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An optimized LC-MS/MS method as a pivotal tool to steer thiamine biofortification strategies in rice

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Abstract

In developing countries, people mainly depend on rice as their primary source of calories. However, the thiamine content of rice is below minimal requirements. Biofortification, via genetic engineering, is a cost-effective strategy to increase thiamine content in rice. We report on the optimization of a matrix-specific method, including extensive optimization of the sample preparation to ensure maximal sensitivity and stability. The LC-MS/MS method was fully validated for the simultaneous quantification of thiamine, its precursors 4-methyl-5-(2-hydroxyethyl) thiazole (HET) and 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP) and its diphosphate derivative (TDP) in both polished and unpolished rice. Bias was below 9% for all analytes and total imprecision (CV%) was within pre-set acceptance criteria ($\leq 15\%$) for both QCs and real samples. Thiamine monophosphate (TMP), for which no labeled analogue was available at the time of analysis, was determined without internal standard. Although both accuracy and precision criteria were met (bias and CV $< 12\%$), the determination of TMP was considered semi-quantitatively. Moreover, TMP was found to be only a minor thiamine form ($< 1\%$ of total thiamine in all lines analyzed, both wild-type and genetically engineered), with measurable levels only present in unpolished rice. Finally, the validity and applicability of the procedure were demonstrated via its successful application on rice lines, genetically engineered to enhance thiamine content. Consequently, this method allows to evaluate the success of biofortification strategies in rice.

Keywords. Thiamine, rice, biofortification, liquid chromatography, tandem mass spectrometry

1. Introduction

Thiamine or vitamin B1 is an essential micronutrient for human health. Following dietary ingestion, thiamine is converted to its biologically active form thiamine diphosphate (TDP), which serves as a cofactor of several key enzymes involved in energy metabolism. Since thiamine stores in the body are relatively small and its half-life is only 9-18 days, humans primarily rely on the diet for their daily supplementation [1]. Acute thiamine deficiency manifests as beriberi, which can cause detrimental neurological and/or cardiovascular effects with a fatal outcome when not treated [2]. Thiamine is mainly obtained from animal products (meats, liver, eggs, dairy products), beans, peas, nuts and whole grains [3].

In the past half century many efforts have been made to tackle hunger (macronutrient malnutrition), which resulted in low-priced calorie-rich staple crops such as rice. Rice (*Oryza sativa*) is the most important staple crop for human consumption. However, the thiamine content in rice is below minimal requirements (0.02 mg/100 g cooked white rice, RDA (recommended daily allowance) = 1.2-1.4 mg/day) [2]. This is mainly caused by polishing and cooking of the rice, which depletes the thiamine content. Therefore, thiamine deficiency is widespread in developing countries, where a varied diet is lacking and people mainly rely on polished, white rice for the majority of their energy supply. Several strategies to improve the nutritional quality of rice have been reported, such as dietary diversification, supplementation, fortification and parboiling of rice. However, these strategies are less appropriate for developing countries as they are expensive and often involve behavioral changes. On the other hand, biofortification, via genetic engineering, has been stated as a sustainable and cost-effective strategy to increase the thiamine content in rice [4,5].

The evaluation of the effectiveness of such engineering strategies relies to an important extent on the bio-analytical determination of the intermediates (4-methyl-5-(2-hydroxyethyl) thiazole (HET) and 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP)) and end products (thiamine, thiamine mono- and diphosphate (TMP, TDP)) of the biosynthesis pathway in rice. Several methods for thiamine quantification in food matrices by using HPLC coupled to fluorescence detection have been

reported [6-14]. However, considering the fact that in the early genetic engineering phase only minute quantities of rice are available, the sensitivity offered by LC-MS/MS analysis makes it the method of choice to accurately determine the distribution of the analytes of interest (HET, HMP, thiamine, TMP and TDP). Moreover, the above-mentioned HPLC-fluorescence methods did not determine the precursors of thiamine biosynthesis, HET and HMP, although insight into their modulation is essential information when aiming at implementing a rational engineering strategy. We previously reported on the chromatographic challenges we have overcome during the set-up of an LC-MS/MS method for the determination of thiamine, its intermediates and phosphate derivatives in *Arabidopsis thaliana* [15]. However, determination of these analytes in the starch-rich rice matrix implies a substantially different work-up, requiring extensive optimization of the sample preparation, taking into account factors such as matrix effect, recovery and stability. Furthermore, the different vitamer distribution in the mature, dry rice seeds (both polished and unpolished) necessitates the adaptation of the calibration range. Therefore, we here report on the optimization, validation and application of an HPLC-MS/MS method for the simultaneous quantitative determination of thiamine, TDP, HET and HMP in both unpolished and polished rice and the semi-quantitative determination of TMP in unpolished rice, for comparative purposes. This method allows to evaluate the effectiveness of ongoing genetic modifications in rice and will help to steer future genetic engineering strategies.

