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Optimisation of the determination of thiamin, 2-(1-hydroxyethyl)thiamin, and riboflavin in food samples by use of HPLC

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Abstract

The aim of this study was first to optimise and validate a method using an enzyme-mixture to liberate protein- and phosphate-bound thiamin and riboflavin in food by the use of ultrasonication and HPLC, and second to include the quantitation of the vitamin B_1 active compound 2-(1-hydroxyethyl)thiamin (HET).

The enzyme-mixture consisted of α-amylase, proteinase, and phosphatase. The use of ultrasonication in the enzyme treatment enabled the results for vitamin B_1 and B_2 to be obtained in 1 day. In consequence of an incomplete release of phosphate-bound thiamin of some of the batches of enzymes used, thiamin was quantitated as the sum of thiaminmonophosphate and thiamin. The vitamin B_1 active compound, HET was detected and quantitated separately. The standard deviations for the method were 3.7%, 4.7%, and 13.3% for thiamin, riboflavin, and HET, respectively. The relative bioactivity of HET is similar to the bioactivity of thiamin. In the samples of animal origin the content of HET represented 7–24% of the content of thiamin, while in dried yeast the content of HET was 37% of the content of thiamin. Quantitation of vitamin B_1 in food by a post-column derivatisation is recommended to include separate quantitation of thiamin and HET.

Keywords: Vitamins; Thiamin; HET; Riboflavin; Ultrasonication; HPLC; Food composition

1. Introduction

Essential for the calculation of dietary intake of nutrients from food is the use of reliable, accurate and precise analytical methods for nutrients in the foods. For vitamin B_1 and vitamin B_2 European standards were set in 2003 (European Standard, 2003a, 2003b). The principle of these methods is the release of thiamin as well as riboflavin from proteins, and the conversion of phosphorylated compounds, including thiaminmonophosphate (TMP), thiamindiphosphate (TDP) and thiamintriphosphate to thiamin, and flavinmononucleotide (FMN) and flavin adenine dinucleotide (FAD) to riboflavin. It is possible to quantitate each of these vitamers separately (Fox, Ackerman, & Thayer, 1992; Russell & Vanderslice, 1992; Viñas, Balsalobre, López-Erroz, & Hernández-Córdoba, 2004). However, since the bioavailability of these vitamers is regarded as equal, the quantitation of each of the vitamers is not important for the information in food composition tables (Bates, 1997; Gregory, 1997).

The critical part of the analysis is the enzymatic treatment necessary to liberate protein- and phosphate-bound thiamin and riboflavin (Arella, Lahély, Bourguignon, & Hasselmann, 1996; Defibaugh, 1987; Hasselmann, Franck, Grimm, Diop, & Soules, 1989; Hollman et al., 1993; Ndaw, Bergaentzle, Aoude-Werner, & Hasselmann, 2000; Ollilainen et al., 1993).

The time-consuming part of the analysis is the acid hydrolysis followed by enzymatic hydrolysis. The optimum duration of the latter was found to be 4–18 h for vitamin B_1 and 18 h for vitamin B_2 (van den Berg & van Schaik, 1995).

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Since 1950, the chemical determination of thiamin has used the derivatisation of thiamin to thiochrom by alkaline $K_3Fe(CN)_6$, followed by extraction with 2-methyl-1-propanol. Finally, fluorescence detection was used for quantitation (AOAC, 1984). The introduction of HPLC enabled the derivatisation step to be performed either pre-column (Arella et al., 1996; Bognar, 1981; Finglas & Faulks, 1984; Hasselmann et al., 1989; Ndaw et al., 2000; Ollilainen et al., 1993) or post-column (Bognar, 1992; Defibaugh, 1987; Ujiie, Tsutake, Morita, Tamura, & Kodaka, 1990; Wimalasiri & Wills, 1985). Development of all the methods has focused on the detection and the quantitation of thiamin and thiaminphosphates. However, in 1990 the metabolite 2-(1-hydroxyethyl)thiamin (HET) was identified running a post-column HPLC-method for thiamin (Ujiie et al., 1990). Comparison between a manual thiochrom method similar to AOAC (1984) and a post-column derivatisation HPLC-method showed that the manual thiochrom method quantitated the sum of thiamin and HET. Especially in fish and meat samples this was noticeable due to a relatively high content of HET in these products (Ujiie, Tsukake, Morita, Matsuno, & Kodaka, 1991).

