DETERMINATION OF THIAMINE IN PREMIXTURE AND COMPOUND FEED BY LIQUID CHROMATOGRAPHY METHOD

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Abstract

The analytical procedure of thiamine quantification in premixtures and compound feedingstuffs by HPLC method is presented. Thiamine was extracted from feed samples with 0.1M hydrochloric acid at 100°C for 30 min. In the case of compound feedingstuffs, 10% Taka-diastase solution was added to the samples, and then the samples were incubated at 37°C for 17 h. Afterwards, thiamine was oxidised to thiochrom, using 1% alkaline potassium ferricyanide (III). The filtrated solution was analysed by HPLC with isocratic flow of eluent chloroform-methanol 90:10 (v/v). The measurement was done by a fluorescence detector. This method has been applied for the quantification of the total content of thiamine in compound feedingstuffs, as well as added thiamine in the form of hydrochloride or nitrate salt. The limit of the quantification of this method was determined on the level of 1 mg/kg. The coefficient variation of thiamine quantification results in premixtures samples was 3.7%, and in compound feedingstuffs was 5.56%. The Horrat value for premixture samples reached a value of 0.60, whereas in the case of compound feedingstuffs it was 0.63, thus confirming acceptable precision of the procedure. The recovery rate for thiamine added in the form of hydrochloride was 102.3% in premixes and 98.9% in compound feedingstuffs. The recovery rate for thiamine added in the form of nitrate salt to compound feedingstuffs was similar and reached a value equal to 98.7%.

Key words: thiamine, quantification, HPLC, premixtures, compound feedingstuffs.

Thiamine (vitamin B1) plays main functions in tissue respiration, participating mainly in carbohydrate transformation processes. The bio-active form of this vitamin is thiamine pyrophosphate, which is a coenzyme of decarboxylases, transketolases, and enzymes, which catalyse oxidative decarboxylation of ketoacids. The thiamine deficiency is harmful for animal organisms. In feed materials of plant origin, vitamin B1 is present in small quantities, mainly in pericarp (3-5 mg/kg) and germs (10-20 mg/kg) of cereal grains, yeasts (40-120 mg/kg), potatoes (5-10 mg/kg dry matter), and 3-4 mg/kg is found in powdered milk (17). From many years, Polish industrial feed production has been based on recipes, which contain defined amounts of vitamin B1, according to the requirements of the animal species. A recommended thiamine doses to compound feedingstuffs for poultry, varies from 1-2 mg/kg in case of compound feedingstuffs for laying hens to 3-4 mg/kg for turkeys (16). The main thiamine sources are its synthetic form like hydrochloride and nitrate salts, available in the form of preparations and vitamin premixtures. To compound feedingstuffs, thiamine is added as a premixture for the supplementation of its level, according to the animal’s requirements. Thiamine is characterised by low stability, especially in neutral or alkali environment. The quantitative losses of thiamine can occur particularly during thermal and pressure processing of compound feedingstuffs (6). Therefore, the possibility of quantification of vitamin B1 as an additive to premixtures or supplementary feed, and of quantification of total thiamine content in feed, are both important for monitoring analyses of feedingstuffs in the frames of the official control, as well as internal control carried out by feed producers.

Polish feed laws do not contain any standard methodology for quantitative analyses of vitamin B1 in animal feedingstuffs. Likewise, international (ISO), European (EN), or Polish (PN) norms do not contain any methods for the quantification of this vitamin, concerning the total amount or as additive in its synthetic form. In the past, recommended official methods were based on fluorometric measurement of thiochrom, which was received from vitamin B1 oxidation after extraction, enzymatic hydrolysis, and extract purification (2, 15). These methods were sensitive to interference with some disturbing substances. However, the fluorometric method could not be used in case of the presence of materials, which absorb thiamine or influence the thiochrom fluorescence. Taking this into account, the fluorometric method for vitamin B1 quantification was withdrawn by
a Commission Directive 98/54/EC from the registry of standard methods for animal feedingstuff analysis. Classical microbiology methods for the quantification of thiamine in food and feed in spite of sufficient sensitivity are characterised by small selectivity, long execution time, high work consumption, and low precision (3, 5).