2. Materials and methods

2.1 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 Suppl. 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) and acid phosphatase (P1435) 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). Samples were ultrafiltered in 3 kDa molecular weight membrane filters from Merck using a Centrifuge 5804 R from Eppendorf. Homogenization in extraction solvent (0.1 M HCl) was carried out with a Retsch Mill MM 301 (Aartselaar, Belgium). Rice samples were dehusked by a portable rice husker TR-130 and to obtain white (polished) rice, samples were further polished with the Pearliest Grain Polisher during 1 min. Both instruments were obtained from Kett (California, USA). Wild-type rice (*Oryza sativa* subsp.

japonica var. Nipponbare) seeds were used for the optimization of the sample preparation (unpolished) and validation (both polished and unpolished). During the first two weeks, rice plants were grown on half strength Murashige and Skoog medium supplemented with 10 g/L sucrose. Thereafter, plants were transferred on soil and further grown at 28°C and 80% humidity, under a photoperiod of 12h of light alternated with 12h of darkness. Finally, mature seeds were harvested after 4 months of growth on soil. Subsequently, the rice material (both polished and unpolished) was stored at -80°C until analysis.

2.2 Preparation of calibrators and QCs

Due to the absence of blank matrix, calibrators and QCs were prepared in charcoal-treated rice (unpolished) 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 0.82 – 205.40 µg/100 g for TDP; 0.53 – 52.65 µg/100 g for TMP; 2.69 – 2686.17 µg/100 g for thiamine; 0.16 – 82.05 µg/100 g for HET and 0.34– 84.90 µg/100 g for HMP (full overview in Suppl. Table S-1 and explanation of calculations in Suppl. Text S-1). QC solutions (LLOQ, LQC, MQC, HQC, respectively) were prepared similarly to the calibrators at the following concentrations: 0.82, 2.19, 20.65, 183.59 µg/100 g for TDP; 0.53, 1.44, 5.30, 47.08 µg/100 g for TMP; 2.69, 7.07, 266.08, 2365.17 µg/100 g for thiamine; 0.16, 0.45, 8.26, 73.4 µg/100 g for HET and 0.34, 0.97, 9.02 and 80.18 µg/100 g for HMP.

2.3 Sample preparation

Due to the complexity of the matrix, several parameters needed to be re-evaluated and optimized compared to our method in *Arabidopsis thaliana* [15]. 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, initial heating (with evaluation of temperature and time) was evaluated to deactivate endogenous

phosphatase. Moreover, since rice is a starch-rich matrix, the addition of amylase was evaluated to further release the analytes of interest. In addition, as the analytes of interest might be bound to proteins (e.g. TDP serves as a cofactor of several enzymes), the addition of protease to liberate the vitamers from their binding proteins was tested as well. There are no commercially available reference standards for the direct phosphorylated precursors of thiamine, phosphorylated HET (HET-P) and (di)phosphorylated HMP (HMP-P(P)). Therefore, samples are split in two; to one part exogenous phosphatase is added to determine total HMP, total HET and total thiamine levels (i.e. the sum of both non-phosphorylated 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 number of phosphatase units and time of phosphatase incubation were evaluated. For each of the tested conditions, wild-type rice 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, using amber tubes and vials.

2.4 Instrumental conditions

Similar to our method in *Arabidopsis* [15], a Shimadzu Prominence HPLC system (Shimadzu, Kyoto, Japan) coupled to a SCIEX API 5500™ triple quadrupole mass spectrometer (SCIEX, Framingham, MA, USA) was used for all analyses. The hardware system was controlled by the SCIEX Analyst® 1.6.2 software. 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 were adjusted for the analysis in rice and are listed in Suppl. Table S-2. Briefly, all 5 analytes were separated via a Gemini NX®- C18 column (100

mm x 4.6 mm 110 Å, 3 µm particle size) from Phenomenex (Utrecht, the Netherlands). 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 was 10 µL. A chromatogram at LLOQ level is shown in Figure 1.