In the certification procedure for the reference materials for vitamin B_1 and B_2 from the Institute for Reference Materials and Measurements, Geest, Belgium (IRMM) the takadiastase from Phaltz & Bauer was applied (Finglas, Scott, Witthöft, van den Berg, & de Froidmont-Göritz, 1998). However, subsequent problems with newer batches of this enzyme did crop up as the results for certified reference materials as well as recovery of spiked samples failed quality control. Only the recovery tests of standard solutions were in accordance with the specification of the accredited procedure.

The aim of this study was first to optimise and validate a method using an enzyme-mixture to liberate phosphate- and protein-bounded thiamin and riboflavin in food by use of ultrasonication and HPLC, and second to include quantitation of the vitamin B_1 active compound HET. The study was based on the work presented by Ndaw et al. (2000), and our accredited method performed according to CEN-methods, EN14122 and EN14152 (European Standard, 2003a, 2003b).

2. Materials and methods

The optimisation and validation study was performed in our laboratory, which is accredited for the analyses of vitamin B_1 and B_2 according to ISO17025.

2.1. Reagents

2.1.1. Standards

The purity of the standards was >99% unless otherwise stated. For vitamin B_1 the standards were as follows: thiamin as thiamin hydrochloride, a USP reference from Rockville, MD ($M_w = 337.28$), thiaminmonophosphate (TMP) as thiaminmonophosphate chloride dihydrate No. 88340 from Fluka, Sigma–Aldrich ($M_w = 416.82$), thiamindiphosphate or cocarboxylase (TDP) No. C8754 from Sigma, Sigma–Aldrich ($M_w = 460.8$), and 2-(1-hydroxyethyl)thiamin (HET) from Wako Chemicals GmbH, Neuss, Germany ($M_w = 381.33$). The standards for vitamin B_2 were riboflavin from the WHO centre for Chemical reference, Solna, Sweden ($M_w = 376.36$), and riboflavin-5′-monophosphate sodium salt, dihydrate (FMN) No. 83810 (>89% by HPLC) from Fluka, Sigma–Aldrich ($M_w = 514.36$).

2.1.2. Enzymes

The following enzymes were used: takadiastase from *Aspergillus oryzae* (No. T00040, Phaltz & Bauer), α-amylase from *A. oryzae* (No. A6211, Sigma, Sigma–Aldrich), proteinase as papain from papaya latex (No. P3375, Sigma, Sigma–Aldrich), and acid phosphatase, type IV-S from potato (No. P1146, Sigma, Sigma–Aldrich).

2.2. Samples

For the optimisation and validation study of the method presented 16 samples were included, representing the food groups dairy, meat, vegetables, cereals and yeast. The specific samples were milk powder, baby formula, liver paté, raw liver, fried liver, chopped pork, pork meat balls, and lyophilized white cabbage and lyophilized broccoli, oat flour, whole meal flour, wheat bran, and dried yeast. All the samples were stored at −20 °C and analysed within a year. For the validation study the certified reference materials milk powder, CRM 421 and lyophilized pigs liver, CRM 487 from IRMM were analysed.

2.3. Enzyme optimisation

The enzymatic activity given in the certificate from the manufacturer was taken into account when estimating the amount used for the samples.

2.3.1. α-Amylase

Raw liver was used for the optimisation of α-amylase at pH 4.5 and a temperature of 45 °C for 18 h. The amounts tested were 0, 210, 420, 1050, 2100, 4200, and 10,500 U α-amylase in duplicate combined with 12 U papain and 100 U phosphatase.

2.3.2. Phosphatase

For the optimisation of acid phosphatase, broccoli and oat flour were used for the thiamin determinations, while broccoli, oat flour, and dried yeast were used for the riboflavin determinations. The amounts of phosphatase tested were 0, 22, 55, 77, 99, and 121 U combined with 4200 U α-amylase, and 12 U papain. The incubation conditions were pH 4.5 and a temperature of 45 °C for 18 h.

2.3.3. Papain

Proteinase as papain was optimised in wholemeal flour and dried yeast. The amounts of proteinase tested were 0,

3, 6, 9, 15, and 21 U combined with 4200 U α-amylase, and 55 U phosphatase. The incubation conditions were pH 4.5 and a temperature of 45 °C for 18 h.

