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# A simple, sensitive, and specific method for the extraction and determination of thiamine and thiamine phosphate esters in fresh yeast biomass

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ABSTRACT

Thiamine is an essential vitamin for most living organisms, of which yeasts are a rich nutritional source. In this study we developed a thiamine extraction and determination method to detect thiamine in fresh yeast biomass. The thiamine determination method combines the derivatization of thiamine to a highly fluorescent product, with chromatographic separation (HPLC) and fluorescence detection. The method specifically detects free thiamine (T), thiamine phosphate (TP), and thiamine pyrophosphate (TPP). It has a high sensitivity of 2 ng/ml for TPP and TP, and 1 ng/ml for T, excellent instrumental repeatability, and low day-to-day variation in retention time of the different phosphate forms. We demonstrated the robustness of the method by proving that the fluorescence signals of the derivatised samples are stable for at least 82 h after derivatization, and by showing that the final pH of the samples does not influence the fluorescent response. In addition, we developed and validated a thiamine extraction method consisting of beads beating the fresh yeast biomass in 0.1 M HCl using a lysing matrix composed of 0.1 mm silica spheres. The performance of this method was compared to extraction via heat treatment at 95 °C for 30 min, and a combination of beads beating and heat treatment carried out in different order. We demonstrated that thiamine extraction via beads beating is the only method that prevents the biologically active form thiamine pyrophosphate to be degraded to thiamine phosphate, therefore, the extraction method developed and described in this study is preferred when the different thiamine vitamers need to be detected in their actual proportions. The combination of the extraction via beads beating, the conversion of all vitamers to the thiochrome derivatives, and the separation of these compounds on the reversed phase HPLC with a fluorescence detector, yielded a sensitive, specific, repeatable, and robust method for extraction and determination of vitamin $B_1$ in fresh yeast biomass.

## 1. Introduction

Thiamine pyrophosphate (TPP) is the biologically active form of thiamine (vitamin $B_1$), it is an important co-factor of different enzymes that catalyse metabolic reactions occurring in living organisms. Yeasts are a well-known rich source of vitamin B1 (Tylicki et al., 2017). They can salvage thiamine from the environment and synthesize it de novo (Fitzpatrick and Thore, 2014). In yeast species like *Saccharomyces cerevisiae,* thiamine pyrophosphate is known to be a co-factor of enzymes active in the pentose phosphate pathway, the citric acid cycle, and the metabolism of amino acids (Hohmann and Meacock, 1998). Although TPP represents the major thiamine form detected in yeast cell extracts, vitamin $B_1$ is also found in its unphosphorylated form (T), and in the mono and tri-phosphorylated forms, namely thiamine phosphate (TP) and thiamine triphosphate (TTP), the latter being found in very low concentrations (Perli et al., 2020).

Thiamine is stable at low pH, but easily degrades once exposed to alkaline pH (Voelker et al., 2021). For this reason, thiamine is often extracted through acid hydrolysis with either hydrochloric, sulfuric, or trichloroacetic acid (TCA) (Edwards et al., 2017). Moreover, as it is a co-factor, this vitamin has a non-selective protein-binding capacity (Edwards et al., 2017), therefore, to aid the acid hydrolysis, extraction is usually carried out at high temperatures with samples that are either heat-treated at around 80–100 °C, or autoclaved to reach even higher

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temperatures. When thiamine extracts are heat-treated, degradation of thiamine diphosphate to thiamine monophosphate can occur (data shown in this paper), therefore, it is not possible to use heat treatment for extraction when the concentrations of all $B_1$ vitamers need to be determined specifically.

Following the extraction, vitamin $B_1$ can be quantified using different assays. Microbiological assays measure thiamine indirectly as a function of microbial growth. In general, these methods are very sensitive but laborious and time-consuming. Moreover, the most commonly used microbiological assays are not specific for the different vitamers of thiamine, although the use of specific auxotrophic strains can be used to detect specific phosphates forms and precursors (Strobbe et al., 2022). Standard HPLC-UV methods are used instead to measure thiamine directly, in addition these methods have high specificity but lower sensitivity. A sensitive and specific method is obtained by combining the derivatization of thiamine and thiamine phosphates to the highly fluorescent thiochrome derivatives, with liquid chromatographic separation (HPLC) of the phosphate esters and finally detection of fluorescence. Potentially such a method, in combination with an extraction procedure that preserves the thiamine phosphate forms from degradation, could be applied to yeast biomass extracts with low purity and low concentration of vitamin $B_1$ vitamers for their specific detection.

In this paper, we describe an optimised method for the extraction and quantification of thiamine, thiamine phosphate, and thiamine pyrophosphate in fresh yeast biomass. The extraction method is based on the use of silica beads for cell disruption in the presence of hydrochloric acid. The method for the determination of thiamine in the extracts is based on the pre-column derivatization of thiamine and phosphates esters to the fluorescent thiochrome products as described by Gerrits et al. (1997), using potassium hexacyanoferrate as an oxidizing reagent in alkaline conditions, followed by pH adjustment of the extracts. This derivatization method was combined with liquid chromatographic separation (reversed-phase HPLC) and fluorescence detection as described by Sander et al. (1991). By combining the extraction and determination procedures mentioned above, we developed a fast, reproducible, sensitive, and specific method for detection and quantification of thiamine and phosphorylated thiamine forms.