2.5 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 [16,17]. Since no analyte-free matrix was available, rice material 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 authentic non-spiked samples on the other hand, for both polished and unpolished rice. The absence of interferences was accepted when the mean transition ratio observed in the spiked blanks and samples (spiked and native) was within the tolerated window of that observed when analyzing neat standard solutions [18] (see Suppl. 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.* [19]. 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 three different days ($n=3 \times 2$). The intra- and total-batch precision were determined using ANOVA [20], 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 $\pm 15\%$ for the QC samples, except for the LLOQ, where they should be within $\pm 20\%$. Matrix effect (ME) and recovery (RE) were evaluated based on the method suggested by Matuszewski *et al.* [21]. 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 rice (both polished and unpolished) 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 rice. Low concentrations were adjusted depending on the endogenous level of the specific matrix (Suppl. Table S-4). The low 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%. 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=5). The results of the diluted samples were compared to the results of the non-diluted 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°C, 4°C and 28°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 (48h at 4°C) of processed samples (both QC and real samples) and stability after 3 freeze-thaw cycles after 2 weeks of storage at -20°C and -80°C was determined (n=3).

2.6 Application

To objectively evaluate the performance of our method, the method was applied on wild-type and genetically modified rice samples (4 lines; Strobbe *et al.*; unpublished data), obtained from plants grown under the same conditions, as described in section 2.1 'chemicals and materials'. Each line was evaluated as polished and unpolished, all assessed in triplicate.

3. Results and Discussion

3.1 Sample preparation

The sensitive and specific detection of the thiamine vitamers and precursors in a specific food matrix necessitates a dedicated sample preparation procedure. Using our previously described extraction procedure for the analysis of *Arabidopsis thaliana* samples as a basis (Suppl. Figure S-2) [15], we established and optimized an extraction protocol for rice. Importantly, the sample preparation procedure should ensure the stability of the labile thiamine vitamers. Since TDP, which can possibly be converted to all other analytes, is the most labile form, the sample preparation was optimized to obtain the highest TDP signal. This allows the most accurate determination of the vimer distribution in rice samples. First, we had to anticipate that we would only have access to minute amounts of engineered rice samples. We therefore evaluated the amount of starting material (50-100-150-200-250 mg) that would still allow adequate quantification of all analytes in unpolished rice, and, if possible, in polished rice. TMP and TDP, both present at low levels in unpolished and polished rice, were the critical analytes in this evaluation. In unpolished rice, 200 mg allowed the determination of both TDP and TMP, while in polished rice no signal at all was obtained for TMP, even when starting from 250 mg of material. Similar to unpolished rice, 200 mg of polished rice was sufficient to obtain a signal for TDP. As higher amounts of starting material were not considered an option, we opted for 200 mg as the minimal sample weight for both polished and unpolished rice samples. Next, an initial heating step (30 min) was applied for three reasons: 1) the heating step will denature and consequently inactivate the endogenous phosphatase; 2) it will release the thiamine vitamers from their binding proteins, and 3) it will soften the hard rice seeds before homogenization. Although heating at 74°C was sufficient to completely liberate TDP from the *Arabidopsis* matrix, evaluation of the heating temperature (95 vs. 74°C) revealed that heating the rice samples at 74°C yielded only half the TDP signal compared to heating at 95°C. Although the stability of TDP was higher at 74°C (which could be derived from the higher D₃ TDP signal following the 30 min incubation), less TDP was released during extraction. Consequently, to limit TDP degradation at 95°C

