analytical chemistry

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Article

The First Comprehensive LC–MS/MS Method Allowing Dissection of the Thiamine Pathway in Plants

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Cite This: *Anal. Chem.* 2020, 92, 4073–4081

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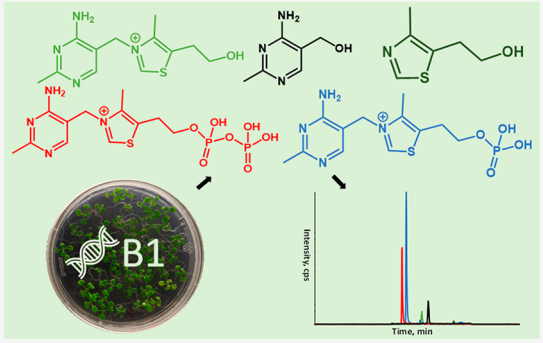
ABSTRACT: *Arabidopsis thaliana* serves as a model plant for genetic research, including vitamin research. When aiming at engineering the thiamine (vitamin B1) pathway in plants, the availability of tools that allow the quantitative determination of different intermediates in the biosynthesis pathway is of pivotal importance. This is a challenge, given the nature of the compounds and the minute quantities of genetically engineered material that may be available for analysis. Here, we report on the first LC–MS/MS method for the simultaneous quantification of thiamine, its mono- and diphosphate derivatives and its precursors 4-methyl-5-(2-hydroxyethyl) thiazole (HET) and 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP). This method was optimized and validated for the quantitative determination of these analytes in *Arabidopsis thaliana*. All analytes were chromatographically separated within less than 2.5 min during an 8 min run. No unacceptable interferences were found. The method was fully validated based on international guidelines. Accuracy (%bias) and total imprecision (%CV) were within preset acceptance criteria for all analytes in both QC and real samples. All analytes were stable in extracted samples when stored for 48 h at 4 °C (autosampler stability) and when reanalyzed after storage at −80 °C and −20 °C for 2 weeks (freeze/thaw stability). We demonstrated the start material should be stored at −80 °C to ensure stability of all analytes during short- and long-term storage (up to 3 months). The validity and applicability of the developed procedure was demonstrated via its successful application on *Arabidopsis* lines, genetically engineered to enhance thiamine content.



Thiamine, the first compound characterized within the group of B vitamins (vitamin B1), is an essential micronutrient for human health.[1] Thiamine diphosphate (TDP), the biologically active form of thiamine, serves as a cofactor of several key enzymes involved in glucose metabolism, the Krebs cycle and branched-chain amino acid biosynthesis. As humans are not able to synthesize thiamine themselves, they entirely rely on their dietary intake for its supply. When this intake is restricted, deficiency can occur within a couple of weeks due to the limited storage in the body. An acute deficit of vitamin B1 results in the disease beriberi, which can be fatal due to neurological and cardiovascular complications.[2] Plants represent the major source of thiamine in the human diet. However, the content of vitamin B1 in five main staple crops (rice, potato, maize, cassava, and plantain) is below minimal requirements. Thiamine deficiency is therefore especially a problem in developing countries, where a varied diet is lacking and the population often depends on one single crop for the majority of their energy supply. Biofortification via genetic modification is a cost-effective strategy to improve the nutritional quality in staple crops.[3] However, such strategies require a detailed knowledge of thiamine biosynthesis in plants (depicted in Figure 1[4−6]) to select the most promising candidates for metabolic engineering. Due to its small, diploid, and fully sequenced genome, *Arabidopsis thaliana* is known to serve as a reference for plant molecular genetics.[7] It is thus perfectly suited for genetic research to gain new insights into (regulation of) thiamine biosynthesis. In turn, evaluation of the impact of genetic engineering strategies largely relies on the availability of validated bioanalytical strategies to determine the intermediate (HMP & HET) and end products (thiamine, thiamine monophosphate (TMP) and TDP) of the biosynthesis pathway. To limit the workload, ideally one easy-to-apply methodology should be available to analyze in one single run both the intermediates and the thiamine vitamers. The determination of thiamine, its derivatives and precursors presents some important analytical challenges. Thiamine is one of the most unstable B vitamins, only present in low amounts in biological matrices.[8] Although numerous methods for thiamine quantification using HPLC coupled to fluorescence detection have been reported,[9−16] most methods do not use an appropriate internal standard and require pre- or postcolumn derivatization to form the thiamine oxidation

Received: December 18, 2019
Accepted: February 14, 2020
Published: February 14, 2020

https://dx.doi.org/10.1021/acs.analchem.9b05717
Anal. Chem. 2020, 92, 4073–4081

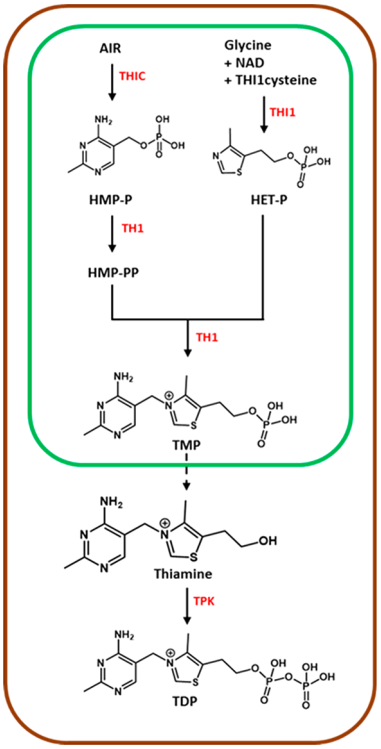


Figure 1. Simplified overview of thiamine biosynthesis in *Arabidopsis*. TDP is synthesized from two building blocks, which are formed via parallel pathways in the chloroplast of the plant. The first building block is the pyrimidine moiety 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P), which is formed from aminoimidazole ribonucleotide (AIR) via the THIC (phosphomethylpyrimidine synthase) enzyme. The second is the thiazole moiety 4-methyl-5-(2-hydroxyethyl) thiazole phosphate (HET-P), formed from glycine, nicotinamide adenine dinucleotide (NAD) and the sulfur of a backbone cysteine residue of THI1 (THI1cysteine) by the THI1 (thiamine thiazole synthase) enzyme. After an additional phosphorylation of HMP-P, the two precursors are condensed to thiamine monophosphate (TMP). These two latter reactions are catalyzed by the bifunctional enzyme TH1 (HMP-P kinase/TMP pyrophosphorylase), which possesses both HMP-P kinase and TMP synthase activities. Once TMP is dephosphorylated, thiamine is converted to its active diphosphate derivative (TDP) in the cytosol of the plant via thiamine pyrophosphokinase (TPK).

product thiochrome. This is not only time-consuming, but also involves the handling of noxious agents. Moreover, due to their highly polar character, the compounds of interest are not retained on regular C18 columns. In addition, the phosphate derivatives, TMP and TDP, may chelate metal ions, typically present in the LC−MS/MS instrument, e.g., in tubings, columns, etc., resulting in peak tailing. Last, due to the ionogenic properties of thiamine, peak tailing caused by secondary interactions with, e.g., silanol groups poses an additional challenge to cope with. To date, no HPLC−MS/MS method exists for the simultaneous determination of thiamine, its phosphate derivatives and its precursors. We developed a method that overcomes all the above-mentioned challenges and report here, for the first time, on the setup and application of a straightforward and fully validated HPLC−MS/MS method for the simultaneous determination of thiamine, TMP, TDP, HET, and HMP. This method, which offers sufficient sensitivity to be applied on minute amounts of *A. thaliana* or other plant samples, will allow to gain new insights into thiamine biosynthesis in plants. In addition, this will help to steer the effectiveness of future biofortification strategies in major food crops.

EXPERIMENTAL SECTION

Chemicals and Materials. Thiamine, ^{13}C labeled thiamine, thiamine diphosphate (TDP), thiamine monophosphate (TMP), 4-methyl-5-(2-hydroxyethyl) thiazole (HET), 2-amino-4-hydroxy-6-methylpyrimidine (HMP analogue), and 2-(1,3-thiazole-5-yl)-ethanol (HET analogue) were obtained from Sigma (Overijse, Belgium). 4-Amino-2-methyl-5-hydroxymethylpyrimidine (HMP) was purchased from TCI Europe (Zwijndrecht, Belgium). Deuterated TDP (D_3 TDP) was acquired from TRC (Ontario, Canada). The HET and HMP analogues were purchased as possible internal standards (ISs) for HET and HMP, due to the absence of commercially available labeled analogues. The structures of all analytes and ISs are given in Supporting Information (SI) Figure S-1. All stock solutions were prepared in a concentration of 10 mg/mL, except for ^{13}C thiamine and D_3 TDP, which had a concentration of 2 and 1 mg/mL, respectively. 2-amino-4-hydroxy-6-methylpyrimidine stock solution was prepared in 1N NH_4OH. HMP, HET, and 2-(1,3-thiazole-5-yl)-ethanol stock solutions were prepared in MeOH. Thiamine, ^{13}C thiamine, TDP, D_3 TDP, and TMP stock solutions were prepared in 0.1 M HCl. All standard and IS solutions were stored at −80 °C. Working solutions were prepared daily by appropriate dilution of the stock solutions in 0.1 M HCl. Amylase (A6255), protease (P5147), acid phosphatase (P1435), NaF, and β-glycerophosphate were purchased from Sigma. Hydrochloric acid (37%) and ammonia (25%) were obtained from VWR (Leuven, Belgium). Sodium acetate and ammonium bicarbonate (NH_4HCO_3) were acquired from Merck (Overijse, Belgium). Activated charcoal (untreated, granular, 8−20 mesh) was obtained from Honeywell (Bucharest, Romania). Ultrapure water was generated using a Synergy UV water purification system from Merck. LC−MS grade methanol and acetonitrile were obtained from Biosolve (Valkenswaard, The Netherlands). For ultrafiltration, 3 kDa molecular weight membrane filters from Merck were used. Homogenization was carried out with a Retsch Mill MM 301 (Aartselaar, Belgium). The leaves of wild-type (genetically unmodified) *Arabidopsis thaliana* (Colombia-O) material were used for the optimization of the sample preparation and validation. Plants were grown on soil at 22 °C and 60% humidity, and 16 h of light was alternated with 8 h of darkness. Finally, the plants were harvested after 50 days. Subsequently, plant material was stored at −80 °C until analysis.

Preparation of Calibrators and QCs. Due the absence of blank matrix, calibrators and QCs were prepared in charcoal-treated *Arabidopsis* as an alternative to the standard addition method. For each compound, the endogenous signal was below 20% of the LLOQ, therefore no subtraction of the endogenous level was performed. Calibrator concentrations ranged from 6.58−1316.75 μg/100 g for TDP; 0.85−169.16 μg/100 g for TMP; 3.36−1344.92 μg/100 g for thiamine; 0.08−41.38 μg/100 g for HET; and 0.16−81.64 μg/100 g for HMP (full overview in SI Table S-1 and explanation of calculations in SI Text S-1). QC solutions (LLOQ, LQC, MQC, HQC, respectively) were prepared similarly to the calibrators at the following concentrations: 6.52, 17.39, 115.90, 1159.05 μg/100 g for TDP; 0.84, 2.24, 14.92, 149.21 μg/100 g for TMP; 3.37, 8.98, 144.83, 1196.95 μg/100 g for thiamine;

https://dx.doi.org/10.1021/acs.analchem.9b05717
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0.08, 0.22, 3.59, 35.92 μg/100 g for HET; and 0.16, 0.44, 7.31, and 73.09 μg/100 g for HMP.