2.3.4. Ultrasonication

Use of an ultrasonic bath (Ultrasonic cleaner 8890, Cole–Parmer Instrument Company, Vernon Hills, IL, USA) was tested with pork liver and oat flour, as well as with recovery tests. The two enzyme-mixtures tested were 55 U phosphatase combined with 4200 U α-amylase; and 55 U phosphatase and 4200 U α-amylase combined with 12 U papain. Furthermore, two different time-intervals were tested, 20 and 60 min, combined with five different pH levels, 4.0, 4.5, 4.8, 5.2 or 5.6.

2.4. Enzymatic extraction protocol

2.4.1. Standardised HPLC method: use of takadiastase at 45 °C for 18 h

In a 100 ml conical flask, 50 ml 0.1 M hydrochloric acid (HCl) was added to 1–5 g sample. The solution was autoclaved at 121 °C for 30 min, and then cooled and adjusted to pH 4.0 with 4 M sodium acetate. Subsequently, 100 mg takadiastase was added, and the solution was incubated at a temperature of 45 °C for 18 h. Finally, the solution was transferred to a volumetric flask and diluted to 100 ml with 0.01 M HCl. If necessary the solution was diluted to an appropriate concentration prior to filtration with 0.2 μm filter and injection into the HPLC-system.

2.4.2. The method presented: use of enzyme mixture and ultrasonication

In a 50 ml conical flask, 25 ml 0.1 M HCl was added to a test sample with a content maximum of 100 μg phosphorylated compounds of riboflavin and thiamin i.e. 0.2–15 g. The solution was autoclaved at 121 °C for 30 min, and subsequently cooled followed by adjustment to pH 4.5 with 4 M sodium acetate. For enzymatic treatment 4.5 ml of a solution containing 4200 U amylase, 12 U papain and 55 U acid phosphatase, and 500 μl 1% glutathione was added. This solution was placed in the ultrasonic bath for 1 h. The solution was then transferred to a volumetric flask and diluted to 50 ml with 0.01 M HCl. If necessary the solution was diluted to an appropriate concentration prior to filtration through a 0.2 μm filter and injection into the HPLC-system.

2.5. Chromatographic quantitation

2.5.1. Apparatus

The HPLC system (Waters Milford, MA, USA) was equipped with a pump (600), an autosampler (717plus), a fluorescence detector (2475), an In-Line Degasser, and a Reagent Manager for the post-column derivatisation reagent. Waters software Millennium, Client Server version No. 4 was used for quantitation of the peak areas.

2.5.2. Chromatographic conditions

The HPLC-system was equipped with a reversed phase column (Supelcosil LC-18-DB, 5 μm particle size, 250 × 4.6 mm, Supelco, Bellefonte, PA, USA) and was run isocratic using methanol:buffer (35:65) as the mobile phase. The modifiers in the buffer were sodium heptasulfonate (12.7 mM), tetraethyl ammonium chloride (0.1%) and potassium dihydrogenphosphate (50 mM) adjusted to pH 3.3. The injection volume was 50 μl and the flow rate 1 ml/min. Riboflavin was measured directly with fluorescence detection using excitation and emission wavelengths at 468 nm and 520 nm, respectively. Thiamin, TMP, and HET were measured indirectly with fluorescence detection after post-column oxidation to thiochrom, thiochrommonophosphate and hydroxyethylthiochrom, respectively. The post-column oxidation utility was a plastic tube (2 m × 0.33 m), while the derivatisation reagent phase consisted of 0.15 mM potassium hexacyanoferrat (III) and 3.5 M potassium hydroxide. The settings of the fluorescence detector were 368 nm for excitation and 420 nm for emission. Separate injections from the same extract were run for the determination of thiamin and HET or riboflavin, respectively.

2.6. Data analysis

For clarification the results for HET are expressed as thiamin hydrochloride (thiamin, HCl), and the contents of HET multiplied by 0.884, which is the relation between the two compounds molecular weight. The sum of thiamin and HET are expressed as vitamin B_1. For the detection and quantitation of TMP in the chromatogram the content was multiplied by 0.809 to be given as thiamin, HCl, and the sum of thiamin, HCl detected as TMP and thiamin are given as the content of thiamin. In the recovery test the added TDP and FMN was calculated as the equivalent thiamin, HCl and riboflavin, respectively before calculation of the %-recovery.

The linearity of the calibration curves was tested with the studentized residuals of linear regression. The linearity was checked by evaluation of the residuals i.e. if the residuals showed no significant difference from zero. ANOVA and GLM-procedures were performed for the results from the optimisation of amounts of enzymes and the ultrasonication conditions, respectively. To identify individual differences Tukey's Studentized Range test was used. The Statistical Analysis System software package, version 9.1 (SAS Institute, Cary, NC, USA) was used. Agreement between the presented method and the standardised HPLC method was assessed according to (Bland & Altman, 1986). A *P*-value of less than 0.05 was considered significant. All values are mean ± standard deviation (SD), unless otherwise specified.