while maintaining a similar recovery, we shortened the heating step from 30 to 10 min. This resulted in a higher absolute TDP and D₃ TDP signal, demonstrating an improved recovery and stability when heating for only 10 min (Suppl. Figure S-3). Next, we evaluated whether the subsequent addition of amylase and protease would further liberate the analytes from the carbohydrate-rich matrix and their binding proteins, respectively. However, treatment with these enzymes did not yield increased levels. We therefore concluded that liberation of the vitamins from the starch-rich matrix was maximal and complete after 10 min heating at 95°C in 0.1 M HCl. Thereafter, homogenization of the samples requires milling in a Retsch Mill. The minimal milling time was set at 30 min at 30 Hz. Subsequently, the pH was adjusted to 4.5 with 145 µL 2.5 M sodium acetate to ensure optimal phosphatase activity. As described previously [15] and in section 2.3 'sample preparation', the addition of acid phosphatase to one half of the sample (the other half is left untreated) enables us to distinguish between total and non-phosphorylated HMP and HET. First, we evaluated the incubation time, following the addition of 10 units of phosphatase to all samples. However, we didn't observe any increase in HMP or HET level in function of the incubation time, instead levels rather decreased upon prolonged incubation (Suppl. Figure S-4). This could not be explained by a lack of activity of the added phosphatase as in the same samples TMP and TDP were fully converted, and D₃ thiamine (which is the conversion product of D₃ TDP by phosphatase) was formed after 1 hour incubation with phosphatase (Suppl. Figure S-5). Therefore, we concluded that in the mature rice seeds all HMP-P(P) and HET-P was already converted to HMP and HET, respectively, during maturation. Yet, we decided to maintain the phosphatase treatment step, as we cannot predict what will happen in the genetically modified samples. Based on our results for TMP and TDP, we conservatively set the incubation time at two hours. Last, the number of phosphatase units (2-4-6-8-10-15 and 20 units) was evaluated to obtain maximal HMP and HET levels during this 2h incubation step. As expected, there were no significant differences in HMP and HET levels in function of the number of phosphatase units added (Suppl. Figure S-6). We decided to continue with 8 units of phosphatase per sample, as from that point both TMP and TDP were completely converted. The final sample

preparation can be summarized as follows (schematic overview is shown in Figure 2): to 200 mg of rice material (unpolished or polished), 1.5 mL 0.1 M HCl, containing the ISs (D_3 TDP, 20 ng/mL; ^{13}C thiamine, 100 ng/mL; HET analogue, 60 ng/mL; HMP analogue, 7.5 ng/mL), was added. After heating at 95°C for 10 min, the samples were milled in a Retsch mill for 30 min at 30 Hz. Subsequently, the pH was adjusted to 4.5 with 145 μ L 2.5 M sodium acetate. Next, the samples were split in two halves (2 x 500 μ L): to one part 8 units of acid phosphatase were added, followed by incubation at 37°C for 2h. To the other part, the same volume of water was added, followed by incubation at 4°C for 2h. Finally, all samples were ultrafiltered (3 kDa filters) for 20 min at 12 000 g and transferred to vials for analysis.

3.2 Method validation

Although the chromatography is the same as the one published for *Arabidopsis thaliana* samples [15], the analysis of a completely different matrix, namely rice, might influence several parameters. First, due to a difference in vitamer distribution, the calibration range needed to be re-set, with calibrators prepared in the appropriate matrix. This in turn necessitates the re-evaluation of accuracy and precision of the method. Second, different matrices might present particular interfering compounds. Third, due to the difference in sample preparation and the different matrix as such, matrix effects and recovery need to be re-assessed. Last, stability of the analytes in the starting material is expected to be matrix-specific. Therefore, a full validation of the developed methodology is required.

As described previously [15], no labeled ISs for HET and HMP are commercially available, therefore, an HET and HMP analogue were evaluated during validation. Similar to the results obtained for *Arabidopsis thaliana* [15], the HET analogue was found the best suited IS for both HET and HMP. Since for TMP no labeled analogue was commercially available at the time of analysis, D_3 TDP was evaluated as potential IS. However, matrix effects in unpolished rice were not compensated by D_3 TDP (130-140%) since the ionization of D_3 TDP was more suppressed than the ionization of TMP. Moreover, D_3 TDP-corrected matrix effects were different between charcoal-treated rice and native

rice. Consequently, we can conclude that D₃ TDP is not an appropriate IS for the quantification of TMP in rice, especially when quantification is based on a calibration curve prepared in charcoal-treated 'blank' rice matrix. These latter results emphasize the importance of the development of a matrix-specific LC-MS/MS method, as for *Arabidopsis thaliana* D₃ TDP was deemed appropriate for the quantification of TMP. Furthermore, these results show that the impact on (D₃)TDP and TMP ionization is matrix dependent, and more pronounced when analyzing rice material. Continuing validation, TMP results were further evaluated without IS (absolute data), to possibly serve as semi-quantitative results.