Sample Preparation. Due to the complexity of the matrix and the (in)stability of the compounds of interest, several parameters needed to be evaluated and optimized. Given that for the eventual application the availability of sample material would be limited, the minimal sample weight was assessed. Next, endogenous phosphatase activity should be inhibited at an early stage of the procedure to allow a correct assessment of the distribution of the different vitamers in the sample. Therefore, several approaches, such as initial heating (with evaluation of temperature, pH and time) and the addition of phosphatase inhibitors were evaluated for their ability to deactivate endogenous phosphatase. Subsequently, the minimal milling time to homogenize the sample was determined. As TDP serves as a cofactor of several enzymes, the addition of protease to liberate the analytes from their binding proteins was tested as well. There are no commercially available reference standards for the two main building blocks in thiamine biosynthesis, HET-P and HMP-P(P). Therefore, samples are split in two; to one part exogenous phosphatase is added to determine total HMP, HET and thiamine levels (i.e., the sum of both nonphosphorylated and phosphorylated). To the other part, the same volume of water is added to determine the individual vitamer levels (HET, HMP, thiamine, TMP and TDP). In that way, maximal information is obtained despite the absence of the appropriate standards. The optimal pH and temperature during incubation, number of phosphatase units, and time of phosphatase incubation were all evaluated. For each of the tested conditions, wild-type *Arabidopsis* samples were analyzed in triplicate and the final sample preparation method was selected based on a comparison of the peak area ratios obtained for each condition. Due to light sensitivity of the thiamine vitamers, all experiments were performed under subdued light.

Instrumental Conditions. A Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) coupled to a SCIEX API 5500 triple quadrupole mass spectrometer (SCIEX, Framingham, MA, U.S.A.) was used for all analyses. The hardware system was controlled by the SCIEX Analyst 1.6.2 software. A Phenomenex (Utrecht, The Netherlands) Gemini NX- C18 column (100 mm × 4.6 mm, 3 μm particle size), equipped with the corresponding SecurityGuard guard cartridge, was used for the separation of all 5 analytes. The column oven was set at 30 °C. The mobile phase consisted of 10 mM NH_4HCO_3 pH 8.8 (solvent A) and methanol (solvent B) and was pumped at a flow rate of 0.6 mL/min. The proportion of B was increased linearly from 0% to 50% in 3 min, followed by a rapid increase in 0.5 min to 95%, where it was kept for 2 min. Subsequently, the mobile phase was adjusted to its initial composition and held for 2.5 min for re-equilibration, resulting in a total run time of 8 min. The injection volume depended on the calibrator or the sample treatment (to prevent detector saturation); for the high calibrators and from phosphatase-treated samples only 5 μL was injected (due to the higher total levels), while from nonphosphatase-treated samples 10 μL was injected. On the mass spectrometer, all experiments were performed in positive electrospray ionization (ESI) mode. The source temperature was set to 500 °C and the ion spray voltage to 4500 V. Nitrogen was used as gas 1 (55 psig), gas 2 (50 psig), curtain gas (30 psig), and collision-activated dissociation gas ("high"). Detection of the analytes was carried out in the multiple reaction monitoring mode (MRM) mode by monitoring two characteristic precursor-to-product ion transitions with a dwell time of 30 ms. Compound-specific parameters, as well as the MRM transitions, are given in SI Table S-2.

Method Validation. Method validation was based on U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) guidelines for bioanalytical method validation and covered selectivity, accuracy, precision, carry-over, calibration model, dilution integrity, matrix effect, recovery, and stability.[17,18] Since no analyte-free matrix was available, *Arabidopsis* was treated with activated charcoal to obtain a surrogate "blank" matrix with an endogenous level below 20% of the LLOQ of all analytes. To assess selectivity, transition ratios were compared between neat standard solutions on the one hand and spiked blanks, spiked samples, and original, nonspiked samples on the other hand. The absence of interferences was accepted when the mean ratio of the spiked blanks and samples (spiked and native) was within the tolerated window[19] of the ratio of the neat standard solutions (see SI Table S-3). Carry-over was examined by analysis of three blank samples after measurement of four times the highest calibrator. Carry-over for the analytes should not exceed 20% of the peak area found for the LLOQ and 5% for the IS. The calibration model was evaluated by the analysis of six calibration curves over a total of three different days ($n = 3 \times 2$). An F-test was performed to investigate homoscedasticity ($\alpha = 1\%$) at the highest and lowest calibrator level. Selection of the calibration model was based on the calculation of the sum % residual error (%RE) and the plot of the %RE against the nominal concentrations. A linear and a quadratic regression model were evaluated with or without weighing factors ($1/x$, $1/x^2$, $1/y$, $1/y^2$, $1/\sqrt{x}$, and $1/\sqrt{y}$). The calibration model with the lowest %RE and no trend in the %RE plot should be chosen. Statistical analysis to substantiate this choice was performed using an R-script developed by Desharnais et al.[20] To test the selected model for goodness of fit, the %bias of the back-calculated concentrations of the calibrators from their nominal value was determined. The %bias should be less than 15% (and 20% for the LLOQ) for 75% of all calibrators to accept the chosen model. Accuracy (%bias) and precision (% CV) were assessed by analyzing QCs (LLOQ, LQC, MQC, and HQC) in duplicate on four different days ($n = 4 \times 2$). The intra- and total-batch precision were determined using ANOVA,[21] whereas the accuracy was calculated by dividing the difference between the mean calculated concentration and the nominal value by the nominal value, and multiplying by 100. The %bias and %CV should be within ±15% for the QC samples, except for the LLOQ, where they should be within ±20%. The measurement uncertainty for every analyte was calculated by the sum of the total CV (based on the analysis of native samples), the impurity of the analytical standard, the uncertainty on the weighing (powder and solvent) and possible unattributed variances.[22] Matrix effect (ME) and recovery (RE) were evaluated based on the method suggested by Matuszewski et al.[23] Due to the absence of blank matrix, calibrators and QCs were prepared in charcoal-treated matrix. Therefore, ME was evaluated for both charcoal-treated matrix, and *Arabidopsis* itself, with and without phosphatase treatment. Extracts ($n = 6$) from these different matrices were spiked at low and high concentrations. High concentrations corresponded to HQC level in charcoal-treated *Arabidopsis*. Low concentrations were adjusted depending on the endogenous level of the specific matrix (SI Table S-4). The low