3. Results

3.1. Enzymatic optimisation

The optimisation results for α-amylase and phosphatase are given in Tables 1 and 2, respectively. The use of different amounts of α-amylase showed no difference in content of riboflavin ($P = 0.26$), but for thiamin and HET the quantitated content depended on amount of α-amylase ($P < 0.001$ and $P = 0.02$, respectively). The use of varying amounts of phosphatase showed a significant effect on thiamin in broccoli ($P = 0.003$), but not on thiamin in oat flour ($P = 0.67$). The content of riboflavin in broccoli, oat flour and dried yeast showed a significant effect ($P = 0.003$, $P < 0.001$, and $P < 0.001$, respectively). The results for optimisation of papain from 0 to 21 U per g sample identified no significant difference in the amount of thiamin or riboflavin for either whole meal flour or dry-yeast (results not shown). The results of the tests of different conditions for the ultrasonication procedure are summarised in Table 3. The enzyme used, pH, and duration of ultrasonication showed a significant effect on the recovery of thiamin ($P < 0.001$), but no effect on the recovery of riboflavin ($P = 0.14$).

Table 1
Effect on quantity of thiamin, HET, and riboflavin by use of varying amounts of α-amylase, 55 U phosphatase, and 12 U papain per g sample of liver

U (α-amylase/g sample)	Thiamin (mg/100 g[A])	HET (mg/100 g[A])	Riboflavin (mg/100 g[B])
0	0.166[c]	0.0050[b]	2.40
210	0.184[c]	0.0061[a]	2.57
420	0.206[b]	0.0078[a]	2.66
1050	0.218[b]	0.0094[a]	2.72
2100	0.236[a]	0.0104[a]	2.73
4200	0.238[a]	0.0104[a]	2.75
10,500	0.242[a]	0.0106[a]	2.76

[A] Values within a column with unlike superscript were significantly different ($P < 0.05$).
[B] No significant difference ($P = 0.26$).

Table 2
Effect on quantity of thiamin in broccoli and oat flour, and of riboflavin in broccoli, oat flour, and dried yeast by use of varying amounts of phosphatase, 4200 U α-amylase and 12 U papain per g sample

Phosphatase	Thiamin[A]		Riboflavin[A]		
U/g sample	Broccoli	Oat flour[B]	Broccoli	Oat flour	Yeast
0	0.840[c]	0.201	1.01[b]	0.34[b]	2.16[b]
22	0.893[bc]	0.205	1.35[a]	0.65[a]	3.82[a]
55	0.952[ab]	0.207	1.45[a]	0.65[a]	3.88[a]
77	0.958[ab]	0.207	1.47[a]	0.68[a]	4.02[a]
99	1.001[a]	0.208	1.48[a]	0.69[a]	4.04[a]
121	1.005[a]	0.209	1.57[a]	0.70[a]	4.07[a]

[A] Values within a column with unlike superscript were significantly different ($P < 0.05$).
[B] No significant difference ($P = 0.67$).

3.2. Performance characteristics for the validated method

3.2.1. Linearity

The calibration curve in the concentration range for injection of 50 μl 0.01–0.10 μg/ml of thiamin, TMP, riboflavin and FMN, and 0.001–0.01 μg/ml for HET was characterized linear as the studentized residuals of the linear regression analysis were not significantly different from zero. The relative responses between TMP/thiamin, HET/thiamin, and FMN/riboflavin were 0.92, 0.86 and 0.66, respectively.

3.3. Detection and quantitation limits

The quantitation limit for thiamin, TMP, HET and riboflavin was 20 pg corresponding to 50 μl 0.4 ng/ml. However, the quantitative determination limit also depended on the content of the vitamer in the added enzymes, and on the weight of the samples. In the injection solution, the content of thiamin and riboflavin from the enzymes added to each sample corresponded to 0.0013 μg/ml and 0.003 μg/ml, respectively. The weight of the samples varied between 0.2 and 15 g, with a quantita-

Table 3
Recovery of TDP added to milk powder and FMN added to white cabbage with different conditions for the enzymatic hydrolyses