Among instrumental methods, the high performance liquid chromatography (HPLC) method is sufficient as regards to its selectivity and sensitivity for the thiamine quantification in pharmaceuticals, food, and feedingstuffs. Analytical procedures, elaborated some years ago, base on electrochemical and UV detection (14, 18) or fluorometric detection of the vitamin (1, 18, 19). Each of the elaborated and validated procedures contains precisely described extraction stage, which allows quantification of the total thiamine amount in the analysed sample.

Taking these into account, the study was undertaken with the aim of applying the HPLC method with fluorometric detection for the quantification of thiamine, which was added to premixture, or quantification of total thiamine amount in compound feedingstuffs. The validation parameters of the method were evaluated for both premixture and compound feedingstuff sample matrices.

**Material and Methods**

**Materials.** The study was carried out on premixture and compound feedingstuff samples, which contained vitamin B1. The parameters of this method were also evaluated on certified reference materials: CRM 121 (Wholemeal flour) and CRM 421 (Milk powder) (3). The following reagents were used in the study: chloroform and methanol (HPLC grade), hydrochloric acid, trichloroacetic acid, sodium hydroxide, isobutanol saturated with water, potassium ferricyanide (III), and sodium acetate (POCH Gliwice, Poland). Enzyme Taka-diastase from *Aspergillus oryzae* was delivered by Fluka. Deionised water was prepared on Milli-Q system (Millipore, France).

**Standards and standard solutions.** The standard stock solutions of thiamine hydrochloric salt and thiamine nitrate salt (100 µg/mL in 0.1 M hydrochloric acid) were stored at 4°C in darkness up to one month. The working solutions from 0.1 to 1.0 µg/mL were prepared each time just before use.

**Liquid chromatography - fluorescence detection.** High pressure liquid chromatograph (Dionex P-680) with fluorescence detector (Dionex RF 2000), extinction length $\lambda=365$ nm and emission wavelength $\lambda'=435$ nm was used for the analysis. The separation was done on the column LiChrophor 100 NH$_2$ (250 mm x 4.6 mm x 5 µm), or a similar column. The solution of chloroform and methanol (90:10, v/v) was a mobile phase for LC. The isocratic eluent flow was 2 mL/min, at a controlled temperature of 25°C for the separation of interfered elements, which could be in the sample extract.

**Extraction.** Because Vitamin B$_1$ is sensitive to light, all actions during this phase of analysis were conducted without exposure to daylight (by using amber glass flask or flask protected by aluminium foil).

1. **Preparation of premixture sample:**
   The 5 g of the premixture was weighed in Erlenmeyer flask, and then 98 ml of 0.1 M hydrochloric acid was added and the flasks were agitated for 30 min. Afterwards 2 ml of 50% trichloroacetic acid was added and that solution was boiled for 10 min in a water bath. Next, the samples were cooled to room temperature. The extracts were then transferred to centrifuge tubes and centrifuged for 10 min. If it was necessary, an additional dilution was done, in order to fit the concentration of the solution to the calibration curve.

2. **Preparation of compound feedingstuff sample:**
   The 5 g of the compound feed was weighed in an Erlenmeyer flask, then 60 ml of 0.1 M hydrochloric acid was added and the flasks were placed into an ultrasound bath at 50°C for 30 min. After that, they were agitated for 30 min, and then boiled for 30 min in a water bath. Afterwards, they were cooled to room temperature and the pH was adjusted to 4.3-4.7 by 2.5 M sodium acetate solution. Then, 5 ml of 10% Taka-diastase enzyme was added to the solutions, which were then incubated at 37°C for at least 16 h, but no longer than 17 h. After that time, the enzymatic reaction was stopped by an addition of 2 ml of 50% trichloroacetic acid. The samples were once more boiled in a water bath for 10 min, and cooled to the room temperature. The whole amount of the extract was then transferred into a measuring flask, then completed with deionised water to 100 ml volume, and mixed. The part of the extract was then spun in the centrifuge.

**Derivatisation.** The 5 ml of the extract was transferred into a new flask and 5 ml of water-saturated isobutanol was added to the sample. The whole volume was agitated for 30 s. Afterwards 3 ml of 1% potassium ferricyanide (III) in 15% sodium hydroxide was added, and the samples were once more agitated for 30 s. Then the samples were left for the phase separation. Then, the upper phase was taken, filtered by a syringe filter, and 20 µl of the solution was dosed on a chromatographic column.