https://dx.doi.org/10.1021/acs.analchem.9b05717
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concentration was chosen to be at least 100% of the endogenous level. To correct for endogenous levels, samples were split in two, post extraction. To one part of the sample a mixture of analytes with ISs was spiked (B), while to the other part only a mixture of ISs was spiked (B0). Neat solvent (water) was spiked with the same low and high concentrations (A), as well as with the IS mixture. The absolute ME was calculated by first correcting the peak area of (B) for the endogenous peak area (B0). Thereafter, the corrected area [(B)−(B0)] was divided by the area of (A). The comparison of the absolute ME of an analyte with the absolute ME of its IS results in the relative ME, of which the %CV should not exceed 15%. RE was assessed within the same experiment as matrix effect and evaluated by spiking samples before (C) and after extraction (B) (n = 6). To correct for endogenous levels, 6 samples were included which were only spiked with an IS mixture prior to extraction (C0). Recovery was calculated by dividing the peak area ratio of [(C)−(C0)] by the peak area ratio of [(B)-(B0)]. Although no acceptance criteria were set for RE, %CV should be within 15%. The purpose of the analytical method is to quantify metabolites related to thiamine biosynthesis in different conditions. This includes metabolic engineering approaches, where a calibration model encompassing the widest possible range is needed to evaluate the effectiveness of genetic modification strategies. However, as genetic modifications may lead to concentrations even higher than the upper limit of quantification (ULOQ), we investigated whether final extracts of samples can be safely diluted. Samples were processed and final extracts were diluted 5- and 10-fold with water (n = 6). The results of the diluted samples were compared to the results of the undiluted samples, which were analyzed in the same run. As dilution of samples should not affect the accuracy and precision, the set %bias and %CV should be within 15%. Short- and long-term stability of the start material was evaluated for 2 weeks, 1 month and 3 months at −20 and 4 °C, whereby storage at −80 °C was used as the reference (n = 3 per condition). The mean concentration of the samples at a given storage condition should be within 15% of the mean concentration of the samples stored at −80 °C. Three-month stability of the stock solutions at −80 °C was evaluated against freshly prepared stocks (n = 3). Additionally, autosampler stability (48 h at 4 °C) of processed samples (both QC and real samples) and stability after 3 freeze−thaw cycles after 2 weeks of storage at −80 °C and −20 °C was determined (n = 3).

Application. To objectively evaluate the performance of our method, the method was applied on wild-type and genetically modified *Arabidopsis* samples (4 lines; Strobbe et al.; unpublished data) which were grown on half strength Murashige and Skoog medium supplemented with 10 g/L sucrose at 22 °C and 100% humidity. In the growth chamber, 16 h of light was alternated with 8 h of darkness. Plants were harvested 15 days after germination. Subsequently, the seedlings were stored at −80 °C until analysis. Three biological replicates were assessed for each line.

RESULTS AND DISCUSSION

Chromatography. The chromatographic separation of thiamine, its precursors and phosphate derivatives coupled to MS/MS detection is an extremely challenging task. Due to the highly polar character of these compounds, HILIC is often recommended to increase retention.[24,25] However, TDP and TMP were insoluble in the high organic injection solvent which is required. The Phenomenex Gemini C18 column (100 mm × 4.6 mm, 3 μm particle size), claimed to withstand moderately alkaline conditions, demonstrated sufficient retention and separation of the phosphate derivatives with a mobile phase between pH 8.8−10. Most published methods, based on HPLC-fluorescence determination of thiamine, make use of a phosphate buffer as mobile phase. However, due to its nonvolatility, this buffer is incompatible with MS/MS detection. Therefore, the final mobile phase A consisted of 10 mM NH_4HCO_3. Although the highest sensitivity and resolution of TMP and TDP were obtained at pH 10, this high pH proved detrimental for the stability of the silica-based column. Although bringing the pH to 8.8 initially yielded promising results, also this pH turned out to be problematic for the Gemini C18 column, with a clear deterioration of the column (as evidenced by substantial peak broadening) after only a few hundred injections. Subsequently, a Gemini NX-C18 column (100 mm × 4.6 mm, 3 μm particle size) was evaluated at the same settings (pH 8.8). This column, in which stabilizing ethane cross-linking is incorporated onto the silica surface, showed sufficient robustness, with more than 5000 injections up till now. During chromatographic development, we observed in-source fragmentation of thiamine to HET. Therefore, baseline separation of thiamine and HET was essential. Following optimization of the gradient, all five compounds could be simultaneously analyzed and baseline separated within 2.5 min, in an 8 min run (Figures 2 and SI S-2).

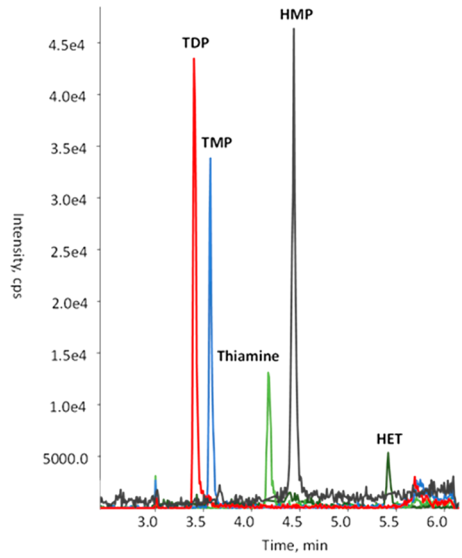

Figure 2. Chromatogram of TDP, TMP, thiamine, HMP, and HET in charcoal-treated *Arabidopsis* matrix at LLOQ level.

Sample Preparation. The complexity of the sample matrix, combined with the (in)stability of some of the target analytes, posed an additional challenge. As TDP, the most labile form, can be converted to all other analytes of interest, the sample preparation was optimized to obtain the highest TDP signal. This allows the most accurate determination of the vitamer distribution in *Arabidopsis* samples. First, as we needed to anticipate that the method was to be applied on minute amounts of genetically engineered plant material, we evaluated different sample weights (50, 100, 150, 200, and 250 mg) to minimize the amount of required material. Mainly due

https://dx.doi.org/10.1021/acs.analchem.9b05717
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to low HET levels, 100 mg was set as the minimal sample weight to still allow accurate quantification of all target analytes. Originally, samples underwent an initial cooking step for 30 min in 1 mL 0.1 M HCl to deactivate endogenous phosphatase and liberate the vitamers from their binding proteins via acid hydrolysis. However, due to degradation of TDP during this step, alternatives were investigated. First, the addition of phosphatase inhibitors NaF (1 M) and β-glycerophosphate (200 mM) was tested. Neither of these could inhibit the endogenous acid phosphatase, which is in accordance with findings by Hälvin et al.[26] Therefore, we considered fine-tuning the heating step to inactivate phosphatase, while limiting TDP degradation. First, extraction solvents at different pHs (0.75−3−4.5−7) were evaluated. However, upon heating more HET and HMP were formed from TDP in H_2O (pH 7) and at pH 3 and 4.5 in aqueous NH_4 formate buffer, with the highest levels found at pH 4.5. Second, shortening the duration of the heating (10 min) at 100 °C did not result in complete liberation from the binding proteins: TDP levels were almost 3 times lower than when samples were cooked for 30 min, despite the instability. Finally, reducing the heating temperature to 74 °C during 30 min resulted in the highest absolute TDP signal. Next, we evaluated whether subsequent addition of protease (265 units) would further liberate the vitamins from their binding proteins. To this end, the pH of the samples was adjusted to the optimal pH for protease activity (pH 7.2). Yet, there was no significant difference between samples treated or not with protease. Consequently, we concluded that the liberation of the vitamins from their binding proteins was maximal and complete after 30 min heating at 74 °C in 0.1 M HCl. Homogenization of the samples requires milling in a Retsch Mill. We observed that this process (originally 30 min), when performed in the absence of matrix, resulted in the degradation of D_3 TDP to D_3 thiamine (which we also included in our method). Moreover, we noticed that the degree of degradation and the variability in degradation increased with the milling time (SI Figure S-3). Therefore, we reduced the milling time to 10 min, which was still sufficient to homogenize the samples without any significant degradation (SI Figure S-4). Thereafter, the pH was adjusted to 5.5 with 170 μL 2.5 M NaAcetate to ensure optimal phosphatase activity. The addition of acid phosphatase to one-half of the sample (the other half is left untreated) enables us to distinguish between total and nonphosphorylated HMP and HET (cfr. section "sample preparation"). First, the incubation time was evaluated, following the addition of 10 units of phosphatase to all samples. However, even after 72h incubation at 45 °C no plateau for HMP and HET was reached (SI Figure S-5.A). Simultaneously, samples were incubated without phosphatase to gain more information about the stability of HET and HMP at these incubation conditions (SI Figure S-5.B). Mainly due to practical considerations we continued with an incubation time of 24 h. Last, the number of phosphatase units (2−4−6−8−10 and 15 units) was evaluated to obtain maximal HMP and HET levels during this 24 h incubation step. For HMP, there was no significant difference between 10 and 15 units. For HET, there was no significant difference between 8, 10, and 15 units (SI Figure S-6). Therefore, we continued with 10 units of phosphatase. The final sample preparation looked as follows: to 100 mg of *Arabidopsis* material, 1 mL 0.1 M HCl, containing the ISs (D_3 TDP, 200 ng/mL; ^{13}C thiamine, 66.67 ng/mL; HET analogue, 30 ng/mL; HMP analogue, 30 ng/mL), was added. After heating at 74 °C during 30 min, the samples were milled in a Retsch mill during 10 min at 30 Hz. Subsequently, the pH was adjusted to 5.5 with 170 μL 2.5 M NaAcetate. Next, the samples were split in two (2 × 500 μL): to one part 10 units of acid phosphatase were added, followed by incubation at 45 °C for 24 h. To the other part, the same volume of water was added, followed by incubation at 4 °C for 24 h. Finally, all samples were ultrafiltered (3 kDa filters) for 15′ at 12 000 *g* and transferred to vials for analysis.

Method Validation. Although no labeled ISs for HET and HMP were commercially available, an HET and HMP analogue were purchased and both were evaluated during validation. Based on the results, the HET analogue was the best suited IS for both HET and HMP. Although also for TMP a labeled IS is lacking, the purchased D_3 TDP contained D_3 TMP as an impurity. Therefore, the results of TMP were evaluated with both D_3 TDP and D_3 TMP as IS. Since initial results were not conclusive for one specific IS, both TMP ISs were considered throughout the validation. Regarding selectivity, no unacceptable interferences were observed. The transition ratios for all target analytes were within the tolerated window of the ratio of the neat standard solutions (SI Table S-3). No unacceptable carry-over was noticed for thiamine, HET, HMP, and all ISs, while carry-over, exceeding the acceptance criterion (20% of LLOQ), was present for TMP and TDP, following injection at 4×ULOQ. This carry-over issue was not located at the level of the needle, as it was observed after repeating the gradient, even without needle intervention (SI Figure S-7). This carry-over issue might be explained by the chelating characteristics of TMP and TDP, which is of high relevance (and intrinsically present) when using a metal-based column.[27] For TMP, carry-over is only relevant starting from a concentration of 34 μg/100 g (which corresponds to ∼3 times the level in wild-type samples) and can be solved by injection of one blank in between samples with concentrations above 34 μg/100 g. For TDP, carry-over is relevant starting from a concentration of 132 μg/100 g (which is ∼4 times the level in wild-type samples) and can be solved by injection of one blank between samples with concentrations between 132 and 658 μg/100 g and two blanks between samples with concentrations above 658 μg/100 g. Blanks can be run in a shorter 5 min run, which benefits the sample throughput. As TMP and TDP levels in genetically modified lines cannot be predicted, this implies that measures should be taken to minimize carry-over. One of these measures is that, within one run, phosphatase-treated samples should be analyzed before the nontreated samples to avoid carry-over issues. As the calibration data (n = 6) for all analytes were heteroscedastic, weighing was required. A linear regression model with $1/x^2$ weighing gave the lowest %RE for thiamine, HET, TMP, and TDP. For HMP, a quadratic regression model with weighing factor $1/x^2$ resulted in the lowest %RE. Statistical analysis to substantiate this choice was performed using an R-script developed by Desharnais et al.[20] Using the selected models, back-calculation of the calibrators met the preset acceptance criteria. To avoid detector saturation (an issue seen at the highest concentrations of HMP), only 5 μL of the highest calibrators was injected. This allows to extend the calibration range,[28] rendering it possible to quantify potentially very high concentrations in the genetically modified lines. For all analytes, a bias below 12.5% was found for all concentration levels. The repeatability and total imprecision for QCs, analyzed in duplicate on four different days, fulfilled the preset criteria (CV% < 15%, 20% for

https://dx.doi.org/10.1021/acs.analchem.9b05717
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Table 1. Accuracy (bias%) of QCs of HET, HMP, Thiamine, TDP and TMP at Four Concentration Levels in Charcoal-Treated *Arabidopsis* Matrix. Repeatability and Total Imprecision (CV%) of both QC and Native Samples.[a]

Accuracy (bias%) (n = 4 × 2)						
	HET (%)	HMP (%)	Thiamine (%)	TDP (%)	TMP (D_3TDP) (%)	TMP (D_3TMP) (%)
LLOQ	−0.70	−0.90	−4.10	−6.30	1.00	−2.00
LQC	−5.70	−0.30	−2.90	3.60	−3.60	1.20
MQC	−1.50	6.10	−12.30	1.40	−1.80	1.90
HQC	−1.90	6.00	−1.60	−5.40	−7.00	−3.60
Repeatability (CV%) (QCs: n = 4 × 2; samples: n = 3 × 3)						
	HET (%)	HMP (%)	Thiamine (%)	TDP (%)	TMP (D_3TDP) (%)	TMP (D_3TMP) (%)
LLOQ	13.00	9.00	10.70	15.30	16.70	12.30
LQC	2.00	7.20	11.40	7.70	8.20	11.20
MQC	8.10	9.50	9.00	6.60	10.90	6.80
HQC	3.70	4.60	11.80	5.20	9.70	11.90
A NP	7.40	7.90	6.90	3.40	6.00	12.80
A P	2.50	6.30	7.80	N/A	N/A	N/A
Total imprecision (CV%) (QCs: n = 4× 2; samples: n = 3 × 3)						
	HET (%)	HMP (%)	Thiamine (%)	TDP (%)	TMP (D_3TDP) (%)	TMP (D_3TMP) (%)
LLOQ	13.00	11.10	13.60	15.30	16.70	13.50
LQC	2.80	7.20	11.40	11.50	8.20	11.20
MQC	8.50	9.50	10.00	6.60	10.90	13.50
HQC	5.20	7.50	11.80	5.20	9.70	11.90
A NP	7.40	7.90	6.90	6.50	6.00	12.80
A P	6.10	6.30	7.80	N/A	N/A	N/A

[a]Native samples were treated both with (A P) and without (A NP) phosphatase. In phosphatase-treated samples TDP and TMP are completely converted, indicated by N/A.

LLOQ), as shown in Table 1. Also for native, nonspiked samples (phosphatase-treated and nontreated), analyzed in triplicate on three different days, the precision criterion was met (Table 1). The measurement uncertainty for all analytes was ≤15%, which can definitely be considered acceptable, given the matrix and the challenging nature of the analytes of interest. Although the calibration range was set to quantify potentially high increases in analyte levels compared to wild-type, the genetic engineering strategies may lead to concentrations even exceeding the upper limit of quantification (ULOQ). The dilution integrity experiment showed that samples could be diluted 5-fold as well as 10-fold without compromising the accuracy and precision (SI Table S-5). The matrix effect was evaluated both in charcoal-treated matrix, as in wild-type *Arabidopsis* samples with and without phosphatase treatment. IS-corrected results should be similar between charcoal-treated and *Arabidopsis* matrix to justify the use of the calibrators, prepared in charcoal-treated matrix, to quantify the native samples. The results in Table 2 show that the IS-corrected matrix effects for HET, HMP, thiamine and TDP were all within 88−106%, indicating that the IS compensates for potential differences in ionization. Additionally, the CV% was ≤15%, meeting the preset acceptance criteria. Furthermore, there were no significant differences in IS-corrected matrix effect between the different matrices (charcoal-treated matrix, phosphatase-treated and nonphosphatase-treated *Arabidopsis* matrix). For TMP, results were evaluated with both D_3 TDP and D_3 TMP as IS. When using D_3 TDP, matrix effects were compensated and there were no significant differences between the different matrices. When applying D_3 TMP, the IS-corrected results did not significantly differ between both matrices. However, D_3 TMP did not completely compensate for the suppression in ionization.