## 2. Materials and methods

### 2.1. Materials and chemicals

Thiamine pyrophosphate, thiamine monophosphate chloride dihydrate, thiamine hydrochloride, hydrochloric acid (HCl), potassium hexacyanoferrate, and glycerol were purchased from Sigma Aldrich (St. Louis, MO, USA). Thiamine triphosphate was purchased form Alfa Chemistry (Ronkonkoma, NY, USA). Yeast nitrogen base (YNB) w/o ammonium sulphate, amino acids, and thiamine was purchased from United States Biologicals (Salem, MA, USA). Pore size 0.2 μm sterile polyethersulfone filters for microbiological media sterilization, and pore size 0.2 μm sterile polyethersulfone filters with 13 mm diameter for filtration of yeast extract were purchased from Thermo Fisher Scientific (Walthman, MA, USA). Membrane filters with a pore size of 0.2 μm used for yeast biomass determination were purchased from Pall Corporation (Ann Arbor, MI, USA). Lysing matrix tubes and Fast Prep were purchased from MP Biomedicals (Irvine, CA, USA). HPLC vials, 0.3 ml TPX Crimp/Snap Vial 32 × 11.6 mm (clear), and Snap Ring Cap (blue) with X-slitted Septa Silicone/PTFE caps were purchased from BGB Analytik (Harderwijk, NL). Agar bacteriological was from OXOID (Wesel, Germany). Glucose was purchased from Merck Millipore (Burlington, MA, USA). Methanol was purchased from Actu-All Chemicals (Oss, NL). Dipotassium phosphate ($K_2HPO4$) was purchased from VWR (Radnor, Pennsylvania, USA).

### 2.2. Yeast strains and media

The *Saccharomyces cerevisiae* 540 used in this study is property of the Food Microbiology Chair group culture collection at Wageningen University & Research and was isolated from rice wine in Vietnam. The yeast strain was preserved in 30% (vol./vol.) glycerol and stored at −80 °C. The liquid growth thiamine free medium minimal medium (MM medium) used in this study was composed of 20 g/l of glucose, 5 g/l ammonium sulphate, and 1.71 g/l of YNB, and filter sterilised using a sterile 0.2 μm polyethersulfone filter (PES). For MM agar plates 20 g of glucose and 15 g of Agar Bacteriological were added to 800 ml of demineralised water and autoclaved at 121 °C for 15 min, the solution was cooled to 50 °C in a water bath and a solution containing 1.71 g of YNB in 200 ml of demineralised water was filter sterilised ad added to the mixture.

### 2.3. Yeast growth

Yeast culture preserved in glycerol and stored at −80 °C was streaked in MM plates and incubated for 72 h at 30 °C. One colony was transferred to an Erlenmeyer flask containing MM medium and cultivated for 24 or 72 or 90 h at 30 °C 180 rpm. The cell cultures were used for thiamine extraction. The specific incubation times are indicated in the figure's caption.

### 2.4. Determination of biomass

Cell dry weight (DW) measurement was used to establish yeast cell biomass. Membrane filters with a pore size of 0.2 μm were dried for at least 24 h and weighted, then used to filter a defined volume of yeast cell culture using a vacuum pump. After filtration of the yeast culture the filters were dried at 80 °C for 24 or 48 h and weighted using an analytical balance to determine the DW.

### 2.5. Extraction of thiamine

In this study we first optimised a thiamine extraction method based on the mechanical cell disruption via beads beating. The influence of the lysing matrix composition on the extraction yield and vitamer percentage was tested using 3 different beads beating matrices from MP Biomedicals: (i) lysing matrix B composed of 0.1 mm silica spheres, (ii) lysing matrix C composed of 1 mm silica spheres and (iii) lysing matrix E composed of 1.4 mm ceramic spheres, 0.1 mm silica spheres, and one 4 mm glass bead. Subsequently, we tested the influence of the number of beads beating rounds on the total extraction yield and vitamer distribution percentage, ranging from a minimum of 3 rounds, to a maximum of 12. Afterwards, we compared the optimised thiamine extraction method via beads beating to hot acid hydrolysis, and a combination of these carried out in different orders.

Aliquots of yeast cultures were centrifuged at 10000 *xg* for 10 min, the supernatant was discarded and yeast pellets were resuspended in the same amount of 0.1 M HCl used for extraction. For the beads beating extraction 1.5 ml of liquid yeast suspension was pipetted into the lysing matrix tubes and extracted with Fast Prep 5.5 m/s for 1 min followed by three other rounds separated by 1 min intervals. For the hot acid hydrolysis extraction, the yeast suspension was heat-treated at 95 °C for 30 min and then cooled on ice until it reached room temperature. The combined extraction via a beads beating and hot acid hydrolysis was carried out by performing the two methods described above together. Extracts obtained by beads beating were centrifuged at 10000 *xg* for 5 min, and filtered through a sterile 13 mm 0.2 μm PES filter, then stored at 4 °C before being derivatised. To assess the degradation of TPP to TP during hot acid hydrolysis, the cells were first extracted by beads beating, then the extracts were centrifuged at 10000 *xg* for 5 min, and filtered through a 0.2 μm PES filter, to be then heat treated at 95 °C for 30 min, subsequently cooled down in ice until they reached room temperature, and finally filtered again as described above. Samples were stored at 4 °C before the derivatization.

In this manuscript we refer to beads beating as BB, to hot hydrolysis as HH, to the process of beads beating followed by hot hydrolysis as BB + HH, to the process of hot hydrolysis followed by beads beating as HH + BB. We finally refer to the samples and process treated via beads beating, followed by filtering, and then hot hydrolysis as BB + F + H.

### *2.6. Preparation of standard solutions and derivatization reagents*

Standard solutions of T, TP, and TPP were prepared freshly before use by dissolving the vitamin in 0.1 M HCl. Mixed standard solutions were obtained by combining the different single standard solutions, and subsequently diluting the solution in 0.1 M HCl to reach an appropriate concentration. HPLC grade methanol was used for derivatization. The oxidation reagent was freshly prepared before use, it was composed of a solution of 12.1 mM $K_3Fe(CN)_6$ in 3.35 M NaOH. After derivatization, a 1.4 M solution of $H_3PO_4$ was used to adjust the pH of the samples before injection.

