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
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.

![Structure of N-(n-butyl) thiophosphoric triamide](image)
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 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 then mixed. It was then processed with ultrasound for 1–2 minutes.

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:

\[ \sigma = \sqrt{\frac{\Sigma}{N-1}} \]

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.
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. 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:

\[
QL = 10 \cdot \frac{\sigma}{S} \\
DL = 3.3 \cdot \frac{\sigma}{S}
\]

where \(\sigma\) 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 \(\Delta\) 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 \((\Delta)\) 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.

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Concentration level, %</th>
<th>Aliquot volume, cm³</th>
<th>Achieved concentration, μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>16</td>
<td>12.8</td>
</tr>
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<td>2</td>
<td>85</td>
<td>17</td>
<td>13.6</td>
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<td>3</td>
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<td>19</td>
<td>15.2</td>
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<td>16.8</td>
</tr>
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<td>7</td>
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<tr>
<td>8</td>
<td>115</td>
<td>23</td>
<td>18.4</td>
</tr>
<tr>
<td>9</td>
<td>120</td>
<td>24</td>
<td>19.2</td>
</tr>
</tbody>
</table>

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.
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., $R^2 = 0.99974$ 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](image-url)
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 ($\sigma_{\text{sqrt}} = 1.03\%$) had a significant effect on the test result. This dependence is explained by the spectral characteristics of NBPT (Fig. 4).

Robustness

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

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

Fig. 4. NBPT spectral characteristics (UV absorption spectrum NBPT)
Table 2. **Robustness study of the proposed HPLC method**

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>4 h</th>
<th>24 h</th>
<th>120 h</th>
<th>$\sigma_{\text{sq rt}}$</th>
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</thead>
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<td>Standard</td>
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<td>863963</td>
<td>864184</td>
<td>870134</td>
<td>0.34</td>
</tr>
<tr>
<td>Sample</td>
<td>745875</td>
<td>749538</td>
<td>746376</td>
<td>742597</td>
<td>1.56</td>
</tr>
</tbody>
</table>

**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.

**FUNDING STATEMENT**

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**References**

5. European standard EN 16651:2015 Fertilizers – Determination of N-(n-Butyl)thiophosphoric acid triamide (NBPT) and N-(n-Pro pyl)thiophosphoric acid triamide (NPPT).