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 (Suppl. Table S-3). No unacceptable carry-over was noticed for thiamine, HET, HMP, TMP and all ISs, while carry-over, exceeding the acceptance criterion (20% of LLOQ), was present for TDP, following injection at 4xULOQ. The exceedance of the acceptance criteria for carry-over might be explained by the wide calibration range (low LLOQ and high ULOQ) and the chelating characteristics of TDP, which we found are more pronounced than for TMP (likely owing to the presence of two phosphate groups in TDP). TDP carry-over is only relevant starting from a concentration of 82 µg/100 g (which is ~ 12 times the TDP level in wild-type unpolished rice, and ~ 56 times the level in polished rice) and can be solved by the injection of two blanks between samples with a concentration > 82 µg/100 g. Finally, similar to our previous method [15], measures should be taken to minimize this carry-over issue. Therefore, within one run, phosphatase-treated samples should be analyzed before the non-treated samples. In addition, polished rice samples should be measured before unpolished samples, due to the higher TDP levels in the latter samples. As the calibration data (n=6) for all analytes were heteroscedastic, weighing was required. A linear regression model with 1/x² weighing was chosen for all analytes. Statistical analysis to substantiate this choice was performed using an R-script developed by Desharnais *et al.* [19]. Using the selected model, back-calculation of the calibrators met the pre-set acceptance criteria. For all analytes, the calibration range was set as wide as possible, allowing

determination of very high levels -which might be present due to the genetic modifications- as well as very low levels. Low levels can be of interest when evaluating e.g. the effect of the degree of polishing, particularly in wild-type rice. All analytes were quantified with a bias below $< 12\%$. The repeatability and total imprecision for QCs, analyzed in duplicate on three different days, fulfilled the pre-set criteria ($CV\% < 15\%$, 20% for LLOQ) for all analytes, as shown in Table 1. Also for real, non-spiked samples (phosphatase-treated and non-treated), analyzed in triplicate on three different days, the precision criterion was met for all analytes (Table 1).

To anticipate on levels exceeding the ULOQ, as a possible result of the genetic modification, we evaluated the effect of dilution of real samples on the accuracy and precision of the measurement. Unpolished rice samples could be diluted both 5- and 10-fold without compromising the accuracy and precision of the analysis (Suppl. Table S-5). For polished rice samples, we were only able to evaluate the effect of dilution on the determination of HET, HMP and thiamine because TDP levels were near the LLOQ and TMP levels were $< \text{LLOQ}$ in the undiluted samples. Polished rice samples could be diluted 5-fold without affecting the accuracy and precision (Suppl. Table S-5).

Importantly, when applying an existing chromatographic method to a different matrix, the evaluation of matrix effects is essential. The results for the IS-corrected matrix effects of all analytes in the different matrices (charcoal-treated polished and unpolished rice, as well as polished and unpolished rice, both with and without phosphatase treatment) are shown in Table 2. For HET, HMP, thiamine and TDP the IS-corrected MEs were all between 97-113% with a $CV \leq 15\%$. Although no IS was applied for TMP, absolute MEs were similar between all matrices with $CVs < 20\%$ (Table 2). Based on these results, we can justify the use of charcoal-treated rice as 'blank' matrix to prepare the calibrators for quantitation of the real, native samples. Moreover, both unpolished and polished rice samples can be quantified using a single calibration curve prepared in charcoal-treated unpolished rice material.

Recoveries for HET, HMP, thiamine and TDP varied from 86 to 123%, and, importantly, were reproducible ($CV \leq 15\%$) (Table 3). Absolute recoveries for TMP from both charcoal-treated rice and native rice material were very similar and varied from 88-96% in charcoal-treated rice matrix and 75-

101% in native, unpolished rice, respectively. Despite the absence of an IS, CVs were still below 20% (Table 3).

The stability of the analytes should be ensured during the entire analytical process, including storage of the starting material, during sample preparation, the analysis itself, storage of the processed samples, and during possible re-analysis. The stability experiments revealed that all analytes were stable in the extracts of both charcoal-treated matrix and rice, when kept in the autosampler for 48h at 4°C (Suppl. Table S-6). As we have no labeled IS for TMP, it could not compensate for possible degradation (> 15% in QC samples) (Suppl. Table S-6). Additionally, we demonstrated that QCs and samples can be re-analyzed after 2 weeks of storage at -80°C, when the original calibration curve was re-run (Suppl. Table S-6). Remarkably, for TMP an apparent increase in signal was observed (126% after 1 freeze/thaw cycle and 168% after 3 freeze/thaw cycles). Absolute TDP signals remained stable, hence degradation cannot explain the increased signal for TMP. Stock solutions of all analytes were stable when stored at -80°C for 3 months.