### *2.7. Derivatization of samples and standard solutions*

Thiamine derivatization and HPLC determination was carried out within 24 h from the extraction. The derivatization procedure was executed as described by Gerrits et al. (1997). The extract (1 ml) was pipetted in a 10 ml polystyrene tube, after which, 100 μl of methanol was added and mixed, and 200 μl of oxidation reagent was added and mixed, to reach a pH above 12. After 30 s of derivatization, 250 μl of 1.4 M phosphoric acid solution was added, and the content of the tubes was mixed again. The final pH of the prepared samples and standards was 6.9 +/− 0.2. Finally, 200 μl of the derivatized sample was pipetted in the HPLC vial.

### *2.8. HPLC- fluorescence detection set-up*

The derivatized samples were separated using a 50 mm × 4.6 mm Cortecs C-18-2,7 μm column, with a pre-column Cortecs C-18 VanGuard cartridge, 3.9 mm × 5 mm, 2.7 μm. The detection was performed by a fluorescence detector, UV-Waters 2575 FLR (Waters Corporation, Milford, MA, USA), with an excitation wavelength of 365 nm and emission of 450 nm. The limit of detection (LOD) was determined using a signal/noise ratio of 3. The limit of quantification (LOQ) was determined by setting a 500 mV lower area rejection limit. Samples were stored in the HPLC autosampler compartment at 5 °C. The chromatographic conditions used in this study have been previously reported by Sander et al. (1991), and are briefly described below, with a modification of the injection volume. A volume of 5 μl of derivatised samples was injected. Two mobile phases were used for the analysis, mobile phase A was a solution of 25 mM $K_2HPO_4$ (4.35 g/l) with a final pH of 8.4, mobile phase B was HPLC-grade methanol, with a flow rate of 0.5 ml/min, details of the gradient elution percentage are described in Fig. 1. The total run time was 16 min per sample, with integration after 1.5 min.

### *2.9. Data analysis*

Data were stored using Excel software v.16.0 (Microsoft Corporation, Redmond, WA, USA), data analysis and figures were done using Rstudio software v. 4.0.2 (RStudio®, Boston, MA, USA). Chromatograms were analysed using Chromperfect v. 6.0.18 (Denville, NJ, USA). Data were visually checked, and statistical significance was assessed via ANOVA, followed by Tuckey *post-hoc* test.

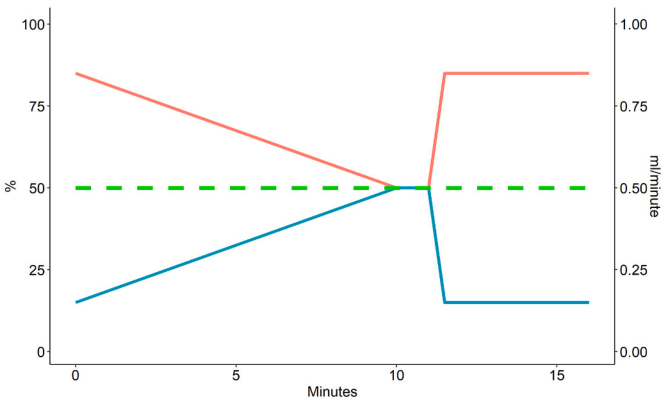

**Fig. 1.** Gradient elution percentage of mobile phases A (red) and B (blue), and flow rate (dashed green line) as ml/min. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## 3. Results

### *3.1. Chromatograms, linearity, LOD and LOQ*

The derivatization of thiamine to thiochrome was executed in this study as reported by Gerrits et al. (1997) which gave adequate chromatographic separation of the vitamers and a high fluorescence response (Fig. 2). The retention times for TPP, TP, and T were 1.94, 2.79, and 5.91 min respectively in the derivatise mixed standard solution, and 1.92, 2.75, and 5.90 respectively in the yeast extract. Minor day to day variation in retention time occurred (Table 1). Peak areas are plotted against the vitamer concentrations and linearity was proven by linear least-square regression (Fig. 3A) with an R value above 0.99 for all the vitamers. The range of linearity was tested by a 10 fold serial dilution (Fig. 3B), starting from 6.38, 5.84, and 5.87 μmol/l, of TPP, TP, and T respectively. Reaching a concentration just above the detection limit of

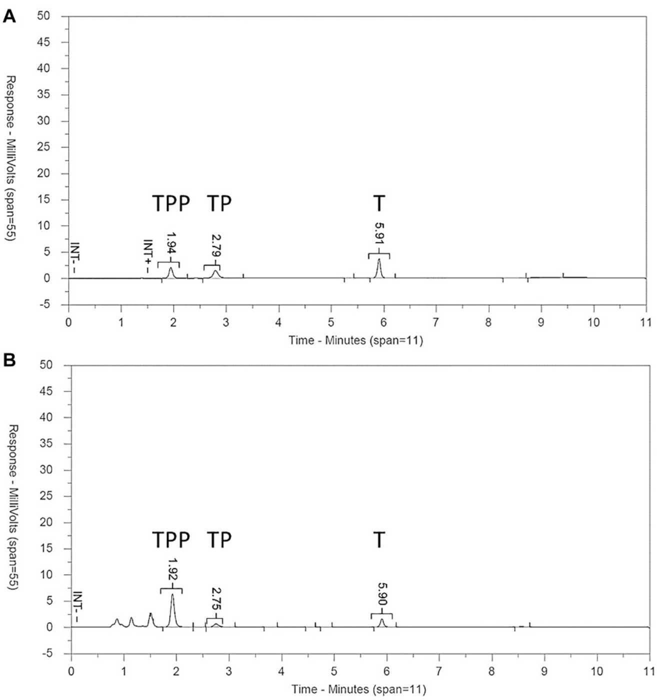

**Fig. 2.** Chromatogram of the standard solution (A) and yeast extract sample (B). The fluorescence response of the different thiamine forms is given in mV and the respective retention times are presented in minutes.

**Table 1**

RSD % ± standard deviation of the area of the derivatised mixed standard solution and single standard solutions after derivatization, and after 36, 58, and 82 h for TPP, TP, and T. Instrumental repeatability, method repeatability, and day-to-day retention time variation expressed as RSD% for TPP, TP, and T, number of samples in brackets.

| | | RSD % | | |
|---|---|---|---|---|
| | | TPP | TP | T |
| RSD% of the area after derivatization, and at 36, 58 and 82 h | Mixed standard solution | 1.04<br>± 0.01 | 0.98<br>± 0.17 | 1.33<br>± 0.42 |
| | Single standard solutions | 1.52<br>± 0.11 | 1.58<br>± 0.30 | 1.40<br>± 0.28 |
| Repeatability | Instrumental repeatability ($n =$ 10) | 1.19 | 1.23 | 1.43 |
| | Method repeatability ($n =$ 10) | 2.95 | 4.07 | 1.66 |
| | Retention time variation ($n = 8$) | 2.32 | 2.29 | 0.8 |

6.38, 5.84, and 5.87 nmol/l respectively.

The LOD was calculated by establishing a 500 mV lower area rejection limit, corresponding to approximately 1.4 ng/ml for TPP and TP, and 0.6 ng/ml for T (3.29 mol/l TPP, 4.05 nmol/l TP, 2.26 nmol/l T). Since T gave a double fluorescent response compared to the other derivatives, the LOQ was calculated to be 1 ng/ml for T, and 2 ng/ml for both TPP and TP (4.7 nmol/l for TPP, 5.8 nmol/l for TP, and 3.8 mol/l T).

### 3.2. *Robustness and repeatability*

To assess the robustness of the method we checked the stability of the derivatised samples, and the influence of the pH adjustment after the derivatization on the fluorescence response. The first experiment aimed at assessing a possible loss of fluorescence in the derivatised samples over time when the samples are stored at 5 °C. As the run time is 16 min per sample, when many samples are analysed, several hours pass between the derivatisation and the injection. Single vitamer standards and mixed standard solution were derivatised in triplicates and injected right after derivatization, after 36, 58, and 82 h. No significant change in peak area occurred over time ($p$-value $>0.1$) for all the tested vitamers and time points, indicating no loss of fluorescence for the various samples over time. The relative standard deviation (RSD) calculated as a percentage was below 1.6% for every sample tested (Table 1). To investigate the influence of the pH of the samples before injection on the fluorescent response, an increasing amount of 1.4 M phosphoric acid was added to the mixed standard solutions in triplicates. Peak areas of the standard solutions were normalised by the amount of phosphoric

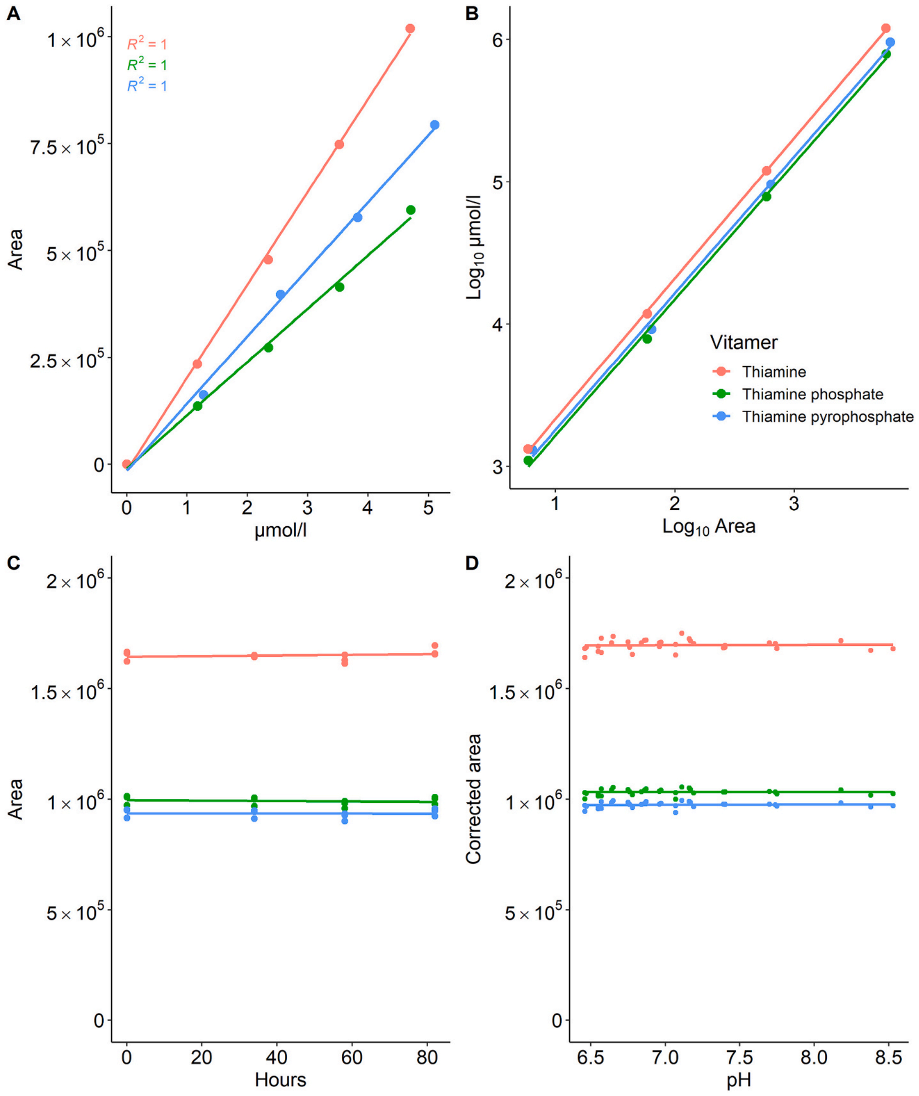

**Fig. 3.** T is depicted in red, TP is depicted in green, and TPP is depicted in blue. Peak area as a function of vitamer concentration in the analysed single standard solution of each vitamer, the linearity was proved by linear least squares regression (A). $\log_{10}$ peak area as a function of $\log_{10}$ vitamer concentration in the analysed single standard solutions, the range of linearity was tested for different concentrations of TPP, TP, and T, the analysis was carried out in triplicates (B). Peak area as a function of time, samples were analysed at $t = 0$, after 36, 58, and 82 h, the analysis was carried out in triplicates (C). Corrected peak areas as a function of the pH of the derivatised samples treated with an increased amount of 1.4 M phosphoric acid, the analysis was carried out in triplicates (D).

