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Development and validation of the high-performance liquid chromatography method for estimating N-(n-butyl) thiophosphoric triamide in granular fertilisers

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N-(n-butyl) thiophosphoric triamide is widely used as a urease inhibitor that can reduce nitrogen loss by ammonia evaporation from urea. It is used as a fertiliser additive in agricultural applications. The recommended doses of inhibitors are not dangerous to animals and the environment, but high doses can cause changes in small animals. Therefore, the amount of inhibitors in the fertiliser must be determined before fertilising the soil.

This study developed and validated a reliable, sensitive, rapid, and precise high-performance liquid chromatography method for the quantification of N-(n-butyl) thiophosphoric triamide in granular fertilisers. The estimation was carried out using a YMC-Triat C18 column (150 × 4.6 mm, 3 mm) and the mobile phase of acetonitrile: deionised water (25:75, v/v). Inhibition efficiency was monitored by a UV detector at 205 nm. The total run time was 10 min with a flow rate of 0.8 mL/min. The parameters considered for validation were linearity, detection and quantification limit, sustainability, robustness, precision, and stability. The newly created innovative method for quantitative estimation of N-(n-butyl) thiophosphoric triamide in granular fertilisers improves the solubility of samples and standard substance the repeatability and reproducibility of the results obtained.

Keywords: NBPT, HPLC, fertilisers, validation

INTRODUCTION

Granular urea is the best-known and most widely used nitrogen fertiliser. According to estimates in the EU agricultural market briefs, the total volume of fertiliser produced globally, measured by nutrient weight, was 181 million tonnes in 2016, of which nitrogen made up 108 million tonnes (60%) and urea 60 million tonnes (EU agricultural markets briefs, 2019). Unfortunately, after application to the soil, urea undergoes hydrolysis to form ammonium carbonate (Raun, Johnson, 1999; Zuki et al., 2020). This process leads to an increase in pH around the urea granules and increases the

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ikelihood of ammonia emissions (usually 10–20% of the applied nitrogen for a typical coarse urea surface application system, depending on soil temperature and moisture) (Economic and Social Council, 2020). Urea inhibitors are used to reduce ammonia emissions from urea-based fertilisers. There are many compounds that produce inhibitory effects, but the one that stands out and has been launched on the market with the greatest success is N-(n-butyl) thiophosphoric triamide (NBPT) (Ramspacher, 2017). NBPT, CAS No. 94317-64-3, is a urease inhibitor which can reduce nitrogen loss by ammonia volatilisation from urea (Watson et al., 1994; Forrestal et al., 2015). It is used as a fertiliser additive in agricultural applications. Compared with urea, NBPT-treated urea produces an NH_3 loss of around 53%, while the yield gain from NBPT usage is around 6%, ranging from 0.8 to 10.2%, depending on the crop (Cantarella et al., 2018). NBPT reduces the level of ammonia emissions into the atmosphere, slowing down the hydrolysis of urea, and extending the duration of leaching of urea into the soil, which protects the released ammonia. By increasing the duration of hydrolysis, NBPT slows down the rate of the increase in the pH value of the soil near urea granules and thus reduces the level of emissions of ammonia into the atmosphere (Economic and Social Council, 2020).

The final fertiliser preparations containing 0.038 to 0.064% NBPT are not classified as hazardous based on the percentage of NBPT content (Australian Government, Department of Health and Ageing, NICNAS, 2011). However, studies of repeated doses in animals at high doses showed changes in animal bodyweight and bodyweight gains, liver and kidney weights with histopathological evidence at necropsy, and changes in biochemical assay results. A significant negative impact on the reproductive system of male and female rats was also detected. In addition, salivation and languid behaviour, decreased erythrocyte cholinesterase levels, and lower grip strength were observed at high doses (Australian Government, Department of Health and Ageing, NICNAS, 2011). Therefore, before applying fertilisers containing NBPT to the soil, it is necessary to establish the quantitative content of the inhibitor. A new method was developed to allow an increase in the solubility of the samples and standard substance and increase the convergence and reproducibility of the results obtained. The objective was to validate a new high-performance liquid chromatography (HPLC) method for quantitative estimation of NBPT in granular fertilisers using other chromatographic conditions and a different sample preparation procedure.

MATERIALS AND METHODS

For NBPT determination by HPLC in granular fertilisers, this study applied the method described in the European standard EN 16651:2015 – 'Fertilizers – Determination of N-(n-Butyl)thiophosphoric acid triamide (NBPT) and N-(n-Propyl)thiophosphoric acid triamide (NPPT) - Method using high-performance liquid chromatography (HPLC)' as a basis (European standard, 2015).