In unpolished rice, the stability of all analytes was ensured during both short-and long term storage at both -20°C and 4°C (Table 4). However, when unpolished rice seeds were stored at 28°C, we saw a decrease in TMP and TDP signal, even after 2 weeks of storage. TMP and TDP levels further decreased during longer storage at 28°C (1 and 3 months), which was accompanied by an increase in HET levels. The degradation of TMP and TDP might result in the formation of HET, which would explain the higher HET levels upon storage at 28°C. Thiamine itself appears to be stable during short-and long term storage at 28°C. However, we cannot exclude the scenario that on the one hand some thiamine is formed due to degradation of TMP and TDP, and on the other hand, some thiamine is degraded, with formation of HET, with as a net result an apparently stable thiamine level. For polished rice, it was more difficult to come to a conclusive result, as the intrinsic variation introduced by the polishing step may contribute to the biological variation. However, a trend towards increasing levels of HET and thiamine upon storage could be discerned, which might be the result of (non-quantifiable) degradation of TDP and/or TMP. Anyway, it can be concluded that, to ensure stability of

the starting material, the rice seeds in their unpolished form should be stored cool and should only be polished shortly before the actual analysis.

3.3 Application

A proof-of-concept of the method's applicability was demonstrated by determining thiamine vitamers and precursors in wild-type (WT) and genetically modified (M lines) rice seeds (Strobbe et al., unpublished data), both unpolished and polished (Figure 3A and B). The plants were grown and the rice seeds polished as described in section 2.1 'chemicals and materials'. Following sample preparation, one aliquot was treated with acid phosphatase to determine total HET, total HMP and total thiamine levels, while the other aliquot was left untreated, allowing the determination of the individual vitamers and precursors. The levels of all analytes in both unpolished and polished rice were within our pre-set calibration range, therefore no dilution of the samples was required. Although carry-over issues for TDP were observed during validation, these were not relevant during the application: TDP levels in the different samples were all from the same magnitude and were all $<82 \mu\text{g}/100 \text{ g}$ (the concentration from which on carry-over becomes relevant). As mentioned above, our validation data indicated that D_3 TDP was not appropriate as IS for the quantification of TMP in rice. Nevertheless, we were able to obtain *semi-quantitative data* (bias $<12\%$, total imprecision $<12\%$, absolute ME CV $<20\%$ and absolute RE CV $<20\%$) when no IS was applied. Consequently, this approach still allows us to guide the effectiveness of the biofortification strategies, by expressing the evolution of TMP levels, *relative* to a control (wild-type rice). Very recently, during the completion of this work, D_3 TMP became commercially available. It will be relevant to evaluate in future experiments whether inclusion of D_3 TMP will improve TMP quantitation. However, based on our findings, TMP quantitation seems to be of limited importance, representing less than 1% of total thiamine in all analyzed unpolished rice lines (including both wild-type and genetically engineered) (Figure 3A and Strobbe et al., unpublished data). Moreover, as recent biofortification strategies aim at specifically targeting the edible endosperm, we mainly focused on the quantification of the vitamins and precursors present in white, polished rice. Up till now, no detectable TMP levels were

observed in polished rice seeds of any of the hitherto analyzed lines (either wild-type or genetically engineered) (Figure 3B and Strobbe *et al.*, unpublished data). Finally, comparison of our results with literature reveals that our thiamine data are in line with those previously reported by Dong *et al.* [22] (based on fluorescence detection): 320 µg/100 g vs. 270 µg/100 g in unpolished wild-type rice, and 66 µg/100 g vs. 30 µg/100 g in polished wild-type rice. Differences might be explained by the different rice variety which was studied (Nipponbare vs. Kitaake) and possible variations in the polishing procedure. As we are the first to report on HET and HMP levels in rice, no comparison with other studies can be made.

4. Conclusion

We successfully optimized and validated a sensitive and specific LC-MS/MS method for the simultaneous determination of thiamine, its precursors (HET and HMP) and phosphate derivatives (TDP quantitatively; TMP semi-quantitatively) in rice (both polished and unpolished). We clearly showed the necessity for a matrix-specific method, including the development of a matrix-specific extraction protocol, the re-evaluation of the calibration range and finally the re-validation of the method to accurately determine the different analytes in rice. The method fulfilled all pre-set acceptance criteria and the application on genetically modified rice lines demonstrated its validity. This method will allow to evaluate the effectiveness of genetic modifications and will help to steer future genetic engineering strategies in rice.

