Annex D.1

Organo-mineral fertilizers — Determination of the N-(nbutyl)thiophosphoric triamide (NBPT) urease inhibitor content

Draft method

1 Scope

This document specifies a method for the determination of the urease inhibitor N(nbutyl)thiophosphoric triamide (NBPT) and its oxidate form NBPTO in urea based organo-mineral fertilizers, using the liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MSQQQ).

It is applicable to organo-mineral fertilizers,

NOTE: It is possible to apply this method to inorganic fertilizers; in this case a validation is carried out by the laboratory for the procedure and data generated.

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.

EN 1482-1[1], *Fertilizers, liming materials and inhibitors - Sampling and sample preparation - Part 1: General sampling provisions*

EN 1482-2[2], *Fertilizers, liming materials and inhibitors - Sampling and sample preparation - Part 2: General sample preparation provisions*

EN 1482-3[3], *Fertilizers, liming materials and inhibitors - Sampling and sample preparation - Part 3: Sampling of static heaps*

EN 12944-1[4], *Fertilizers, liming materials and inhibitors - Vocabulary — Part 1: General terms*

EN 12944-2[5], *Fertilizers, liming materials and inhibitors - Vocabulary — Part 2: Terms relating to fertilizers*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 12944-1 and EN 12944-2 and the following shall be applied.

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

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

[1] Under preparation

[2] Under preparation

[3] Under preparation

[4] Under preparation

[5] Under preparation

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

4 Principle

This analytical method is based on the principles of liquid chromatography coupled with triple quadrupole mass spectrometry (LC-MSQQQ) for determination of the separated inhibiting compounds NBPT and NBPTO.

WARNING — Persons using this document should be familiar with normal laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably trained staff.

5 Reagents

5.1 General

Use only reagents of recognized analytical grade and distilled water or ultrapure water for LC-MS.

5.2 Reagents for liquid chromatography

5.2.1 Methanol, LC-MS grade.

5.2.2 Formic acid, LC-MS grade.

5.2.3 Ammonium formate, p.a.

5.2.4 Water, ultrapure LC-MS grade or distilled water.

5.2.5 N-(n-butyl)thiophosphoric triamide (NBPT), CAS n. 94317-64-3, minimum 98 %.

5.2.6 N-(n-butyl)phosphoric triamide (NBPTO), CAS n. 25316-39-6, minimum 80%.

5.2.7 Urea, p.a.

5.3 Calibration standards

5.3.1 Stock solution, mass concentration ρ_{NBPT} = 0,20 mg/ml

Weigh 50 mg NBPT (5.2.5) into a 250-ml-measuring flask and dissolve to volume with water (5.2.3). Store at +4 °C ± 1 °C for no more than 2 days.

5.3.2 Stock solution, ρ_{NBPTO} = 0,20 mg/ml

Weigh 50 mg NBPTO (5.2.6) into a 250-ml-measuring flask and dissolve to volume with water (5.2.3). Store at +4°C ± 1 °C for no more than 2 days.

5.3.3 Blank solution, water (5.2.4).

6 Equipment and consumables

Disposable equipment is acceptable in the same way as reusable glassware if the specifications are similar. Ordinary laboratory equipment, and particularly the following.

6.1 Analytical scale, capable of weighing to the nearest 0,0001 g.

6.2 Graduated pipettes, for volumes 5 ml, 10 ml, 15 ml, 20 ml, 25 ml.

6.3 One-mark volumetric glass flasks Class A [3], capacity 100 ml and 250 ml.

6.4 Horizontal shaker agitator

6.5 Qualitative filter paper

6.6 LC-MSQQQ

6.6.1 Operative Conditions

- Column: a non-endcapped silica-based HSS PFP/Fluoro-Phenyl Reversed Phase HPLC column, 3,5 µm 4,6 mm x 150 mm (designed for low pH separations that require alternative selectivity compared to a fully endcapped, high coverage C18 phase, usable for UPLC separations)
- Mobile phase A: Water (5.2.4) with 0,1 % formic acid (5.2.2) and 5 mmol ammonium formate (5.2.3)
- Mobile phase B: Methanol (5.2.1) with 0,1% formic acid (5.2.2)
- Flow rate: 0,6 mL/min
- Column temperature: 30 °C
- Injection volume: 20 µL
- Run time: 15 min
- Expected retention time, t_R of NBPT: 8 min.
- Expected Retention Time, t_R of NBPT-O: 6,9 min

t	A	B
min	%	%
0	90	10
2	90	10
7	5	95
10	5	95
10,1	90	10
15	90	10

- Detector: MS triple quadrupole (MSQQQ)
- Electrospray ionization (ESI)
- Positive polarity
- Gas temperature: 135 °C
- Gas flow: 12 l/min
- VCap: 4 000 V

	Molecular ion	Fragment ion	CollisionEnergy
Qualifier	168	151	15
Quantifier	168	74	15

7 Sampling and sample preparation

Sampling is not part of the method specified in this document. A recommended sampling method is given in EN 1482-1. Sample preparation shall be carried out in accordance with EN 1482-2.