%	TDP added to milk powder[A]					FMN added to white cabbage[B]				
	20 min		60 min			20 min		60 min		
pH	Amyl[C]	Phos[D]	Amyl[C]	Phos[D]	Mix[E]	Amyl[C]	Phos[D]	Amyl[C]	Phos[D]	Mix[E]
4.0	55[d]	7[f]				85	83			
4.5			96[ab]	94[abc]	103[a]			76	74	85
4.8	86[c]	12[f]	100[ab]	94[abc]		89	79	81	70	
5.2			99[ab]	93[bc]	97[ab]			80	69	84
5.6	39[e]	5[f]				62	85			

Enzyme was α-amylase, phosphatase or the mixture of enzymes, pH between 4.0 and 5.6, ultrasonication for 20 or 60 min.
[A] Values with unlike superscript were significantly different ($P < 0.05$).
[B] No significant difference ($P = 0.14$).
[C] 4200 U α-amylase/g sample.
[D] 55 U phosphatise/g sample.
[E] 4200 U α-amylase, 55 U phosphatase, and 12 U papain per g sample.

tion limit of 0.004 mg/100 g for thiamin and 0.01 mg/100 g for riboflavin. None of the enzymes contained TMP or HET, hence the quantitation limit was assessed to 0.001 mg/100 g. Chromatograms of vitamin B_1, a standard and an extract of pigs liver, and for vitamin B_2, a standard and an extract of whole milk powder, are shown in Figs. 1 and 2, respectively.

3.4. Precision

For standard solutions, the between-days standard deviation was 3.2–3.7% ($n = 10$) for all the compounds. The triplicate analyses of each of the 16 samples included in the comparison study showed a relative standard deviation of 0.4–13.3% for thiamin, 1.0–13.7% for riboflavin, and 7.9–23.2% for HET in the 7 samples in which HET was detected. For vitamin B_1 (the sum of thiamin and HET) the standard deviation in the 7 samples was 1.2–5.4%.

Pooled standard deviation for all samples was 3.7% for thiamin, 13.3% for HET, and 4.7% for riboflavin.

3.5. Accuracy

3.5.1. Certified reference materials

The results for the analysed certified reference materials were as follows with the certification values shown in brackets (Finglas et al., 1998). The content of thiamin obtained in pigs liver and milk powder were 0.87 ± 0.03 mg/100 g (0.86 ± 0.11 mg/100 g) and 0.71 ± 0.02 mg/100 g (0.651 ± 0.048 mg/100 g), respectively. For riboflavin the similar values were 10.8 ± 0.3 mg/100 g (10.68 ± 0.56 mg/100 g) and 1.50 ± 0.04 mg/100 g (1.45 ± 0.06), respectively. In pigs liver the content of HET was 0.10 ± 0.02 mg/100 g.

3.6. Comparison between standardised method and presented method

The results for the analysed samples for the contents of thiamin, HET, and riboflavin are shown in Table 4. The method by Bland–Altman (Bland & Altman, 1986) was used to compare the results for thiamin and riboflavin assessed by the two methods. No statistical difference was detected for either thiamin or riboflavin.

4. Discussion

4.1. Enzymatic treatment

Many efforts have been made to find the optimum extraction procedure for the enzyme treatment necessary for the release of phosphate bound vitamers of vitamin B_1 and B_2 (Arella et al., 1996; Bertelsen, Finglas, Loughridge, Faulks, & Morgan, 1988; Defibaugh, 1987; Hasselmann et al., 1989; Hollman et al., 1993; Ndaw et al., 2000; Ollilainen, Mattila, Varo, Koivistoinen, & Huttunen,