Reagents and chemicals

The fertilisers containing N-(n-butyl) thiophosphoric triamide (Fig. 1) were provided by Achema, a leading producer of nitrogen fertilisers and chemical products in Lithuania and the Baltic countries. HPLC-grade acetonitrile (45726-2.5L-F, Sigma-Aldrich) and N-(n-butyl) thiophosphoric triamide (91588-25MG, Sigma-Aldrich) were also applied. Water used in the HPLC analysis was prepared using a water purification system (Adrona Crystal EX Trace/ HPLC/ Bio, Latvia). The mobile phase and all the solutions were filtered through a 0.45-mm Chromafil Xtra PTFE-45/13 membrane filter (Macherey-Nagel, Germany) prior to use.

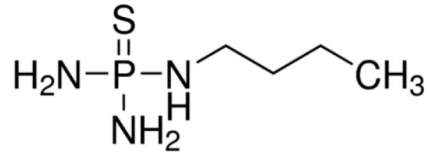

Fig. 1. Structure of N-(n-butyl) thiophosphoric triamide

Instruments and software

The HPLC system (Nexera, Shimadzu) with an autosampler and a PDA detector was used for analysis, guided by LabSolutions software, and an ultra sonicator (Bandelin, Sonorex, Germany).

HPLC analysis was performed using a YMC-Triat C18 (average particle size 3 mm, 120 Å) column (150, 4.6 mm) (YMC Co. Ltd. Japan). The mobile phase consisted of acetonitrile and deionised water at a ratio of 25:75 (v/v %). The eluent was monitored with a UV detector at 205 nm with a flow rate of 0.8 mL/min, and a sample size of 2 µL was carried out at a column oven temperature of 40°C.

Preparation of the stock solution of NBPT

An accurately weighed 40 mg of internal NBPT standard was transferred into a 50-mL volumetric flask, diluted with the mobile phase, and sonicated until completely dissolved. The prepared solution was kept at room temperature for cooling and the volume made up to mark with the mobile phase and then mixed. Subsequently, 1.0 mL of the prepared solution was placed in a 50-mL volumetric flask, made up to mark with the mobile phase and stirred. It was then processed with ultrasound for 1–2 minutes.

Preparation of the sample solution

A sample of 2 g of granular fertiliser containing NBPT was transferred to 50-mL volumetric flasks, diluted with the mobile phase, and sonicated until completely dissolved. The prepared solution was kept at room temperature for cooling; the volume was made up to mark with the mobile phase and mixed.

Validation of the method

The analytical method was validated in order to obtain documented evidence that the applied chromatographic method allowed highly reliable information to be acquired about the qualitative and quantitative composition of the tested samples. The method was validated following ICH Q2A (Validation of analytical methods: Definition and terminology, ICH Q2A, 1994) and ICH Q2B (Guideline on validation of analytical procedures: Methodology) guidelines for linearity, detection, and quantification limit (LOG/LOQ), robustness, precision and sustainability (ICH Q2B, 1996). It was also processed with ultrasound for 1–2 minutes.

Linearity

For linearity, the eligibility criterion was a correlation coefficient in the range of 80–120%, with a step of 5%, and not less than 0.99. For this study, nine different concentrations of NBPT were processed and the calibration curve was constructed in the specified concentration range of 12.8, 13.6, 14.4, 15.2, 16.0, 16.8, 17.6, 18.4, and 19.2 µg/mL, i.e., at concentration levels of 80, 85, 90, 95, 100, 105, 110, 115, and 120% of the nominal concentration of the test solution, respectively. The calibration curve was obtained between the ratios of peak areas of NBPT to the concentration of the analyte in the test solution. All concentration levels and the linear relationship were evaluated and calculated automatically using Microsoft Excel software.

Preparation of working standard solution

Four mL of the prepared stock solution of NBPT was transferred to a 200-mL volumetric flask; the volume was made up to mark with the mobile phase, stirred and also treated with ultrasound. From the stock solution, working solutions were prepared by serial dilution. Nine aliquots of the working solution (in accordance with Table 1) were transferred into nine 50-mL volumetric flasks; the volume in each flask was made up to mark with the mobile phase, and mixed. It was also processed with ultrasound for 1–2 minutes.

Two chromatograms were recorded for each concentration level. The residual standard deviation σ was calculated using the formula:

$$\sigma = \sqrt{\sum /(N-1}$$

where Σ is the sum of the squares of deviations from the regression equation and *N* is the number of points on the regression line.

Table 1. **Preparation of solutions for determination of linearity**

No.	Concentration level, %	Aliquot volume, cm^3	Achieved concentration, µg/ml
1	80	16	12.8
2	85	17	13.6
3	90	18	14.4
4	95	19	15.2
5	100	20	16.0
6	105	21	16.8
7	110	22	17.6
8	115	23	18.4
9	120	24	19.2

Limit of detection (LOD)/Limit of quantification (LOQ)

The LOD is the smallest amount of an analyte in a sample that can be detected, but not necessarily quantified. Acceptance criteria for the LOD should be no more than 0.0015%. The LOQ is the smallest amount of an analyte in a sample that can be quantified with acceptable accuracy. Acceptance criteria for the LOQ determination should be no more than 0.004%.

Five different concentrations of NBPT were processed in the concentration range of 0.5, 1.0, 2.0, 4.0, and 8.0 ppm.

Preparation of stock solution of NBPT

Accurately weighed 40 mg of internal NBPT standard was transferred to a 20-mL volumetric flask, diluted with the mobile phase, and sonicated until completely dissolved. The prepared solution was kept at room temperature for cooling; the volume was made up to mark with the mobile phase and mixed. It was also processed with ultrasound for 1–2 minutes.

Preparation of working standard solution

One mL of the prepared standard was placed in a 100-mL volumetric flask, made up to mark with the mobile phase, stirred, and also treated with ultrasound. Five aliquots of the working solution were transferred into five 20-mL volumetric flasks; the volume in each flask was made up to mark with the mobile phase, mixed, and also treated with ultrasound. Different concentrations were processed at 0.5, 1.0, 2.0, 4.0, and 8.0 ppm. At concentration levels of 1.0 ppm and 4.0 ppm, three chromatograms were recorded, while for the other levels six chromatograms each were recorded. The signal-to-noise ratio (S/N) was calculated at a concentration level of 0.5 ppm.

Calculations

The detection limit (DL) and quantification limit (QL) were calculated using the formulas:

$QL = 10 \cdot \sigma / S$

$DL = 3.3 \cdot \sigma / S$

where **σ** is the residual standard deviation of the regression line and *S* is the slope of the calibration line for the first five levels.

Precision

Precision was defined at two levels: repeatability and reproducibility. Repeatability is defined as a measure of precision when measured under the same conditions over a short period of time. Acceptance criteria for the confidence interval Δ of repeatability should be no more than 0.5%. Six quality control samples containing NBPT and two quality control standards were studied. One injection of each analyte was made and then a further injection was performed in the same sequence within the same day for repeatability. The values of the squared deviations, standard deviation, and confidence interval were calculated using Microsoft Excel software. For reproducibility, the entire procedure described for repeatability was performed on a separate day by a different operator. Acceptance criteria for the confidence interval (Δ) of reproducibility should be no more than 1.0%.

Sustainability

One set of samples was recorded to define the influence of column temperatures of 38°C and 42°C. A second set of samples demonstrated the influence of the detector wavelength. The chromatograms of the samples were recorded at detector wavelengths of 203 and 207 nm.

A third set was recorded with the ratio of solvents in the mobile phase (acetonitrile:water) of 23:77 and 27:73.

Robustness

The quality control standard and the samples were prepared according to paragraphs 2.3 and 2.4. The chromatograms were recorded immediately after preparation, 4, 24, and 120 hours after preparation. All the samples were analysed by standard chromatographic conditions to determine their peak areas.

RESULTS AND DISCUSSION

The HPLC method was developed and validated to determine NBPT in granular fertilisers. Despite NBPT determination by HPLC in granular fertilisers being described by standard LST EN 16651:2015 of the Lithuanian Standards Board, the chromatographic conditions were optimised to provide a better performance. The parameters used for validation of the method were linearity, LOD/LOQ, robustness, precision and sustainability. The results were obtained using a YMC-Triat C-18 column (150 × 4.6 mm, 3 mm) and the mobile phase consisting of acetonitrile:deionised water at the ratio of 25:75 (v/v %), with a flow rate of 0.8 mL/min. The retention time for NBPT was 5.5 ± 0.2 min (Fig. 2). The method was performed and validated for the various parameters as per ICH guidelines.