acid added, and compared to the reference amount of phosphoric acid needed to reach a final pH of 6.9, the preferred pH indicated by Gerrits et al. (1997). No significant difference in corrected peak area was found between samples with different pH before injection (p-value>0.1) (Fig. 3D). Instrumental repeatability, extraction method reproducibility, and day to day variation of retention time were checked to validate the method. The instrumental reproducibility was checked by injecting the same sample 10 times (Table 1), for all the vitamers the RSD was lower than 1.5%. The reproducibility of the method was checked by performing BB extraction of the same yeast culture 10 times, the RSD for each vitamer was found to be below 4.1% for all vitamers. The day to day variation of retention time for the different vitamers in the standard solutions was below 2.4%, indicated as RSD, for a total of 8 separate analyses. The instrumental and method reproducibility, and low day-to-day retention time variation of the different vitamers, indicate that this method is highly robust, repeatable, and reproducible.

### 3.3. *Optimization of beads beating extraction*

To optimize the extraction of thiamine using BB two parameters were considered in this study: the type of lysing matrix and the number of subsequent beating rounds. Three different lysing matrices were tested to extract thiamine from the same yeast batch. Matrix B is routinely used to lyse bacteria, fungal tissues, and spores, matrix C is designed lysis of yeasts and fungi, and matrix E is generally applied for mixed samples. The total thiamine extraction yield and respective vitamer proportions were determined using the different lysing matrices (Fig. 4A and 4B). Lysing matrix B gave a significantly higher extraction yield of 54.1 nmol/g, compared to the matrices C and E which yielded 46.9 and 22.7 nmol/g (*p*-value<0.05) respectively. No significant differences in vitamer proportions were found between lysing matrix B and C for TPP, TP and T (*p*-value>0.1). TPP accounted for approximately 69% of the total thiamine in both matrices. In matrix B, TP accounted for 8.2% of the total thiamine, while T accounted for 22.5%. In matrix C, TP accounted for 7%, and T accounted for 23.8% of the total thiamine. Lysing matrix E yielded significantly less total thiamine, and no TPP was detected in the extracts, T accounted for 66.8% of the thiamine and TP for 33.2%. Therefore, lysing matrix B was chosen as the preferred matrix for thiamine extraction. To further optimize the BB extraction, on the same yeast culture we increased the number of rounds from 3 up to 12 using lysing matrix B. No significant differences were found between the BB rounds and the total thiamine yield (p-value>0.1), also the vitamer distribution percentage did not differ significantly when beating rounds were progressively increased from 3 to 12 (p-value>0.1) (Fig. 4C and 4D). Therefore, 3 beating rounds were considered sufficient to extract thiamine.

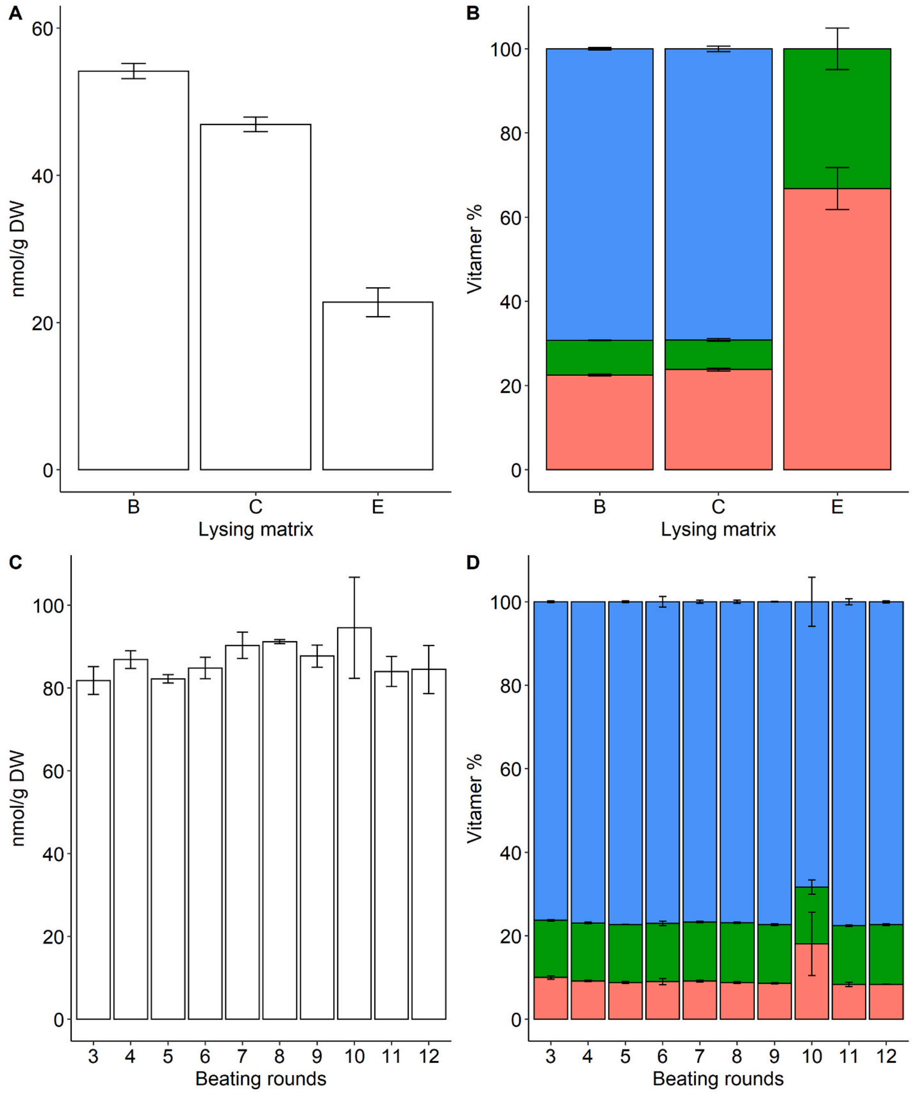

**Fig. 4.** T is depicted in red, TP is depicted in green, and TPP is depicted in blue. Total thiamine yield in nmol/g DW (A) and vitamer distribution percentage of thiamine (B) in yeast extract. Yeast biomass was harvested from *S. cerevisiae* cultures grown in MM for 90 h at 180 rpm and extracted in triplicate using lysing matrix B, C, and E. Total thiamine yield in nmol/g DW (C) and vitamer distribution percentage of thiamine (D), fresh yeast biomass was harvested from *S. cerevisiae* cultures grown in MM for 24 h at 180 rpm and extracted in duplicate using lysing matrix B, BB round tested were increased from 3 to 12.

### 3.4. *Comparing beads beating extraction to hot acid hydrolysis*

Extraction of vitamin $B_1$ via hot acid hydrolysis has been largely used for the thiamine determination in foods and yeast samples (Kenta Yamanaka et al. (1996), Haj-Ahmad et al. (1992), Schweingruber et al. (1991), K. Yamanaka et al. (1994)). In this study, we compared the extraction of vitamin $B_1$ via BB, HH, and a combination of these two treatments carried out in different orders, to assess the effect on the total thiamine extraction yield and the vitamer distribution. Vitamin $B_1$ extraction via BB had the lowest yield compared to all the other methods ($p$-value$<$0.1), with an average total of 55.7 nmol/g DW (Fig. 5A). The highest total thiamine extraction was achieved by BB + HH, yielding on average 71.8 nmol/g DW. HH alone yielded on average 71.2 nmol/ g DW, and HH + BB yielded on average 68.1 nmol/g DW. The vitamer ratios also differed among the samples (Fig. 5B), with T staying virtually unchanged in all samples, accounting for approximately 25% of the total yield, and TPP and TP as the remaining 75% approximately. TPP proportionally decreased as TP increased in all the heat-treated samples. In the samples treated via BB only, TPP accounted for 61.5% of the total thiamine while TP for 10.7% on average. In the BB + HH samples TPP accounted for 49.54% and TP for 23.5%. The decrease in TPP and increase in TP compared to the BB sample was even more remarkable in the samples that were only treated via HH. In these samples TPP represented roughly 39.3% of the total thiamine and TP 36.5%. A similar vitamer proportion was found in the sample treated by HH + BB, in which TPP represented on average 37.6% of the total yield and TP accounted for 38%.

### 3.5. *Degradation of thiamine pyrophosphate during hot acid hydrolysis*

To confirm possible degradation of TPP to TP during hot acid hydrolysis, cells that were first extracted by only BB, then extracts were centrifuged and filtered to remove the biomass, they were then heat treated at 95 °C for 30 min (BB + F + HH). The total thiamine yield and vitamer distribution was compared to the BB samples. We found a significant difference ($p < 0.05$) in the BB + F + HH samples, with a total average yield of 66.9 nmol/g DW, compared to 55.7 nmol/g DW of the BB samples (see Fig. 6A). The vitamer distribution percentage also changed in the BB + F + HH samples. The T content remained roughly unchanged, representing on average 27.9% of the total thiamine in the BB samples, and 26.1% in the BB + F + HH samples. However, the TPP accounted for 28.3% of the total thiamine detected in the BB + F + HH samples, while TPP accounted on average for 61.4% of the total thiamine pool in the BB sample. As a consequence, TP increased in the BB + F + HH samples reaching 45.6% of the total thiamine yield. These results indicate that a partial degradation of TPP to TP occurs during heat treatment. To further confirm the degradation of TPP to TP, a defined mixed solution of TPP, TP, and T was heat-treated at 95 °C for 30 min, similar results were obtained in this case. The total thiamine yield did not differ significantly before and after heat treatment. Instead TPP, which first accounted for 34% of the total thiamine, represented only 19.5% of the total thiamine in the heat treated samples. TP, which accounted for 39.7% of the total thiamine in the untreated sample, increased above 53% after heat treatment. This confirms that heat-treating thiamine extracts leads to a 1:1 conversion of TPP to TMP, while T remains unchanged, thereby leading to a non-specific detection of vitamin $B_1$ vitamers.

## 4. Discussion

HPLC-UV detection methods are often used for thiamine quantification. These methods detect thiamine directly, and are usually applied to products with relatively high concentrations of thiamine, for instance in fortified foods. HPLC-UV methods are not sufficiently sensitive when products contain low amounts of vitamin $B_1$. Another pitfall of these methods is their low specificity when the analysed matrix contains UV absorbing impurities. This will produce chromatograms in which thiamine peaks are overlapping with the UV-detectable impurities, resulting in difficult thiamine quantification. In this study, we propose the derivatization of thiamine to thiochrome to increase both specificity and sensitivity of detection. The pre-column derivatization of thiamine to thiochrome can be both done manually or automatically. Manual pre-column derivatization, although laborious, is easier and cheaper to set up compared to an automated pre-column derivatization system. During derivatization methanol was used as it is reported to increase fluorescence (Bubeshko et al., 2011), potassium hexacyanoferrate was used as

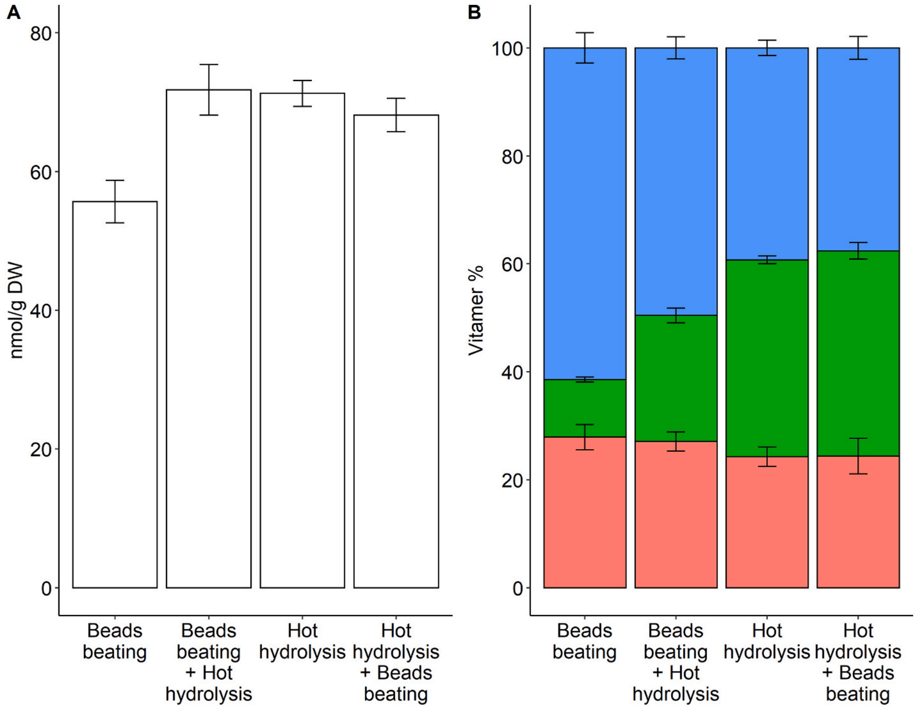

**Fig. 5.** T is depicted in red, TP is depicted in green, and TPP is depicted in blue. Total thiamine yield in nmol/g DW (A) and vitamer distribution percentage of thiamine (B) in yeast extract. Fresh yeast biomass was harvested from three different *S. cerevisiae* cultures grown in MM for 72 h at 180 rpm and extracted in triplicate, yeast biomass was lysed using lysing matrix B and 3 BB rounds of 1 min, heat treated extract were treated for 30 min at 95 °C, these treatments were tested both individually and combined in a different order.

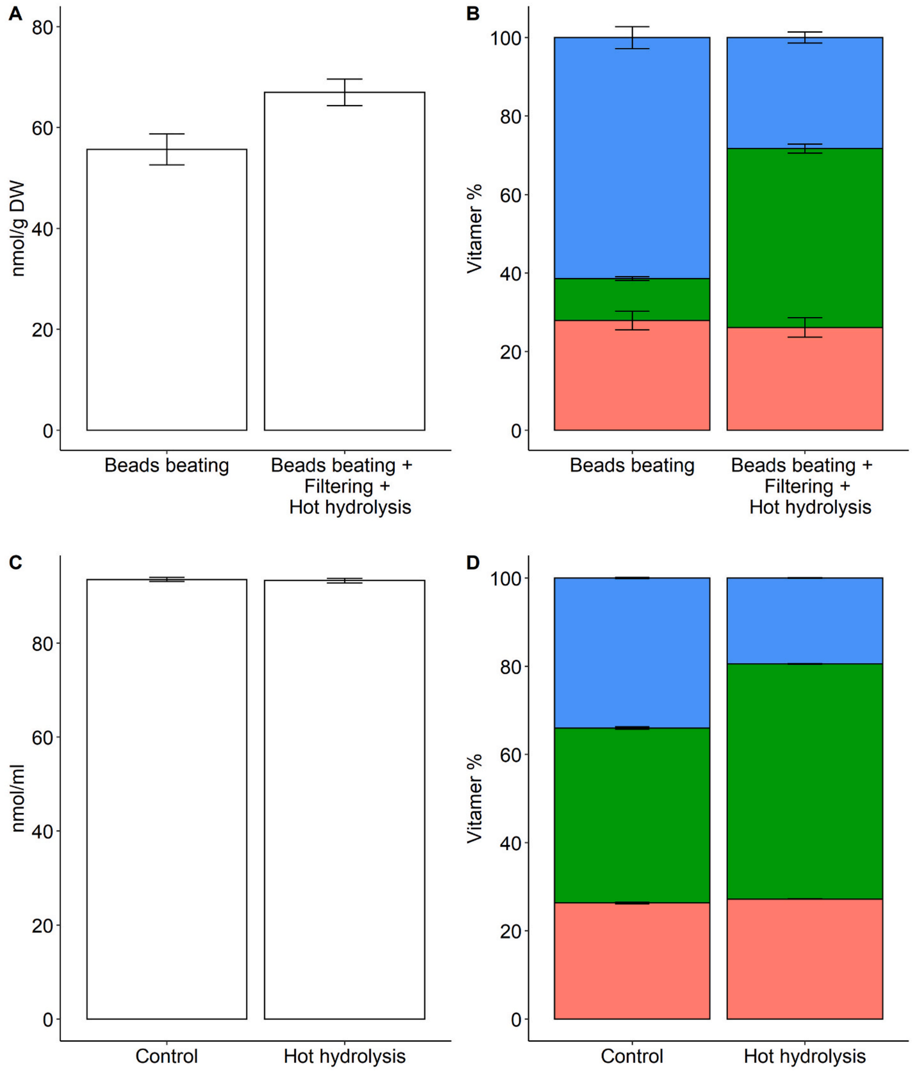

**Fig. 6.** T is depicted in red, TP is depicted in green, and TPP is depicted in blue. Total thiamine yield in nmol/g DW (A) and vitamer distribution percentage of thiamine (B) in fresh yeast extract. Fresh yeast biomass was harvested from three different *S. cerevisiae* cultures grown in MM for 72 h at 180 rpm and extracted in triplicate, yeast biomass was lysed using lysing matrix B and 3 BB rounds of 1 min, afterwards, extracts were filtered. The filtered extract was used as a control (“Beads beating”) or heat-treated for 30 min at 95 °C and filtered again before derivatization (“Beads beating + Filtering + Boiling”). Total thiamine as nmol/ml (C) and vitamer distribution percentage of thiamine (D) in the mixed standard solution. A mixed standard solution of TPP, TP, and T was used as control, the same solution was heat-treated for 30 min at 95 °C (“Hot hydrolysis”), then filtered again, and samples were analysed in duplicate.

an oxidation reagent because of its lower toxicity compared to other reagents such as cyanogen bromide or mercuric chloride (Edwards et al., 2017). The purpose of adding phosphoric acid is to quench the derivatization reaction, and at the same time neutralizes the extracts. The latter prevents wearing and damaging of the pre-column, column, and pipes. An important consideration to have an accurate thiamine determination is to minimize phosphate ester degradation. This can be achieved by working with freshly prepared thiamine standard solutions and by monitoring the possible thiamine phosphates esters degradation by injecting them both in combination and separately. We tested the method for the detection of TTP in 0.1 M HCl, the method is able to detected this thiamine vitamer (Appendix 1).

Studies on thiamine extraction using acid hydrolysis reported low thiamine recovery due to the formation of impurities and precipitates when using sulfuric acid and TCA. Therefore in this study hydrochloric acid was the preferred solvent for thiamine extraction, as it has been extensively used for extracting thiamine from yeast biomass (Edwards et al., 2017). The different extraction methods tested not only showed different thiamine yields, but also different vitamer proportions in each sample. When hot acid hydrolysis was used to extract thiamine from the yeast samples TP increased in proportion compared to BB alone, while T stayed roughly the same in all samples. This decrease in TPP and increase in TP could be due to higher amounts of TP being extracted through hot acid hydrolysis, and/or to the conversion of TPP into TP. As was confirmed by the follow-up experiments, total extraction yield increased when samples were heat-treated after BB, independently of the use of filtered or unfiltered samples before heat-treatment. This indicates that the binding of thiamine to proteins plays a role in the extraction, and consequently this would advocate for the use of heat during the extraction (Chandra-Hioe et al., 2015). However, heat treatment also causes TPP degradation to TP, but not to T, as proven by the boiling of the standard solutions (Fig. 6C and 6D). This heat-induced conversion of TPP into TP should not be neglected for studies that require a specific determination of the vitamers. Therefore, hot hydrolysis alone, or a combination of hot hydrolysis and BB could lead to determination of an incorrect vitamer proportion and thus biologically irrelevant data. This is even more important for studies aimed at better understanding of thiamine biosynthesis and metabolism in yeasts. Furthermore, it is also possible to accelerate protein degradation by using a proteases. This requires an additional step of incubation of the extracts with the enzyme, followed up by enzymatic inactivation, possibly via heat treatment (thereby risking the degradation of TPP to TP). The use of an enzymatic step could therefore potentially increase the thiamine recovery, but lengthen the extraction procedure.

During BB extraction using lysing matrix E no TPP was found in the extract after derivatization, the reason behind this is still unknown. We assume this could be due to some interaction between the TPP and components found only in lysing matrix E, such as ceramic beads and a glass sphere, in addition to the silica beads that are also present in matrix B and C.

The biological state of the yeast and the growth phase determine the vitamer proportions and the intracellular total thiamine. Total thiamine is highest during exponential growth to then decreases during the stationary phase. The vitamer proportions also changes during yeast growth (Kowalska et al., 2012), therefore, the variations in the thiamine yield and the vitamer composition that were observed in this study between the different experiments are due to the incubation time of the yeast culture before extraction.

## 5. Conclusions

In this study we combined two previously known methods for thiamine detection. The thiochrome derivatization method was combined with reversed phase-HPLC coupled to fluorescence detection, to create a new reproducible thiamine-vitamer detection and quantification method of which we proved the robustness, reproducibility, and repeatability. In conjunction to the detection and quantification method, we developed and optimised a thiamine extraction method using beads-beating with silica beads. We proved that the commonly used heat-treatment of fresh yeast biomass for extracting thiamine leads to the degradation of TPP to TP, resulting in a non-selective determination of the thiamine vitamers. Although this mechanical cell disruption extraction method yields lower amounts of thiamine, it can still be used to measure and determine the different thiamine phosphate forms with increased specificity. This new finding is particularly useful for all studies that aim to determining the thiamine proportions in fresh yeast biomass specifically.

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## Declaration of Competing 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.

## Data availability

Data will be made available on request.

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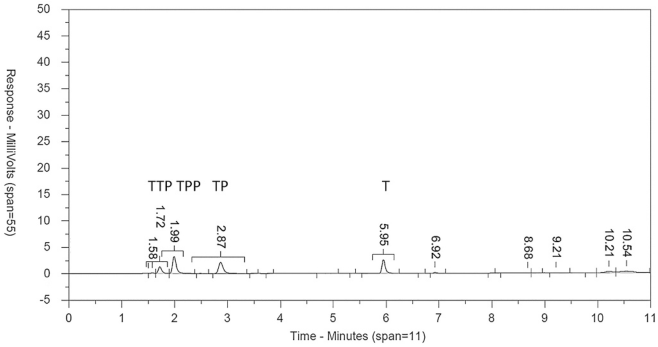
## Appendix

**Appendix 1** Chromatogram of the standard solution containing TTP, TPP, TP, and T. The fluorescence response of the different thiamine forms is given in mV and the respective retention times are presented in min.



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