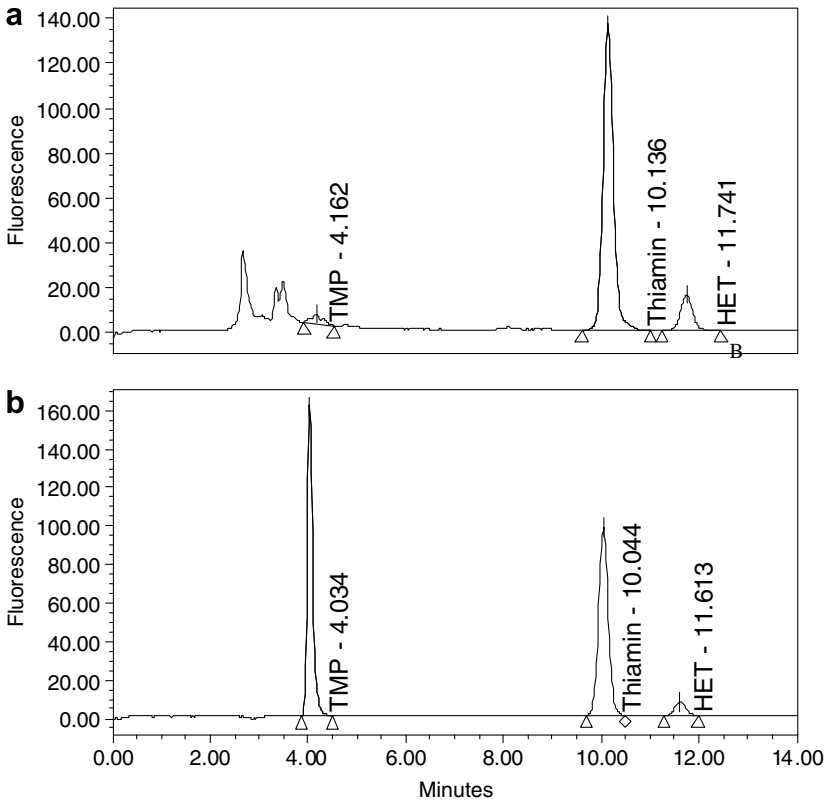

Fig. 1. Chromatogram of pigs liver, CRM 487 (a), and TMP (0.1 μg/ml), thiamine (0.1 μg/ml) and HET (0.01 μg/ml) (b).

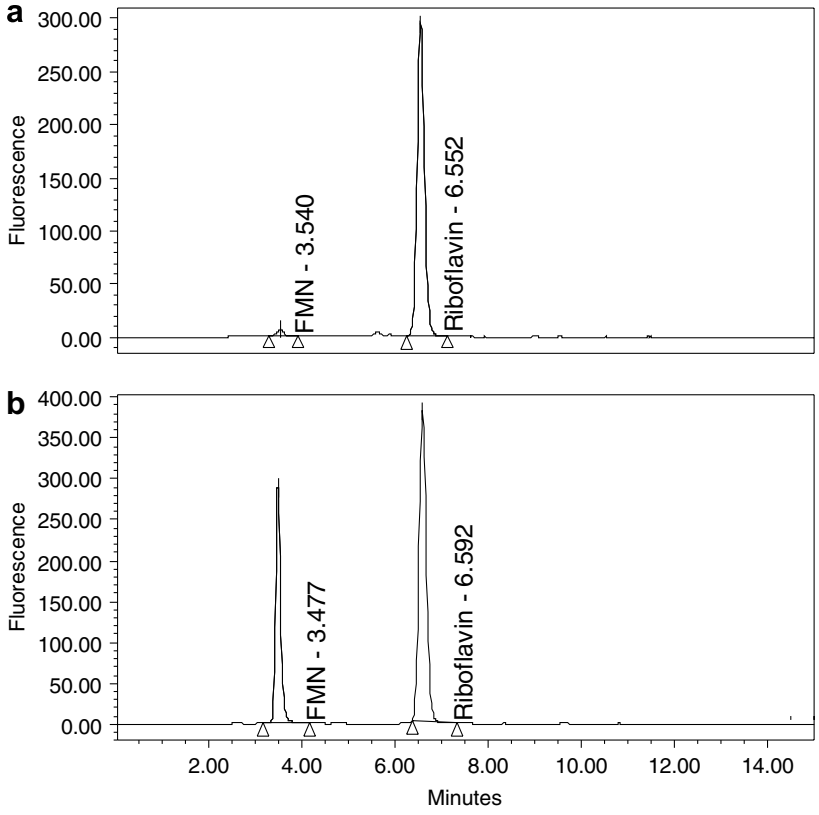

Fig. 2. Chromatogram of whole milk, CRM421 (a), and FMN (0.025 μg/ml) and riboflavin (0.025 μg/ml) (b).

Table 4
Quantity of thiamin, riboflavin, and HET by the presented method and thiamin and riboflavin by a standardised method

	Thiamin[A]				HET[A]		Riboflavin			
Enzyme[B]	Takadiastase		Enzyme-mixture		Enzyme-mixture		Takadiastase		Enzyme-mixture	
Enzymatic condition[B]	Oven		Ultrasonication		Ultrasonication		Oven		Ultrasonication	
Content (mg/100 g[C])	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Whole milk powder, CRM 421	0.656	0.009	0.707	0.016	ND		1.48	0.02	1.50	0.04
Milk powder	0.308	0.012	0.323	0.005	ND		1.30	0.04	1.30	0.08
Baby formula	0.468	0.003	0.447	0.002	ND		0.74	0.03	0.69	0.05
Pigs liver, CRM 487	0.869	0.023	0.869	0.028	0.10	0.02	11.0	0.4	10.8	0.3
Liver, raw II	0.221	0.005	0.227	0.009	0.015	0.003	2.85	0.01	2.73	0.26
Liver, raw I	0.288	0.020	0.270	0.006	0.066	0.013	2.95	0.29	2.98	0.16
Liver, fried I	0.393	0.018	0.371	0.017	0.029	0.002	3.23	0.19	3.29	0.14
Liver paté	0.073	0.008	0.071	0.003	ND		1.14	0.08	1.08	0.02
Chopped pork meat	0.730	0.037	0.713	0.036	0.093	0.008	0.20	0.01	0.18	0.01
Pork meat balls	0.442	0.018	0.450	0.022	0.071	0.006	0.22	0.01	0.22	0.01
White cabbage, freeze-dried	0.430	0.015	0.391	0.013	0.007	0.003	0.26	0.01	0.24	0.01
Broccoli, freeze-dried	1.30	0.05	1.32	0.01	ND		1.54	0.04	1.51	0.02
Oat flour	0.213	0.004	0.230	0.020	ND		0.06	0.00	0.07	0.01
Whole meal flour	0.400	0.023	0.465	0.062	ND		0.07	0.00	0.07	0.01
Wheat bran	1.06	0.08	1.29	0.10	ND		0.21	0.00	0.22	0.01
Yeast, dried	7.87	0.19	8.09	0.10	0.301	0.070	4.12	0.18	3.76	0.08

[A] Results given as thiamin, HCl.
[B] Detailed description of the condition, see Section 2.
[C] Mean and standard deviation (SD) based on 3–11 determinations. Not detected (ND).

1990; Ötles, 1991). Mainly the enzymes used have been an α-amylase characterized by impurity activity of phosphatase and proteinase e.g. chlaradiastase and takadiastase. The takadiastase from different producers showed a difference in the capability to liberate phosphate bound thiamin and riboflavin (van den Berg & van Schaik, 1995). The use

of a mixture of specific enzymes seemed beneficial as this might diminish future needs for replacement of enzymes. The amount necessary for each of the specific enzymes was in this study optimised by ordinary incubation in an oven at a temperature of 45 °C, and included addition of glutathione as a reducing agent to secure full activity of papain (Bergaentzle, personal communication, 2003). The adequate amount of the rather expensive phosphatase was assessed as the lowest level, which did not significantly differ from the highest amount of enzymes tested i.e. 55 U. But for α-amylase the level next to the lowest level, which did not significantly differ from the highest amount of enzymes was chosen i.e. 4200 U. No effect on quantitated thiamin; HET, or riboflavin was observed for varying amounts of papain, therefore the amount chosen was equivalent to the amount assessed by Ndaw et al. (2000) i.e. 12 U. These amounts differed slightly from the amounts used by Ndaw et al. (2000), which were 1800 U α-amylase, 100 U phosphatase and 10 U papain. The ultrasonication procedure was assumed to have the same optimum amounts of enzymes, as the ultrasonication did not change the efficiency of the enzymes e.g. the same amounts of TMP were quantitated in the samples. Results showed that recovery of FMN did not statistically depend on either pH or enzymes (α-amylase, phosphatase or enzyme mixture) used for the enzymatic hydrolysation. In contrast, the recovery of TDP depended on pH, enzyme, and duration of the ultrasonication procedure. However, if ultrasonication for 60 min no significant difference was observed between enzymes and pH 4.5 and 4.8.

When the enzymatic treatment at 45 °C for 18 h was replaced with enzymatic treatment for 1 h in an ultrasonic bath no differences in the results were observed. Actually, if the ultrasonication process was performed by the more efficient ultrasonic probe (Microson XL2007, Misonix Inc, Farmingdale, NY) satisfactory release of thiamin and riboflavin within 2–60 s was observed. Previously, a similar quick release was found for selenium from yeast (Capelo, Ximenez-Embun, Madrid-Albarran, & Camara, 2004). The reason for the fast reaction rate is not fully understood (Bermejo, Capelo, Mota, Madrid, & Camara, 2004; Capelo et al., 2004).