**Evaluation of the procedure.** The precision of this method was checked by the evaluation of the repeatability and reproducibility. In case of precision, the Horwitz coefficient called Horrat value (Hor) was calculated. The Hor is the ratio of the reproducibility of standard deviation SD$_r$ calculated from the data, to the target standard deviation $\sigma_m$ calculated from the Horwitz formula (9): $\sigma_m=0.02 C^{0.8495}$, where C means concentration expressed as denominated mass fraction (e.g. 1 mg/kg = 10$^{-6}$). Acceptable Hor, which describes the precision of measures, are $0.5<Hor<2$ (10, 11). In order to adjust the results to the repeatability conditions, the target standard deviation $\sigma_m$ was multiplied by 0.66 (SD$_r$=0.66 SD$_u$) (1). The accuracy of the method was evaluated by the recovery rate calculation and analysis of certified reference materials CRM 121 (wholemeal flour) and CRM 421 (milk powder). The estimated
expanded uncertainty of the elaborated method was evaluated by within-laboratory approach, according to the technical report of Eurolab No. 1/2007 (7).

Because the within-laboratory approach for standard uncertainty \( (u) \) estimation was used, it was necessary to evaluate the within-laboratory reproducibility (\( s \)), as a measure of precision, and systematic bias (\( b \)), calculated from the CRM analyses. The standard uncertainty was estimated according to formulas from technical report of Eurolab (7) and the direction of Nordtest (8).

\[
u = \sqrt{s^2 + b^2} \quad [1]
\]

Within-laboratory reproducibility was obtained from the range between the repeated analyses of feed samples. According to the Nordtest direction (8), for two individual thiamine analyses in each sample, the mean value, difference between analyses (range), relative difference (%), and mean relative difference (%) for every samples of the same kind of feed were calculated. The mean range divided by d coefficient (for two repetitions \( d=1.128 \)) was equal to the within-laboratory reproducibility standard deviation.

The contribution of statistic bias (\( b \)) in the uncertainty measure was calculated from the mean standard deviation of results CRM \( \Delta \), the uncertainty CRM \( u_{ref} \), and the standard deviation of analysis CRM \( s_{ref} \), based on the following formula (7, 8):

\[
b = \sqrt{\Delta^2 + u_{ref}^2 + \frac{s_{ref}^2}{n}} \quad [2]
\]

\[
\Delta = \sqrt{\frac{\sum (bias_i)^2}{n}} \quad [3]
\]

**Results**

The characteristic chromatograms of reference thiamine solution (a), premixture extract (b), and compound feedingstuffs (c) are presented in Fig. 1. The retention time was about 3 min. For the applied analytical procedure and chromatography conditions of the separation, there were not observed any of the interference substances.

Table 1 contains the validation parameters obtained during studies, such as precision of the method, which was based on repeatability and expressed as a coefficient of variation (3.7-5.6%), intermediate precision, expressed as within-laboratory reproducibility (3.6%), and Horrat value (Hor=0.60-0.63), which are the objective measure of precision for the applied analytical procedure. The calibration curve possessed good linearity in the range of 0.1-1.0 \( \mu g/mL \) with the correlation coefficient \( r=0.9999 \). The recovery rate of thiamine addition to the analysed samples in the form of hydrochloride and nitrate salts ranged from 98.7% to 102.3%. The limit of quantification was 1 mg/kg, combined standard uncertainty was 7.6%, and expanded uncertainty was 15.2%.

The bias of the method was evaluated on the basis of the results from CRM analyses and was expressed as the relative difference between the achieved result and certified reference value. The bias was determined in the range of 4.8%-5.1%.

**Discussion**

In the presented analytical procedure, a high performance liquid chromatography (HPLC) was applied. The procedure allowed the separation and quantification of vitamin B\(_1\) as an additive to premixture or total amount of this vitamin in compound feedingstuffs.

The achieved coefficients of variation (3.7% in case of premixture, 5.6% in case of compound feedingstuffs), and Horrat values, which amounted to 0.60 and 0.63 respectively, confirmed that the applied analytical procedure offers acceptable precision. According to Horwitz (10, 11), acceptable Horrat values should range between 0.5 and 2. The Horrat values below one provided evidence of a high precision of the results. The accuracy of measures was also excellent, and was expressed by the thiamine recovery rate in the range from 98.7% to 102.3%.

The precision of results at the level of 3% (Hor 0.47) and recovery rate ranging from 91% to 103% were obtained by Wollard and Indyk (18) in the analyses of hydrophilic vitamins in baby-foods (6.7 mg/kg) by liquid chromatography combined with fluorometric detection. In interlaboratory studies, where the HPLC method with fluorimetric detection was applied for the determination of total thiamine content in pet foods and compound feedingstuffs for turkey, the repeatability values were achieved, ranging from 3.3% to 3.9% with an acceptable Horrat value of 0.89 for pet foods and 0.44 for compound feedingstuffs for turkey (1). The recovery rate of thiamine hydrochloride in pet-foods analyses, ranged from 77.0% to 98.8% (1).

The obtained chromatographic separation, precision parameters, and accuracy defined by the recovery rate, illustrated that the applied analytical procedure was correct. The accuracy of the elaborated procedure was additionally confirmed in analyses performed on certified reference materials (CRM) and reached a value of 95.0% (uncertainty 5.0%). This method allows the determination of thiamine, which was added to premixtures and compound feedingstuffs in the form of hydrochloride and nitrate salts, i.e. in the form used by feed producers. Because of this, this method can be applied for official control, and allows verifying the producers’ declarations with regard to the amount of thiamine addition to premixtures and compound feedingstuffs.

In order to verify the applied analytical procedure, the levels of thiamine in certified reference materials were determined. The achieved results are listed in Table 2.
Fig. 1. Characteristic chromatograms of thiamine: a) chromatogram of standard extract (concentration 0.1449 µg/mL), b) chromatogram of compound feedingstuff extract (concentration 0.1458 µg/mL), c) chromatogram of premixture extract (concentration 0.3566 µg/mL).
### Table 1
Validation parameters of the method for thiamine determination

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Limit of quantification</td>
<td>1 mg/kg</td>
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<tr>
<td>Standard calibration curve</td>
<td>r=0.9999</td>
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<td></td>
<td>y=23.373x-0.1125</td>
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<tr>
<td>Linearity range, µg/mL</td>
<td>0.1–1</td>
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<tr>
<td>Precision CV, % (n=10)</td>
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</tr>
<tr>
<td>(Repeatability)</td>
<td></td>
</tr>
<tr>
<td>Compound feed</td>
<td>3.12 mg/kg 5.6</td>
</tr>
<tr>
<td>Premixture</td>
<td>35.2 mg/kg 3.7</td>
</tr>
<tr>
<td>Hor (compound feed)</td>
<td>3.12 mg/kg 0.63</td>
</tr>
<tr>
<td>Hor (premixture)</td>
<td>35.2 mg/kg 0.60</td>
</tr>
<tr>
<td>Within-laboratory</td>
<td>7.7 – 800 mg/kg 3.60</td>
</tr>
<tr>
<td>reproducibility CV, % n=11</td>
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<tr>
<td>Recovery rate - thiamine</td>
<td></td>
</tr>
<tr>
<td>hydrochloride, % (n=6)</td>
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<tr>
<td>Compound feed</td>
<td>3.12 mg/kg 98.9</td>
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<tr>
<td>Premixture</td>
<td>35.2 mg/kg 102.3</td>
</tr>
<tr>
<td>Recovery rate-thiamine nitrate,</td>
<td></td>
</tr>
<tr>
<td>% (n=6)</td>
<td></td>
</tr>
<tr>
<td>Compound feed</td>
<td>3.12 mg/kg 98.7</td>
</tr>
<tr>
<td>Combined uncertainty u (%)</td>
<td>7.6</td>
</tr>
<tr>
<td>Expanded uncertainty U = 2 x u</td>
<td>15.2</td>
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<tr>
<td>(% (k = 2)</td>
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### Table 2
The concentration of thiamine in certified reference materials

<table>
<thead>
<tr>
<th>Certified reference material</th>
<th>Declared concentration of thiamine (uncertainty)</th>
<th>Achieved thiamine analyses results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg dry matter</td>
<td>mg/kg dry matter</td>
</tr>
<tr>
<td>CRM 421 Milk powder</td>
<td>6.51 (±0.48)</td>
<td>6.18</td>
</tr>
<tr>
<td>CRM 121 Wholemeal flour</td>
<td>4.63 (±0.39)</td>
<td>4.41</td>
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</tbody>
</table>
References