Sampling, transport of samples and sample preparation have influence on the determination. The laboratory shall receive a truly representative sample. The sample to be measured shall be homogeneous.

8 Procedure

8.1 General

A representative laboratory sample of the product shall be prepared according to the procedure in EN1482-2.

8.2 Preparation of the test portion

An amount of 5 g of the test sample shall be weighted to the nearest 0,0001 g, transferred to a 250 ml volumetric flask (6.3), added a volume of 150 ml of water (5.2.4) and dissolved by shaking with agitator (6.4) for 30 min at a rate that keeps the sample in suspension.
It shall be made up to volume with water, homogenized thoroughly and filtered with filter paper (6.5).
An aliquot portion of the extract in water (5.2.4) shall be diluted in one or more steps so that the final concentration of the molecules to be determined falls within the calibration range.
All samples shall be injected in duplicate.

8.3 Calibration

Calibration shall be performed before the analysis.

Volumes of 0 ml, 5,0 ml, 10,0 ml, 15,0 ml, 20,0 ml and 25,0 ml of the stock solution (5.3.1 and 5.3.2) shall be introduced into a 100 ml volumetric flask (6.3) and made up to volume with water (5.2.4). The mass concentration of each analyte in the calibration standard solutions is 0 mg/l, 10,0 mg/l, 20,0 mg/l, 30,0 mg/l, 40,0 mg/l and 50,0 mg/l, respectively. The solutions shall be prepared at the time of each batch of analytical determination.

The stock solutions (5.3.1 and 5.3.2) shall be used to determine the retention time of NBPT and NBPTO in the LC system.

The response factor of NBPT and NBPTO shall be calculated by analysis of the calibration standards in the LC system. All standards shall be injected in duplicate.

8.4 Blank test

For each series of determinations, a blank test shall be introduced in each analytical batch using urea sample test (5.2.7) free from NBPT and NBPTO, prepared according to the procedure in 8.2.

9 Calculation and expression of the result

For the preparatory steps to the determination of NBPT and NBPTO, clauses 4, 5, 6, 7 and 8 shall be applied.

The concentration of NBPT and NBPTO in the sample solution is determined according to the principle of external standard.

The mass fraction of NBPT w_{NBPT}, and NBPTO w_{NBPTO} in percent shall be calculated according to formulae (1) and (2):

$$w_{NBPT} = 100 \times \frac{A \times D}{RxVxmx4} \quad (1)$$

$$w_{NBPTO} = 100 \times \frac{A \times D}{RxVxmx4} \quad (2)$$

where:

- A is the peak area for NBPT or NBPTO;
- R is the response factor (see formula 4 and 5) (peak area/µg NBPT or NBPTO);
- V is the injection volume in µl (20 µl);
- m is the mass of the test portion weighed into the sample solution (250 ml), in g.
- D is the dilution factor calculated according to the formula (3):

$$D = \frac{V_1}{V_{p1}} \times \frac{V_2}{V_{p2}} \times \ldots \frac{V_n}{V_{pn}} \quad (3)$$

where:

- $V_{p1,2...n}$ withdrawal volumes of the solutions for each dilution step in ml;

$V_{1,2,...n}$ volumes of the flasks in which dilution is carried out in ml.

The result shall be given as the average of two replicated determinations done on two different test portions from the same sample, with an accuracy of two figures, e.g. 12 %, 1,2 %, 0,12 %.

The external standard response factor, R, is calculated from the average of the peak areas and mass concentrations of NBPT and NBPTO of the 5 calibration standards according to formulae (4) and (5):

$$R_{NBPT} = \frac{R_{C1} + R_{C2} + R_{C3} + R_{C4} + R_{C5}}{5} \quad (4)$$

$$R_{NBPT} = \frac{A_{C1} + A_{C2} + A_{C3} + A_{C4} + A_{C5}}{(\rho NBPT_{C1} \times V_{C1}) + (\rho NBPT_{C2} \times V_{C2}) + (\rho NBPT_{C3} \times V_{C3}) + (\rho NBPT_{C4} \times V_{C4}) + (\rho NBPT_{C5} \times V_{C5})}$$

$$R_{NBPTO} = \frac{R_{C1} + R_{C2} + R_{C3} + R_{C4} + R_{C5}}{5} \quad (5)$$

$$R_{NBPTO} = \frac{A_{C1} + A_{C2} + A_{C3} + A_{C4} + A_{C5}}{(\rho NBPTO_{C1} \times V_{C1}) + (\rho NBPTO_{C2} \times V_{C2}) + (\rho NBPTO_{C3} \times V_{C3}) + (\rho NBPTO_{C4} \times V_{C4}) + (\rho NBPTO_{C5} \times V_{C5})}$$

where:

- R_{C1}, R_{C2}, R_{C3} R_{C4}, R_{C5} response factor of calibration standards;
- A_{C1}, A_{C2}, A_{C3} A_{C4}, A_{C5} peak area of calibration standards;
- $\rho NBPT_{CI}, \rho NBPT_{C2}, \rho NBPT_{C3}, \rho NBPT_{C4}, \rho NBPT_{C5}$ mass concentration of NBPT in each calibration standard C1, C2, C3, C4, C5 in mg/ml;
- $\rho NBPTO_{CI}, \rho NBPTO_{C2}, \rho NBPTO_{C3}, \rho NBPTO_{C4}, \rho NBPTO_{C5}$ mass concentration of NBPTO, in each calibration standard C1, C2, C3, C4, C5 in mg/ml;
- V_{C1}, V_{C2}, V_{C3}, V_{C4}, V_{C5} injection volume of calibration standards in µl.

10 Performance characteristics of the method

10.1 Precision

Details on precision data from interlaboratory study are reported in the Annex A.

11 Test report

The test report shall contain at least the following information:

a) a title, e.g. “Test Report”;

b) the name and address of the laboratory, and the location where the test was carried out, if different from the address of the laboratory;

c) unique identification of the test report (such as the serial number) and on each page an identification in order to ensure that the page is recognized as a part of the test report and a clear identification of the end of the test report;

d) all information necessary for the complete identification of the sample;

e) the test method used with reference to this document including its year of publication;

f) date and location of sampling, sampling procedure, sampling operator if known, and details of any environmental conditions during sampling that can affect the interpretation of the test result(s);

g) date when the analysis was started and finished;

h) the test results obtained with its unit of measurement;

i) any operating details not specified in this document, or regarded as optional, together with details of any incidents occurred when performing the method, which influenced the test result(s);

j) the name(s), function(s) and signature(s) or equivalent identification of person(s) authorizing the test report;

k) where relevant, a statement to the effect that the result(s) relate only to the items tested;

l) where applicable, a statement on the estimated uncertainty of measurement; information on uncertainty is needed in the test report when it is relevant to the validity or application of the test result(s), when the uncertainty affects compliance to a specification limit/tolerance.

Annex A
(informative)

Repeatability and reproducibility data

A.1 Materials used in the interlaboratory study

An interlaboratory study was carried out for the determination of NBPT and NBPTO content in organo-mineral fertilizers, using LC-MSQQQ.

XXXX European laboratories participated in the interlaboratory test.

The values derived from these inter-laboratory tests may not be applicable to concentration ranges and matrices other than those given in Annex A.

A.2 Samples

XXX different commercially available samples have been selected as base matrices on which the NBPT is spiked in order to verify the reliability of the method in the real situation that the laboratories will find in their analysis routine; the samples are listed in Table A.1.

Table A.1 — Materials tested in the interlaboratory study

Number of samples	3
Type of samples	

Samples were shaken well and divided into aliquots, labelled with the appropriate anonymous code and were maintained protected from light at +4°C ± 1 °C until shipment.

A.3 Procedure

The procedure was carried out in accordance with Clause 8.

Specific instructions are sent to laboratories only for the preparation of the final sample before the test (NBPT+fertilizer). Furthermore, the NBPT and NBPTO determination shall be done immediately after the mix of the NBPT with the fertilizer (t=0) and after 7 days (t=7d) from the preparation; during this time range the samples shall be stored at 4°C ± 2 °C.

A.4 Replicate determination

The determination was carried out in triplicate, three individual tests for each sample.

A.5 Results

The statistical evaluation was conducted according to ISO 5725-2:2019 [4]. The mean values, the repeatability standard deviation (S_r) and the reproducibility standard deviation (S_R) were obtained (Table A.2) for each level separately.

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Prior to the execution of the statistical analysis, all the information returned by the laboratories was examined to determine compliance with the agreed-upon test protocol and test method.

Table A.2 — Interlaboratory test results

Sample code	Sample	Matrix	*l*	*n*	*o* %	$\bar{\bar{x}}$ (NBPT, NBPTO)	NBPT declared (%)	NBPT value obtained during homogeneity tests (%)	s_R (%)	C_{VR} %	s_r (%)	C_{Vr} %

Key		
	l	number of laboratories after outlier rejection
	n	number of individual test results after outlier rejection
	o	percentage of outliers
	$\bar{\bar{x}}$	overall mean of results (without outliers)
	s_R	reproducibility standard deviation
	$C_{V,R}$	coefficient of variation of reproducibility
	s_r	repeatability standard deviation
	$C_{V,r}$	coefficient of variation of repeatability

Bibliography

[1] Regulation (EU) 2019/1009 of the European Parliament and of the Council of 5 June 2019 laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003.

[2] EN 15688:2018, *Fertilizers - Determination of urease inhibitor N-(nbutyl) thiophosphoric triamide (NBPT) using high-performance liquid chromatography (HPLC)*

[3] EN ISO 1042:1999, *Laboratory glassware – One mark volumetric flasks (ISO 1042:1998)*

[4] ISO 5725-2:2019, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*