Linearity

The concentration, peak area, and retention time for linearity of NBPT, and the regression line relating standard concentrations using regression analysis were evaluated. The calibration curves were linear in the studied range, and equations of the regression analysis were obtained, i.e., R2 = 0.99974 for NBPT. The method produced linear responses in the concentration range of 80–120%. Good linearity was observed across the above-mentioned range, indicating that the method was linear over the concentration range studied. Figure 3 illustrates the linearity data for NBPT.

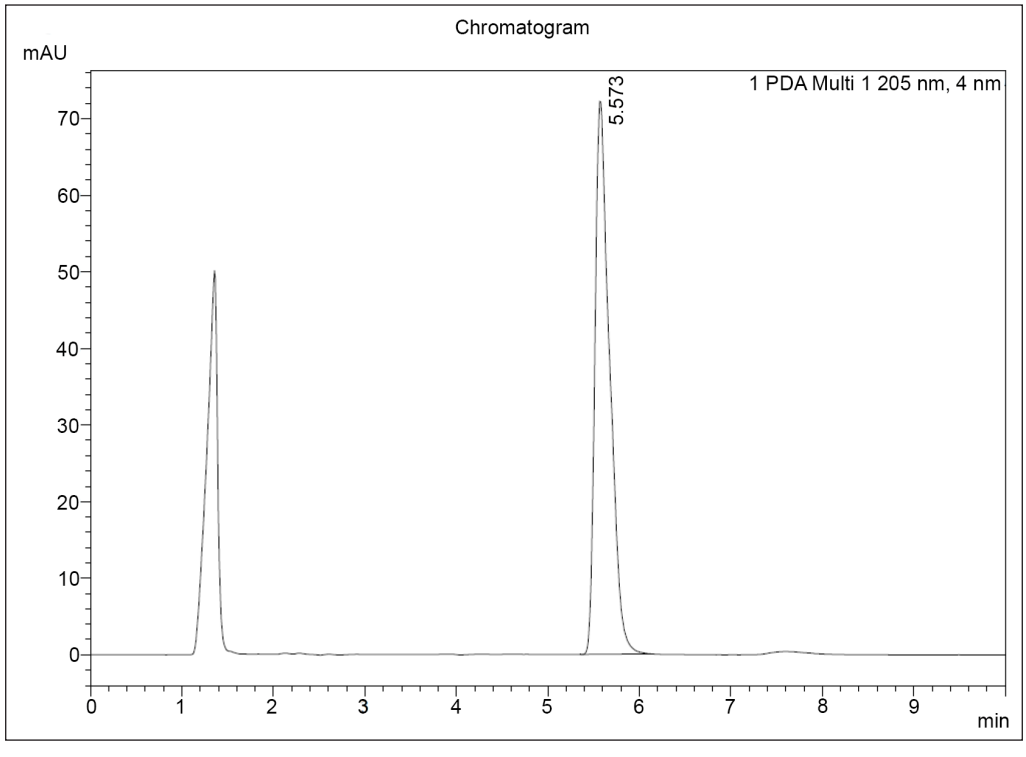

Fig. 2. HPLC chromatogram for NBPT in the standard solution

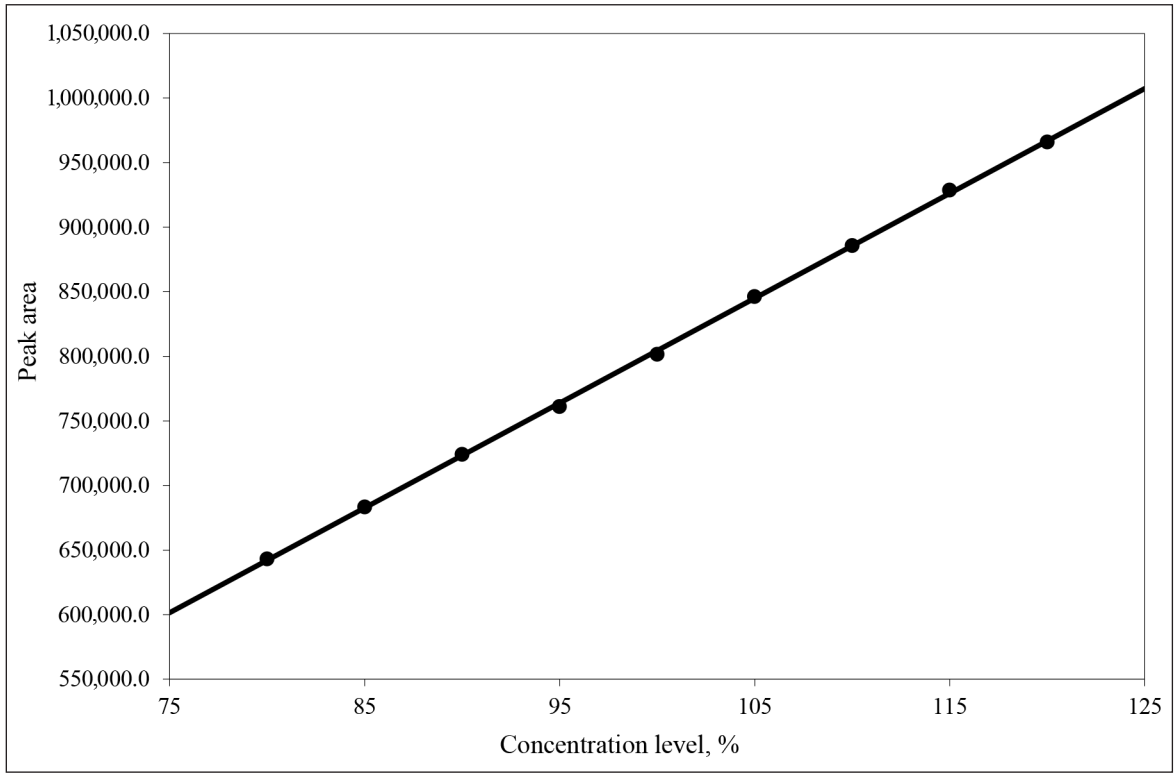

Fig. 3. Linearity graph for NBPT (analyte peak area versus solution concentration)

Sustainability

The sustainability of the method was evaluated by deliberately changing the chromatographic conditions such as flow rate, the ratio of solvents in the mobile phase, detection wavelength, and column temperature. The results showed that only the detector wavelength (σ_{sqrt} = 1.03%) had a significant effect on the test result. This dependence is explained by the spectral characteristics of NBPT (Fig. 4).

Changes to other chromatographic conditions did not lead to significant inaccuracies in the tests (σ_{sqrt} = 0.26%).

Robustness