Figure 1. Chromatogram of TDP, TMP, thiamine, HMP and HET in charcoal-treated rice matrix, spiked at LLOQ level.

Figure 2. Schematic overview of the sample preparation for the determination of on the one hand, total thiamine, total HET and total HMP, and on the other hand the individual vitamers (thiamine, HET, HMP, TMP and TDP) in both unpolished and polished rice.

Figure 3. Distribution of thiamine, its diphosphate derivative (TDP) and precursors (HET and HMP) in wild-type and genetically modified rice seeds, both unpolished (A) and polished (B).

Table 1. Accuracy (bias%) of QCs of HET, HMP, thiamine, TDP and TMP was verified at four concentration levels (LLOQ, LQC, MQC, HQC) in spiked charcoal-treated rice matrix. Repeatability and total imprecision (CV%) of QCs and native samples (polished ("pol") or not ("unpol"), treated with phosphatase ("P") or not ("NP")). In phosphatase-treated samples TDP and TMP are completely converted hence detection is not applicable for these samples, as indicated by N/A.

Accuracy (bias%) (n=3x2)					
	HET	HMP	Thiamine	TDP	TMP
LLOQ	-3.6%	0.2%	2.1%	-2.2%	-1%
LQC	-1.6%	1.9%	2.7%	-4.6%	3.4%
MQC	-2.6%	6.7%	8.5%	1.0%	11.1
HQC	-6.4%	-2.6%	0.4%	1.1%	-3.5%
Repeatability (CV%) (QCs: n=3x2; samples: n=3x3)					
	HET	HMP	Thiamine	TDP	TMP
LLOQ	4.5%	9.8%	15.0%	10.7%	4.8%
LQC	4.2%	3.0%	14.4%	3.7%	7.5%
MQC	5.8%	2.9%	6.6%	10.7%	8.5%
HQC	3.5%	3.5%	6.7%	4.9%	1.8%
unpol NP	2.1%	2.1%	7.5%	7.2%	5.0%
pol NP	1.2%	3.2%	10.4%	9.6%	<LLOQ
unpol P	1.9%	3.4%	7.8%	N/A	N/A
pol P	2.0%	5.4%	14.1%	N/A	N/A
Total imprecision (QCs: n=3x2; samples: n=3x3)					
	HET	HMP	Thiamine	TDP	TMP
LLOQ	8.0%	9.8%	15.0%	10.7%	11.4%
LQC	4.2%	4.8%	14.4%	4.0%	7.5%
MQC	5.8%	2.9%	9.9%	11.3%	6.5%
HQC	3.5%	3.8%	6.8%	6.5%	1.8%
unpol NP	2.1%	2.6%	14.8%	7.3%	5.9%
pol NP	3.4%	5.5%	10.4%	11.9%	<LLOQ
unpol P	2.7%	5.8%	7.8%	N/A	N/A
pol P	4.6%	7.4%	14.1%	N/A	N/A

Table 2. IS-corrected matrix effect (ME) (n=6) for HET, HMP, thiamine and TDP for both charcoal-treated rice (QC) and phosphatase-treated (P) and non-treated rice samples (NP). ME results for TMP are presented as absolute data.

	HET		HMP		Thiamine		TDP		TMP	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
1. QC material										
A. Unpolished charcoal treated matrix										
ME (%)	97	102	96	100	108	104	111	111	88	79
CV (%)	4	3	6	7	15	10	13	12	4	9
B. Polished charcoal treated matrix										
ME (%)	97	102	98	106	111	99	98	102	84	83
CV (%)	6	3	2	6	12	10	4	9	8	10
2. Samples without phosphatase treatment										
A. Unpolished rice										
ME (%)	104	107	99	102	90	94	105	115	86	72
CV (%)	3	3	11	6	11	14	12	13	14	4
B. Polished rice										
ME (%)	101	99	96	103	109	100	102	102	85	90
CV (%)	3	2	6	4	9	12	10	9	12	17
3. Samples with phosphatase treatment										
A. Unpolished rice										
ME (%)	113	112	98	102	96	91	N/A	N/A	N/A	N/A
CV (%)	3	4	6	7	10	13	N/A	N/A	N/A	N/A
B. Polished rice										
ME (%)	103	103	91	98	109	105	N/A	N/A	N/A	N/A
CV (%)	4	3	12	7	12	13	N/A	N/A	N/A	N/A

Table 3. IS-corrected recovery (RE) (n=6) for HET, HMP, thiamine and TDP from both charcoal-treated rice (QC) and phosphatase-treated (P) and non-treated rice samples (NP). RE results for TMP are presented as absolute data.