Recovery was evaluated by spiking either charcoal-treated matrix or native *Arabidopsis* at low and high concentration before and after extraction. Recoveries for the different analytes varied from 51% to 144%, but were reproducible (CV < 15%, except for TMP (D_3 TMP) at HQC in nonphosphorylated *Arabidopsis*, CV = 16%). The IS-corrected results are summarized in Table 2. There is a large difference in IS-corrected recovery for TMP, depending on what IS is applied. However, results are reproducible and similar between charcoal-treated matrix and native *Arabidopsis*. Therefore, we do not expect any dissimilarities in quantitative results caused by this difference in IS-corrected recovery. Stability is an essential aspect when analyzing thiamine, its phosphates and precursors, and particular attention was given to stability during the optimization of the sample preparation. The stability experiments revealed that HET, thiamine, TDP and TMP were stable in the extracts of both charcoal-treated matrix and *Arabidopsis*, when kept in the autosampler for 48 h at 4 °C, as shown in Table 3. However, HMP levels tend to slightly increase, which might be caused by instability of the thiamine vitamers. Consequently, we recommend to minimize the total run time (<48 h) of a sample batch to avoid stability issues. Additionally, we have shown that QCs and samples can be reanalyzed after 2 weeks of storage at either −20 °C or −80 °C, when the original calibration curve was rerun (Table 3). Stock solutions of all analytes were stable when stored at −80 °C for 3 months. The start material should be stored at −80 °C to ensure stability of all analytes during short- and long-term storage (up to 3 months) (SI Table S-6).

Application. A proof-of-principle of the method's applicability was demonstrated by determining thiamine vitamers and precursors in wild-type (WT) and genetically modified (M lines) *Arabidopsis* seedlings, grown on plates (Figure 3). Following sample preparation, one aliquot was treated with

https://dx.doi.org/10.1021/acs.analchem.9b05717
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Table 2. IS-Corrected Matrix Effect (ME) and Recovery (RE) (n = 6) for HET, HMP, Thiamine, TDP, and TMP for Both Charcoal-Treated *Arabidopsis* (QC) and Phosphatase-Treated (A P) and Non-treated *Arabidopsis samples* (A NP).

	HET		HMP		Thiamine		TDP		TMP (D_3TDP)		TMP (D_3TMP)	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
QC												
ME% (CV%)	95 (6)	99 (5)	98 (10)	103 (3)	90 (9)	94 (10)	99 (8)	101 (10)	106 (13)	103 (8)	80 (14)	79 (9)
RE% (CV%)	91 (11)	99 (4)	101 (4)	100 (5)	92 (7)	98 (6)	89 (10)	111 (4)	144 (13)	129 (12)	53 (8)	51 (13)
A NP												
ME% (CV%)	95 (8)	101 (4)	88 (12)	102 (4)	101 (13)	100 (4)	102 (8)	102 (11)	111 (9)	107 (6)	85 (8)	80 (7)
RE% (CV%)	102 (7)	101 (5)	109 (11)	107 (7)	119 (9)	100 (8)	103 (15)	10 (9)	114 (13)	131 (12)	57 (15)	66 (16)
A P												
ME% (CV%)	105 (9)	106 (5)	92 (6)	100 (4)	100 (14)	95 (7)	N/A	N/A	N/A	N/A	N/A	N/A
RE% (CV%)	98 (8)	95 (7)	91 (6)	102 (3)	100 (13)	96 (10)	N/A	N/A	N/A	N/A	N/A	N/A

acid phosphatase to determine total thiamine, HET and HMP levels, while the other aliquot was left untreated, allowing the determination of the individual vitamers and precursors. Although method validation had revealed some inherent carry-over for TMP and TDP, we found this not to be a relevant issue during application. First, phosphatase-treated samples were analyzed before the nontreated samples, and second, TMP and TDP levels of the nonphosphatase treated samples were all of the same magnitude. Since the method validation did not unequivocally indicate what IS was best used for TMP, its concentrations were calculated using either D_3 TDP or D_3 TMP, revealing no significant differences (SI Figure S-8). In the end, we opted for D_3 TDP as IS for TMP, primarily because of practical considerations: as D_3 TMP was present as an impurity, we cannot predict whether D_3 TMP will always be present in every lot of purchased D_3 TDP. Since our method has a total measurement uncertainty of ≤15% for all analytes, we can only make statements about changing vitamer levels when the differences between the different lines are higher than 30%. From Figure 3, it is clear that the distribution of the vitamers and precursors in the M lines differs from that in the WT line. This demonstrates the fit-for-purposeness of the developed methodology to allow selection of the most promising lines, based on the analysis of thiamine and its precursors in minute amounts of plant material. It should be noted that discussing the actual selection of the M lines is not within the scope of this work, but will be discussed elsewhere (Strobbe et al., manuscript in preparation). An interesting observation we made is that the mean, non-phosphorylated thiamine level in seedlings grown on plates (<3.36 μg/100 g) was lower than that observed during optimization and validation of the method, using leaf material from plants grown in soil (12 μg/100 g). While not hampering evaluation of the M lines, it will be interesting to pursue this observation in future experiments, evaluating different growth and stress conditions, and plant materials. Noteworthy, the different nonphosphorylated thiamine levels from *Arabidopsis* grown on plates and in soil bracketed those reported in literature for wild-type leaves (5.5 μg/100g; Dong et al. 2015[6]) and seedlings (3.6 μg/100 g; Pourcel et al. 2013[29]). TDP and total thiamine levels (both 30−40 μg/100 g) in leaves were similar to those reported by Dong et al. (both approximately 40 μg/100 g). TDP results in seedlings (80 μg/100 g were 3× lower than those reported by Pourcel et al. (255 μg/100g), while TMP results were very comparable. Also here, growth conditions may explain the observed differences—again, the methodology presented here is ideally suited for such evaluation. To the best of our knowledge, this report is the first to quantify HET and HMP in plant material, therefore, no reference values are available.

CONCLUSIONS

Thiamine is an essential vitamin and plays a crucial role in energy metabolism. Thiamine deficiency is mainly a problem in developing countries, where a varied diet is lacking and people mainly rely on staple crops, e.g., rice, with low thiamine content for their energy supply. Biofortification, via genetic engineering, is a cost-effective strategy to improve the nutritional quality of staple crops.[30,31] However, insights in thiamine biosynthesis in plants are essential to successfully setup novel engineering strategies. Therefore, modulation of the thiamine biosynthesis pathway in *Arabidopsis thaliana*, the model plant for genetic research, was investigated. The

https://dx.doi.org/10.1021/acs.analchem.9b05717
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Table 3. Stability Data for HET, HMP, Thiamine, TDP, and TMP in Both QCs at High and Low Concentration and Real Samples, Treated (A P) and Non-treated (A NP) with Phosphatase (n = 3).

	HET (%)	HMP (%)	Thiamine (%)	TDP (%)	TMP (D_3TDP)	TMP (D_3TMP)
autosampler stability (48h at 4 °C, n = 3)						
LQC	111	116	93	93	108	108
HQC	113	117	102	105	112	104
A NP	109	116	111	110	89	102
A P	114	113	106	N/A	N/A	N/A
freeze/thaw stability (2 weeks at −80 °C, n = 3)						
LQC	98	112	105	116	105	92
HQC	95	92	98	98	103	83
A NP	102	110	93	100	85	93
A P	113	99	104	N/A	N/A	N/A
freeze/thaw stability (2 weeks at −20 °C, n = 3)						
LQC	102	103	105	103	90	104
HQC	100	98	95	100	102	99
A NP	95	102	101	105	85	102
A P	112	96	106	N/A	N/A	N/A

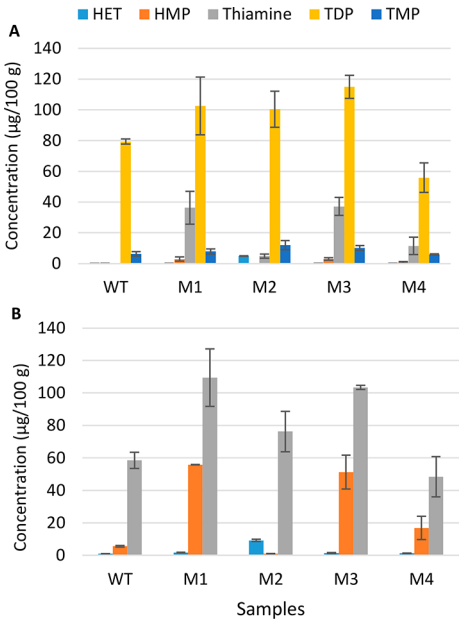

Figure 3. Distribution of thiamine, its phosphate derivatives and precursors in genetically modified *Arabidopsis* seedlings, with (B) and without (A) phosphatase treatment.

bioanalytical determination of the intermediate and end products of the biosynthesis pathway not only allows a better insight into thiamine biosynthesis and its regulation, it also serves as a guidance for the effectiveness of the engineering strategy. This study is the first to report on the development, validation, and application of an LC−MS/MS method for the determination of thiamine, its phosphate derivatives (TMP and TDP) and intermediates (HET and HMP). Thorough optimization of the sample preparation procedure enabled an accurate determination of the vitamer distribution in *Arabidopsis* samples. The method fulfilled all preset acceptance criteria and the application on genetically modified *Arabidopsis* lines demonstrated its validity. This method may serve as a basis to investigate (modulation of) the thiamine pathway in other plants, including different staple crops, low in thiamine content, e.g., rice.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.9b05717.

Text S-1: Concentration of the calibrators and samples (µg/100 g vs ng/mL); Figure S-1, chemical structures of all analytes and ISs; Figure S-2, chromatogram of all analytes in *Arabidopsis*; Figures S-3 and S-4, influence of milling time on the stability of D_3 TDP in extraction solvent (3) and samples (4); Figure S-5, HET and HMP signals in function of phosphatase incubation time; Figure S-6, HET and HMP ratios in function of number of phosphatase units; Figure S-7, extracted chromatogram of TDP when gradient was repeated 3 times within one analytical run; Figure S-8, TMP level in genetically modified *Arabidopsis* samples, calculated with both ISs; Table S-1, concentration of the calibrators (µg/100 g); Table S-2, MRM transitions and compound-specific MS parameters; Table S-3, results selectivity; Table S-4, spiked concentrations matrix effect and recovery; Table S-5, results dilution integrity; and Table S-6, results long- and short-term stability start material (PDF)

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https://dx.doi.org/10.1021/acs.analchem.9b05717
Anal. Chem. 2020, 92, 4073−4081

Author Contributions

The manuscript was written through contributions of all authors. All authors have approved the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

J.V. would like to thank the Research Foundation Flanders (FWO) for granting her a PhD fellowship (1S61617N). S.S. would like to thank the Bijzonder Onderzoeksfonds (BOF) for granting him a Post-Doc fellowship (BOF.P-DO.2019.0008.01). D.V.D.S. and C.S. are grateful to Ghent University for financial support (GOA 01G00409; Bijzonder Onderzoeksfonds UGent).

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https://dx.doi.org/10.1021/acs.analchem.9b05717
Anal. Chem. 2020, 92, 4073−4081