By re-analysing the prepared solutions, it was established that the standard solution was suitable for research within 120 hours and the sample solution within 24 hours (Table 2).

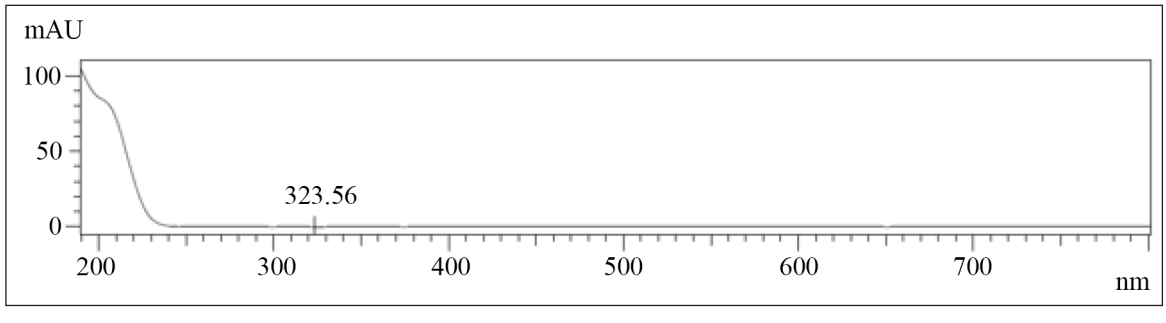

Fig. 4. NBPT spectral characteristics (UV absorption spectrum NBPT)

Table 2. **Robustness study of the proposed HPLC method**

	0 h	4 h	24 h	120 h	σ_{sqrt}
Standard	864218	863963	864184	870134	0.34
Sample	745875	749538	746376	742597	1.56

LOD/LOQ

QL and DL were found to be 0.15 ppm and 0.05 ppm, respectively.

CONCLUSIONS

Compared with the current method EN 16651:2015, this study allowed error reduction in the sample preparation due to the use of external standard methods and changes in the conditions of sample preparation to increase the solubility of the samples. The value of the validated parameters met the acceptance criteria and the solutions were fairly stable over time.

Minor changes in the parameters of the chromatographic system did not affect the results of the study, with the exception of the detector wavelength. During the validation of the analytical method, the parameters of the chromatographic system were established: the number of theoretical plates was not fewer than 7000 and the symmetry coefficient was 0.8–1.4. The HPLC method developed in this study can be used successfully for the quantitative determination of NBPT in granular fertilisers.