The optimisation study was run with a single batch of the enzymes, which showed 100% dephosphorylation of TDP/TMP and FAD/FMN. However, in a chromatogram of sample extracts analysed with other batches of enzymes, TMP was identified. In such cases, the amount detected and quantitated as TMP was converted to content of thiamin, HCl and added to the amount of thiamin, HCl detected and quantitated as thiamin. The amount of thiamin quantitated as TMP depended on the enzyme batch and the matrix. Though the presented method includes quantitation of thiamin detected as TMP, the specific value for TMP is not given, as the method does not quantitate the specific content of TTP, TDP, TMP and thiamin originally available in the samples.

4.2. Precision and accuracy

The internal reproducibility was 3.7% for thiamin and 4.7% for riboflavin. These values were comparable to those of the accredited standardised HPLC-method.

The accuracy of the presented method was satisfactory as the results for certified reference materials, pigs liver CRM 487 and milk powder CRM 421 were in agreement with the certified values. The recovery tests for TDP and FMN performed in standard solution as well as added to the samples showed recoveries for TDP of 97–103% and 99–104%, respectively, and for FMN, 81% and 77%, respectively. The lower recovery for FMN was in agreement with the control chart for the accredited method, which is judged acceptable due to the lower purity of the FMN standard. The comparison of the developed method with the HPLC standardised method showed a significant difference only for thiamin in wheat bran by the test of Bland and Altman (1986). However, no explanation has been found for this discrepancy.

4.3. HET

Hydroxyethylthiamin pyrophosphate (HET-PP) was identified as an "active acetaldehyde" in the function of thiamin as a coenzyme for carboxylase. Decarboxylation of pyruvate serves as an acetaldehyde donor in the enzymatic reaction (Carlson & Brown, 1961; Jordan & Nemeria, 2005; Kluger, Stergiopoulos, Gish, & Karimian, 1985). DL-HET was found to have 80% of the bioactivity of thiamin in a microbiological assay using *Lactobacillus fermenti* as well as *Lactobacillus viridescens* (Krampitz et al., 1958). Furthermore D-HET showed a little higher effect than L-HET, but was almost equivalent to that of thiamin in a microbiological assay using *L. fermenti* and *Kloeckera apiculata* (Kawasaki, Kishi, & Shinoda, 1967). Orally administered vitamers to thiamin-deficient rats showed a similar equivalent activity of DL-HET, D-HET and L-HET (Shiobara, Sato, Homma, Hattori, & Murakami, 1965). Even though relative bioactivity between thiamin and HET was conducted with bacteria, the documentation for the necessity to include quantitation of HET as vitamin B_1 active compounds seems adequate.

The present study showed that a post-column derivatisation technique enables the quantitation of HET. The content of HET compared to the content of thiamin in the samples analysed was 2% in broccoli, 7–24% in liver and meat, while in dried yeast the content of HET constitutes 37%. From these results it may be concluded that the content of HET is important in order to quantitate the vitamin B_1 activity, especially in the samples of animal origin and yeast.

Comparable contents were quantitated by Ujiie et al. (1991), e.g. HET constituted 27% of thiamin in bakers yeast, 2–12% in liver and meat, but only traces in broccoli.

Comparison between pre- and post-column derivatisation of thiamin showed no difference, but this study did not include detection and quantitation of HET (Bognar,

1992). Use of post-column enables the possibility to identify HET, while no published information is available concerning detection of HET in pre-column systems.

5. Conclusion

This study introduces ultrasonication in the traditional enzymatic release of protein- and phosphate bound thiamin and riboflavin in the determination of vitamin B_1 and B_2 in foods. Furthermore, in the cases of incomplete conversion to thiamin the quantitation of remaining TMP in the final extract is described. The ultrasonication process did not show any effect on the efficacy of the enzymes but enabled the enzymatic treatment to be performed within 1 h, as a replacement for 4–18 h incubation for vitamin B_1 and 18 h incubation for vitamin B_2 in the standardised methods. The present method was comparable to an accredited version of the standards methods for thiamin as well as for riboflavin. Additionally, the method included quantitation of HET, which is a vitamin B_1 active compound equal in bioactivity to thiamin, and should be added to the quantity of thiamin to give the content of vitamin B_1. Especially in sample of animal origin and yeast the content of HET is important in order to quantitate the vitamin B_1 activity. Determination of vitamin B_1 in food by a post-column derivatisation is recommended to include separate quantitation of thiamin and HET. Further investigations of HET are planned for estimating the effect of cooking, as well as a comparative study between a post- and pre-column derivatisation procedure for the determination of thiamin and HET.

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