	HET		HMP		Thiamine		TDP		TMP	
	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC	LQC	HQC
1. QC material										
A. Unpolished charcoal treated matrix										
RE (%)	101	98	99	106	94	100	87	97	96	94
CV (%)	5	2	8	5	13	7	10	7	12	12
B. Polished charcoal treated matrix										
RE (%)	93	97	104	94	123	106	102	104	91	88
CV (%)	5	3	3	6	13	8	8	6	11	15
2. Samples without phosphatase treatment										
A. Unpolished rice										
RE (%)	108	102	103	101	111	122	110	96	90	75
CV (%)	9	3	14	4	15	13	13	5	17	10
B. Polished rice										
RE (%)	97	100	100	105	95	101	88	100	101	99
CV (%)	10	3	11	3	12	13	10	8	11	16
3. Samples with phosphatase treatment										
A. Unpolished rice										
RE (%)	94	101	86	98	98	96	N/A	N/A	N/A	N/A
CV (%)	8	3	13	3	9	10	N/A	N/A	N/A	N/A
B. Polished rice										
RE (%)	90	99	98	108	89	107	N/A	N/A	N/A	N/A
CV (%)	12	3	10	9	15	7	N/A	N/A	N/A	N/A

435 **Table 4. Long- and short-term stability data of both unpolished (A) and polished rice (B).**

A. Unpolished rice	HET	HMP	Thiamine	TDP	TMP
2 weeks at 28°C	104%	87%	97%	83%	77%
1 month at 28°C	142%	114%	93%	90%	75%
3 months at 28°C	149%	106%	95%	51%	47%
2 weeks at 4°C	95%	94%	101%	103%	80%
1 month at 4°C	88%	108%	105%	118%	91%
3 months at 4°C	97%	116%	98%	107%	101%
2 weeks at -20°C	93%	94%	97%	113%	99%
1 month at -20°C	96%	114%	100%	114%	115%
3 months at -20°C	99%	106%	105%	117%	107%
B. Polished rice	HET	HMP	Thiamine	TDP	TMP
2 weeks at 28°C	135%	93%	123%	<LLOQ	<LLOQ
1 month at 28°C	133%	110%	105%	<LLOQ	<LLOQ
3 months at 28°C	107%	107%	103%	<LLOQ	<LLOQ
2 weeks at 4°C	142%	104%	121%	<LLOQ	<LLOQ
1 month at 4°C	130%	94%	143%	<LLOQ	<LLOQ
3 months at 4°C	165%	138%	130%	<LLOQ	<LLOQ
2 weeks at -20°C	117%	105%	104%	<LLOQ	<LLOQ
1 month at -20°C	132%	106%	145%	<LLOQ	<LLOQ
3 months at -20°C	125%	115%	108%	<LLOQ	<LLOQ

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Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary material

Text S-1: Concentration of the calibrators and samples ($\mu\text{g}/100\text{ g}$ vs. ng/mL); Fig.S-1: Chemical structures of all analytes and ISs; Fig.S-2: Schematic overview of the previously described sample preparation procedure in *Arabidopsis thaliana*; Fig.S-3: Influence of the initial heating conditions on TDP and D₃ TDP levels; Fig.S-4: HET and HMP signals in function of phosphatase incubation time; Fig.S-5: TDP, TMP, D₃ thiamine signals in function of phosphatase incubation time; Fig.S-6: HET and HMP signals in function of number of phosphatase units; Table S-1: Concentration of the calibrators ($\mu\text{g}/100\text{ g}$); 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; Table S-6: Results autosampler and freeze/thaw stability.

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Highlights:

- Determination of thiamine, its phosphates and precursors in complex cereal matrix
- Development of essential tool to steer thiamine biofortification in staple crops
- Optimization of sample preparation, ensuring stability labile thiamine vitamers
- Successful application to genetically modified rice, both unpolished and polished

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: