +/– 1,25	4,63 +/– 0,09	0,93 +/– 0,04	1,14 +/– 0,35
silt loamy soil	5 mm	19,10 +/– 1,04	4,86 +/– 0,40	1,45 +/– 0,06	0,94 +/– 0,14	1,74 +/– 0,29
	2 mm	18,39 +/– 0,66	4,75 +/– 0,10	1,47 +/– 0,08	0,97 +/– 0,09	1,05 +/– 0,22

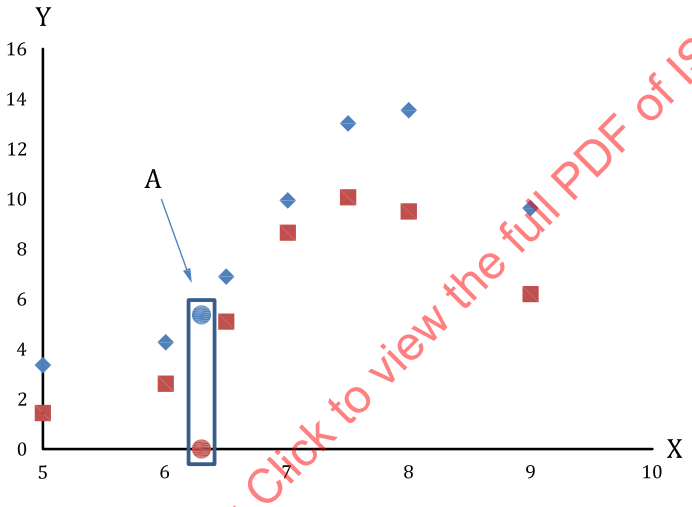
Effect of pH reaction: for PNP enzyme model, several authors point optimal activity around pH 6(see References [5], [22]), arylamidase and urease activity around pH 8. Results showed that for PNP enzyme (β–GLU, α–GLU, β–GAL, PHOS, ARS, and NAG) [see Figure A.1 a)], optimal activities in Tris 50 mmol/l pH[5 to 6,5], but higher activities with distilled water. Results for urease activities are quite similar [Figure A.1 b)]. On the contrary, arylamidase is very sensible to pH solution/soil [Figure A.1 c)], and optimal conditions are Tris 50 mmol/l pH[7,5 to 8].



a) Effect of buffer on activity of GLU, GAL and NAG activity



b) Effect of pH on urease activity



c) Effect of pH on arylamidase activity

Key

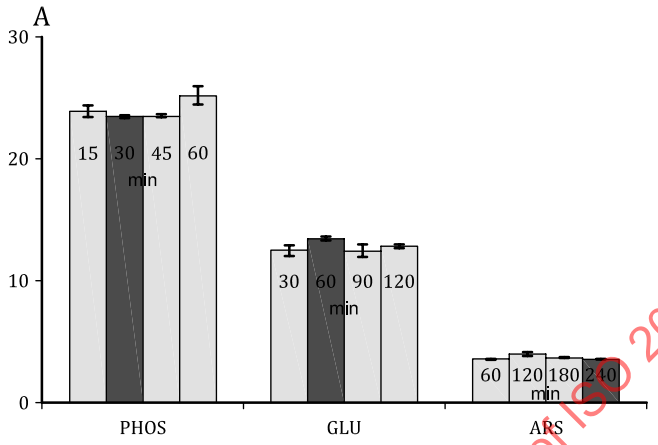
X pH
Y enzymatic activity (mU/g)
A activity in water
▲ clay soil with buffer
▲ silty soil with buffer
● clay soil in water
● silty soil in water
◆ tris buffer 50 mmol/l
■ water
◆ silt loam soil
■ clay loamy soil

Figure A.1 — Effect of pH on enzymatic activities

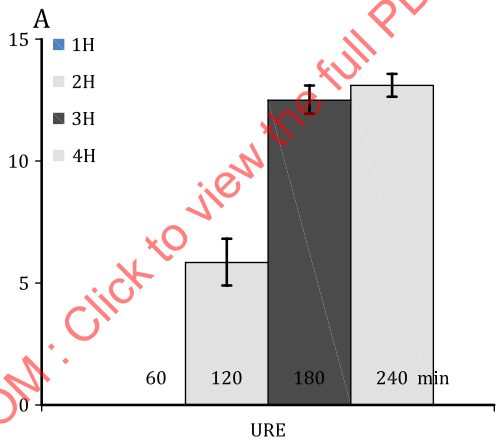
Incubation time effect: results showed that several enzymatic activities are very robust according to incubation time, principally with PNP measurement [Figure A.2 a)], as long as remained in the linear range of PNP quantification. For urease [Figure A.2 b)], short incubations not provide good activities, and for arylamidase (data not shown), long incubation times induce saturation of the reaction. Table A.3 shows open ranges for incubations (bold data are the selected incubation time in ISO standard).

Table A.3 — Incubation time selected for enzymatic activities

Enzymes	ARN	ARS	β-GAL	α-GLU	β-GLU	NAG	PHOS-PAC-PAK	URE
Incubation times (min)	60-**120**	60–120–180-**240**	120-**180**-240	30-**60**-90–120	30-**60**-90–120	30-**60**-90–120	15-**30**-45–60	**180**-240



a) Kinetic of enzymatic activities



b) Kinetic of urease activity

Key

A enzymatic activity (mU/g dry soil); urease activity (mU/g dry soil)

Figure A.2 — Effect of incubation time on enzymatic activities

A.5 Validation parameters

Table A.5 — summary of validation parameters

	ARN	ARS	β-GAL	α-GLU	β-GLU	NAG	PHOS	URE
Specificity	No activity detected in soil after sterilization							
Limit of detection (LOD)	4 pmol	0,7 nmol	0,7 nmol	0,7 nmol	0,7 nmol	0,7 nmol	0,7 nmol	0,2 nmol
Limit of quantification (LOQ)	11 pmol	2 nmol	2 nmol	2 nmols	2 nmols	2 nmol	2 nmol	0,5 nmol
Linearity of the detection	0 µM to 250 µmol/l	0 µM to 600 µmol/l						
Linearity of response (g soil in 25 ml of water)	0,5 g to 8 g	1 to 10 g						0,5 to 12 g
Precision	4 %	4 %	4 %	4 %	4 %	4 %	4 %	3 %
Repeatability (%)	nd	9 %	9 %	9 %	9 %	9 %	9 %	9 %
Intermediate precision (%)°								
Uncertainty range (U95 %)	10 % to 15 %	11 % to 14 %						6 % to 15 %

Annex B
(informative)

International ring test for evaluating soil enzymatic activities with colorimetric method

B.1 Aim

This international ring test aims to evaluate the variability of the method described in this document to assess the soil enzymatic activities in microplates with colorimetric methods.

For each table, legend is: nd is for no data; na is for value not applicable, * experimental error; ** outlier ($p < 0,05$)

B.2 Background

The ring test on this document started in December 2015 and finished in July 2016. eight laboratories participated in the interlaboratory test: i) University of Aveiro and Centre of Environmental and Marine Studies (CESAM), Portugal; ii) Central Institute for Supervising and Testing in Agriculture (CISTA), Czech Republic; iii) Facultad de Farmacia y Madrid, department edafologia, Spain iv) INERIS, France, v) ESITPA, France, vi) UMR Eco&Sols, INRA, Montpellier, France vii) Unit ECOSYS, Biochem-Env Platform, INRA, France, viii) University Complutense of Madrid, Spain.

A movie of experimental schedule was presented at the introductive meeting.

B.3 Test materials and methodology

The participants were provided with 6 soils and another for preliminary tests (two grasslands and four cultured soils), and all testing chemicals, sent to each laboratory. Characteristics of soils were presented in Table B.1.

Upon arrival, the participants were asked to keep reagents at room temperature or 4 °C in the dark, according to the recommendations of this document. Before starting the ring test, participants are inviting to test the standard curve in order to calibrate every microplate reader, and to prepare reageants according to to the test protocols using test soil. Soils were sent and used after air-dried preparation.

Table B.1 — Soil characteristics

SOILS	%SILT	%SAND	%CLAY	pH	OC g/kg	Ntot g/kg	CEC	land use
1	13,0	76,2	10,8	5,6	21,9	1,24	nd	grassland
2	56,2	27,1	16,7	7,4	10,0	1,01	11,5	culture
3	63,5	19,7	16,8	5,5	25,7	2,5	8,1	grassland
5	78,3	6,70	15,0	6,6	10,5	1,0	7,9	culture
6	79,3	16,1	14,6	6,0	11,45	1,19	6,1	culture
7	66,5	9,6	23,4	8,5	14,3	1,2	16,9	culture

Accuracy of standard curves and soils measurement have been evaluated separately according to ISO 5725-2. The repeatability and intermediate precision have been evaluated in Annex A and Reproducibility in Annex B. Averages and relative standard deviations are calculated to estimate the

performance of the method (maximal variation). (OC: organic carbon; Ntot: total nitrogen; CEC: cation exchange capacity; nd: not determided).

B.4 Evaluation of the standard curve results

The data for standardization for PNP or β-naphthylamine or ammonium chloride are given in the Table B.2, B.3 and B4 respectively. The graphs drawn and curves fitted using linear regression plot for PNP are represented in Figure B.1 and for β-naphthylamine in Figure B.2, and for ammonium chloride in Figure B.3.

Results for repeatability of PNP standard curve (see Table B.2) give good precision with $CV_r \leq 5$ % above 29 nmol/ml, and for reproductibility with $CV_R \leq 13$ % under 29 nmol/ml, near limit of quantification. This method allows good reproducibility too, with CV ≤ 10 % for each concentration.Table B.2 — Absorbance measurement results for PNP standards

PNP nmol/ml		14	29	72	140	220	290	360	slope	r^2
LAB1	mean	**0,125**	**0,201**	**0,463**	**0,872**	**1,270**	**1,664**	**2,027**	0,005 5	0,999
n = 3	%CV_r	*1,35 %*	*1,00 %*	*1,64 %*	*1,68 %*	*0,35 %*	*0,48 %*	*0,50 %*		
LAB2	mean	**0,103**	**0,176**	**0,396**	**0,761**	**1,114**	**1,481**	**1,833**	0,005 0	1,000
n = 2	%CV_r	*0,00 %*	*0,00 %*	*0,00 %*	*0,00 %*	*4,23 %*	*0,00 %*	*0,00 %*		
LAB3	mean	**0,114**	**0,192**	**0,416**	**0,755**	**1,145**	**1,509**	**1,862**	0,005 0	1,000
n = 10	%CV_r	*13,09 %*	*5,47 %*	*2,99 %*	*5,85 ss%*	*2,35 %*	*1,41 %*	*1,66 %*		
LAB4	mean	**0,095**	**0,167**	**0,383**	**0,750**	**1,128**	**1,447**	**1,773**	0,004 9	0,999
n = 4	%CV_r	*3,24 %*	*1,72 %*	*1,16 %*	*1,00 %*	*2,76 %*	*0,38 %*	*0,37 %*		
LAB5	mean	**0,124**	**0,185**	**0,380**	**0,713**	**1,040**	**1,372**	**1,696**	0,004 5	1,000
n = 5	%CV_r	*9,43 %*	*2,48 %*	*1,13 %*	*1,09 %*	*0,50 %*	*0,72 %*	*0,96 %*		
LAB6	mean	**0,104**	**0,166**	**0,361**	**0,696**	**1,031**	**1,376**	**1,712**	0,004 6	1,000
n = 1	%CV_r									
LAB7	mean	**0,108**	**0,176**	**0,379**	**0,722**	**1,049**	**1,388**	**1,718**	0,004 6	1,000
n = 12	%CV_r	*10,47 %*	*6,97 %*	*4,01 %*	*2,80 %*	*2,73 %*	*2,34 %*	*1,87 %*		
	mean	**0,110**	**0,180**	**0,397**	**0,753**	**1,111**	**1,462**	**1,803**	0,004 9	
	SD	0,010	0,012	0,031	0,054	0,078	0,096	0,108	0,000 3	1,000
	%CV_R	*9,30 %*	*6,72 %*	*7,84 %*	*7,11 %*	*6,98 %*	*6,58 %*	*6,02 %*	6,20	

(Abbreviations: SD: standard deviation; %CV_r: coefficient of variation for repeatability within laboratory; %CV_R: coefficient of variation for reproducibility inter laboratory; n: number of measures)

For β-naphthylamine precision (see Table B.3), repeatability CV_r is ≤ 10 %, and reproducibility $CV_R \leq 20$ % (except for 10 nmol/ml near limit of quantification).

Table B.3 — Absorbance measurement results for β-naphthylamine standards

β-Naphtylamine nmol/ml		10	20	50	100	200	slope	r^2
LAB1	mean	**0,212**	**0,265**	**0,545**	**1,073**	**2,064**	0,010	0,999
n = 3	%CV_r	*7,10 %*	*7,20 %*	*9,52 %*	*4,08 %*	*5,79 %*		
LAB2	mean	**0,258**	**0,388**	**0,719**	**1,372**	**2,949**	0,014	0,996
n = 2	%CV_r	*2,52 %*	*1,03 %*	*7,65 %*	*7,91 %*	*2,53 %*		
LAB3	mean	**0,135**	**0,211**	**0,432**	**0,889**	**1,828**	0,009	0,998
n = 10	%CV_r	*8,15 %*	*3,35 %*	*1,32 %*	*4,78 %*	*2,12 %*		
LAB4	mean	**0,157**	**0,283**	**0,642**	**1,164**	**2,270**	0,011	1,000
n = 4	%CVr	*3,18 %*	*5,49 %*	*2,88 %*	*0,21 %*	*1,37 %*		
LAB5	mean	**0,144**	**0,268**	**0,593**	**1,251**	**2,431**	0,012	1,000
n = 2	%CV_r	*5,34 %*	*0,67 %*	*0,96 %*	*4,24 %*	*5,67 %*		
LAB6	mean	**0,208**	**0,299**	**0,752**	**1,216**	**2,449**	0,012	0,997
n = 1	%CV_r							
LAB7	mean	**0,186**	**0,279**	**0,632**	**1,248**	**2,417**	0,012	1,000
n = 6	%CV_r	*1,99 %*	*8,10 %*	*3,21 %*	*3,55 %*	*3,69 %*		
	mean	**0,186**	**0,285**	**0,616**	**1,173**	**2,344**	0,011	
	SD	0,040	0,049	0,100	0,143	0,325	0,002	1,000
	%CV_R	*21,81 %*	*17,34 %*	*16,19 %*	*12,22 %*	*13,87 %*	13,54	

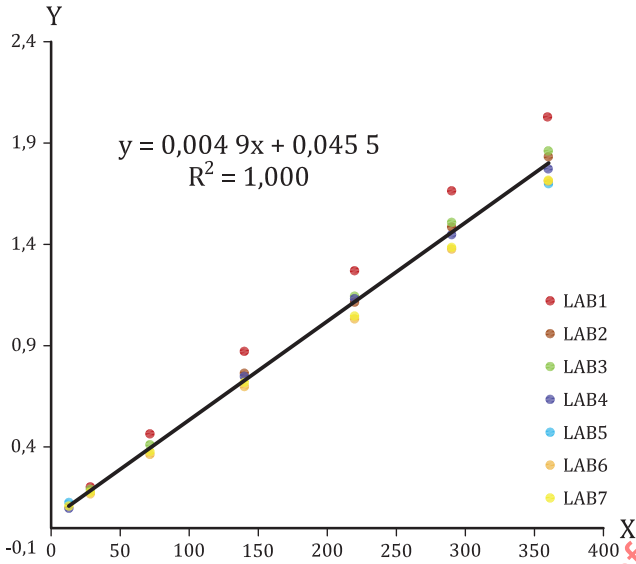
(Abbreviations: SD: standard deviation; %CV_r: coefficient of variation for repeatability within laboratory; %CV_R: coefficient of variation for reproducibility inter laboratory; n: number of measures)

For ammonium chloride precision (see Table B.4), repeatability CV_r is ≤ 6 %, and reproducibility CV_R ≤ 20 % (except at 6 nmol/ml, near limit of quantification).

Table B.4 — Absorbance measurement results for ammonium chloride standards

NH_4Cl nmol/ml		6	16	31	62	155	233	310	slope	r^2
LAB1	mean	**0,085**	**0,129**	**0,207**	**0,376**	**0,859**	**1,284**	**1,667**	0,005 5	0,999
n = 3	%CV_r	*2,07 %*	*2,44 %*	*2,50 %*	*6,40 %*	*1,15 %*	*1,46 %*	*0,23 %*		
LAB2	mean	**0,101**	**0,147**	**0,230**	**0,411**	**0,907**	**1,362**	**1,821**	0,004 1	0,953
n = 2	%CV_r	*0,00 %*	*0,68 %*	*0,22 %*	*1,10 %*	*0,66 %*	*1,95 %*	*0,38 %*		
LAB3	mean	**0,062**	**na**	**0,181**	**0,330**	**0,804**	**1,199**	**1,606**	0,003 8	0,946
n = 10	%CV_r	*13,40 %*		*6,58 %*	*4,54 %*	*1,39 %*	*1,63 %*	*1,19 %*		
LAB4	mean	**0,068**	**0,074**	**0,180**	**0,325**	**0,766**	**1,112**	**1,514**	0,003 5	0,958
n = 4	%CV_r	*3,70 %*	*3,40 %*	*1,11 %*	*0,77 %*	*0,13 %*	*0,45 %*	*1,49 %*		
LAB5	mean	**wrong reading wavelength**								
	%CV_r									
LAB6	mean	**0,078**	**0,118**	**0,176**	**0,278**	**0,682**	**0,977**	**1,317**	0,003 0	0,944
n = 1	%CV_r									
LAB7	mean	**0,072**	**0,113**	**0,183**	**0,327**	**0,781**	**1,176**	**1,572**	0,003 6	0,948
n = 6	%CV_r	*8,19 %*	*5,30 %*	*3,77 %*	*2,35 %*	*1,77 %*	*2,41 %*	*2,36 %*		
	average	**0,078**	**0,116**	**0,193**	**0,341**	**0,800**	**1,185**	**1,583**	0,004 9	
	SD	0,013	0,024	0,019	0,042	0,071	0,122	0,153	0,000 3	1,000
	%CV_R	*16,51 %*	*20,91 %*	*9,96 %*	*12,35 %*	*8,90 %*	*10,33 %*	*9,65 %*	6,20	

(Abbreviations: SD: standard deviation; $\%CV_r$: coefficient of variation for repeatability within laboratory; $\%CV_R$: coefficient of variation for reproducibility inter laboratory; n: number of measures)

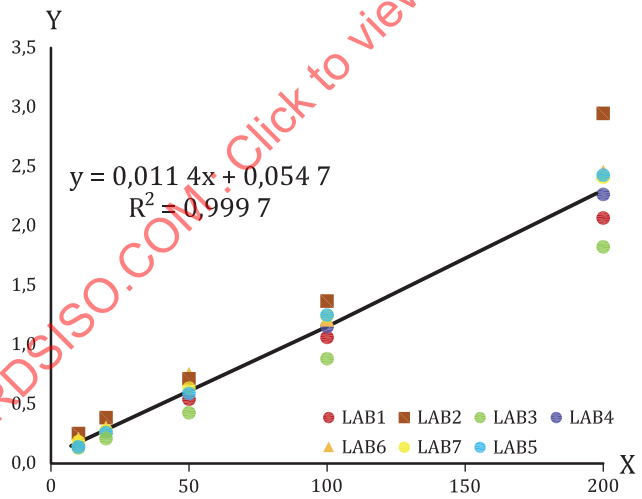


Key

X PNP concentration (nmol/ml)

Y absorbance at 405 nm

Figure B.1 — Calibration curve for PNP



ey

X β-Naphtylamine concentration (nmol/ml)

Y absorbance at 540 nm

Figure B.2 — Calibration curve for β-naphthylamine