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References

1. Australian Government, Department of Health and Ageing, NICNAS. Existing chemical secondary notification assessment NA/467S. N-(n-butyl) thiophosphoric triamide (NBPT), February 2021.
2. Cantarella H, Otto R, de Brito Silva AG. Agronomic efficiency of NBPT as a urease inhibitor: a review. J Adv Res. 2018; 13: 19–27.
3. Economic and Social Council. ECE/EB.AIR/2020/6, ECE/EB.AIR/WG.5/2020/5. Draft guidance document on integrated sustainable nitrogen management.
4. EU Agricultural markets briefs. Fertilisers in the EU. Prices, trade and use, 2019. No. 15 https://ec.europa.eu/info/sites/default/files/food-farming-fisheries/farming/documents/market-brief-fertilisers_june2019_en.pdf.
5. European standard EN 16651:2015 Fertilizers – Determination of N-(n-Butyl)thiophosphoric acid triamide (NBPT) and N-(n-Propyl)thiophosphoric acid triamide (NPPT).
6. Forrestal PJ, Harty M, Carolan R, Lanigan GJ, Watson CJ, Laughlin RJ, McNeill G, Chambers BJ, Richards KG. Ammonia emissions from urea, stabilized urea and calcium ammonium nitrate: Insights into loss abatement in temperate grassland. Soil Use Manag. 2015; 32: 92–100.
7. ICH Q2A (Validation of analytical methods: definition and terminology). 1994.
8. ICH Q2B (Guideline on validation of analytical procedures: Methodology). 1996.
9. Ramspacher A. Assessment of the global market for slow and controlled release, stabilized and water-soluble fertilizers. Presentation at IFA strategic forum 2017. Paris: IFA – International Fertilizer Association. November 2017.
10. Raun WR, Johnson GV. Improving nitrogen use efficiency for cereal production. Agron J. 1999; 91: 357–63.

11. Zuki MMM, Jaafar N. Md, Sakimin SZ, Yusop M Kh. N-(n-Butyl) Thiophosphoric Triamide (NBPT)-Coated Urea (NCU) improved maize growth and nitrogen use efficiency (NUE) in highly weathered tropical soil. Sustainability. 2020; 12: 8780.

12. Watson C, Miller H, Poland P, Kilpatrick D, Allen M, Garrett M, Christianson C. Soil properties and the ability of the urease inhibitor N-(n-BUTYL) thiophosphoric triamide (nBTPT) to reduce ammonia volatilization from surface-applied urea. Soil Biol Biochem. 199; 26: 1165–71.

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SUKURTAS IR PATVIRTINTAS LABAI EFEKTYVIOS SKYSČIŲ CHROMATOGRAFIJOS METODAS N-(N-BUTIL) TIOFOSFORO TRIAMIDUI GRANULINĖSE TRĄŠOSE NUSTATYTI

Santrauka

N-(n-butil) tiofosforo triamidas plačiai naudojamas kaip ureazės inhibitorius, galintis sumažinti azoto nuostolius amoniako garavimo iš karbamido metu. Kaip trąšų priedas naudojamas žemės ūkyje. Rekomenduojamos inhibitorių dozės nėra pavojingos gyvūnams ir aplinkai, tačiau didelės dozės gali pakenkti smulkiems gyvūnams, todėl prieš tręšiant dirvą būtina nustatyti inhibitorių kiekį trąšose. Šio tyrimo metu buvo sukurtas ir patvirtintas patikimas, greitas ir tikslus efektyvios skysčių chromatografijos metodas N-(n-butil) tiofosforo triamido kiekiui granulinėse trąšose nustatyti. Įvertinimas atliktas naudojant YMC-Triat C18 kolonėlę (150 × 4,6 mm, 3 mm) ir judriąją acetonitrilo fazę: dejonizuotą vandenį (25:75, v/v). Slopinimo efektyvumas užfiksuotas UV detektoriumi esant 205 nm bangos ilgiui. Bendras veikimo laikas 10 min., srauto greitis 0,8 ml/min. Buvo tikrinami šie parametrai: tiesumas, aptikimo ir kiekybinio įvertinimo ribos, tvarumas, tvirtumas, tikslumas ir stabilumas.

Naujas N-(n-butil) tiofosforo triamido kiekio granulinėse trąšose nustatymo metodas pagerina mėginių ir standartinių medžiagų tirpumą bei gautų rezultatų pakartojimą ir atkūrimą.

Raktažodžiai: NBPT, HPLC, trąšos