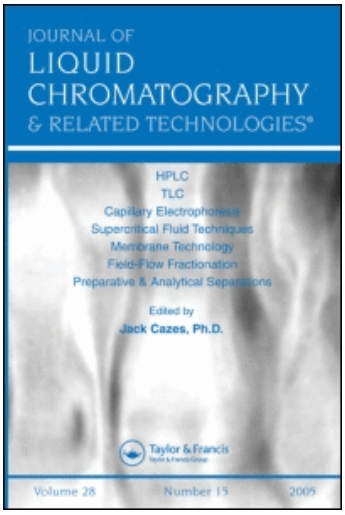

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A Simple Determination of Thiamine in Rice (*Oryza Sativa* L) by High-Performance Liquid Chromatography with Post-Column Derivatization

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To cite this Article Ohta, Hideaki , Maeda, Mari , Nogata, Yoichi , Yoza, Koh-Ichi , Takeda, Yuko and Osajima, Yutaka(1993) 'A Simple Determination of Thiamine in Rice (*Oryza Sativa* L) by High-Performance Liquid Chromatography with Post-Column Derivatization', Journal of Liquid Chromatography & Related Technologies, 16: 12, 2617 — 2629

To link to this Article: DOI: 10.1080/10826079308019597

URL: http://dx.doi.org/10.1080/10826079308019597

JOURNAL OF LIQUID CHROMATOGRAPHY, 16(12), 2617-2629 (1993)

A SIMPLE DETERMINATION OF THIAMINE IN RICE (*Oryza sativa* L.) BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH POST-COLUMN DERIVATIZATION

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ABSTRACT

We report a rapid high-performance liquid chromatographic method for the determination of thiamine in rice with fluorimetric post-column derivatization. The analysis system consisted of a combination of both thiamine extraction with a mixture of 0.1 M hydrochloric acid-40% methanol (0.1 M HCl-40% MeOH) solution from rice flour, and chromatographic separation and determination. To extract thiamine, the rice flour was refluxed for 30 min at 60 °C with 0.1 M HCl-40% HCl. The separation systems constituted of a Sorbax TMS column, column oven (55°C), elution system containing a mixture of 0.01 M sodium dihydrogen phosphate and 0.5 M sodium perchlorate solution, a mixing coil for chemical reaction and a spectrofluorimetric detector. The thiamine derivative compound was detected at 375 nm for excitation and 435 nm for emission. The recovery of thiamine was 94 -101%. The contents of thiamine in rice of six cultivars produced in Japan were determined by the above-mentioned HPLC method and AOAC method. The two methods gave comparable results and a high correlation coefficient (r = 0.958) was obtained.

INTRODUCTION

In plant material thiamine is largely present in the free form (1), although the principal form in the animal products is the pyrophosphate ester. Ultraviolet (UV) detectors have been used for the high-performance liquid chromatographic determination in samples that contain sufficient amounts of thiamine including multivitamin pharmaceutical preparations (2,3) and enriched cereal products (4). However, a fluorescence detector is necessary for the detection in unsupplemented foods that have microgram amounts of thiamine at natural levels, because of the low sensitivity of the UV detector (5). Thiamine is easily converted through oxidation to thiochrome, which exhibits strong fluorescence. An alkaline solution of potassium ferricyanide is generally used as an oxidizing agent. Thiamine can be converted to thiochrome by a pre-column reaction before the HPLC separation (6,7) and a post-column reaction (8,9,10).

The major cereal crop in Asian countries is rice (*Oryza sativa* L.) which provides not only carbohydrates, proteins and lipids but also minerals and vitamins as well. In the case of cereal food takadiastase is generally added to hydrolyze the starch present in the sample (11). This procedure, however, is time-consuming. To examine the thiamine content of rice, which is gathering interest, we used a combination of simple extraction with a mixture of hydrochloric acid-methanol (HCl-MeOH) solution and the determination using a high-performance liquid chromatography (HPLC) with post-column derivatization.

This paper describes a simple analytical method for thiamine in rice which involves extraction from rice flour with a mixture of HCl-MeOH solution followed by HPLC determination with post-column derivatization.

MATERIALS AND METHODS

Materials

Brown and polished rice of six cultivars, including Koshihikari, Sasanishiki, Toyonishiki, Kiyonishiki, Akihikari and Kitahikari, were used. The rice was polished with a commercial polisher (Satake, Hiroshima, Japan) to 90% and 93% of its initial weight to remove the husk and bran. All the rice investigated was

ground into rice flour in a laboratory mill to pass through a 30-mesh screen, mixed by tumbling, then stored in a desiccator.

Apparatus

The system consisted of an LC-3A pump for liquid chromatography, a SIL-1A injector, a Zorbax TMS column (25 cm x 4.6 mm I.D.; Du Pont, Wilmington, Delaware, USA); CTO-2A column oven (55°C), a stainless steel mixing coil (30 cm x 0.8 mm I.D.), a PRR-2A proportioning pump, an RF-530 spectrofluorimetric detector (excitation, 375 nm; emission, 435 nm) and a Chromatopac C-R1B recordor-integrator. All the instruments were purchased from Shimadzu (Kyoto, Japan).

Reagents

Thiamine hydrochloride, sodium dihydrogen phosphate, sodium perchlorate, and potassium hexacyanoferrate (III) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and takadiastase from Sankyo (Tokyo, Japan). All other chemicals were also of an analytical reagent grade quality commercially available.

Thiamine extraction

Two methods for extracting thiamine from rice were used.

HCl-MeOH solution extraction A 3.00 gram portion of rice flour was weighed into a 100 ml volume erlenmeyer flask, and stirred with a glass rod to make a homogeneous mixture after addition of 50 ml of a 0.1 M HCl-40% MeOH solution. Then the flask was connected with a refluxing cooler and refluxed for 30 min in a water bath (60°C). The HCl-MeOH extract was homogenized for 1 min with a Voltex mixer and vibrated for 20 min with an Ultrasonic wave vibrator (Branson Co., CT, U.S.A.), followed by centrifugation at 3 000 x *g* for 20 min. A portion of the extract supernatant was filtered with a 0.45 μm Milipore filter

(Milipore Corporation, Tokyo, Japan). Duplicate injections of 5 µl of filtrate were performed onto the HPLC column and the average value was reported.

Conventional extraction The rice flour was decomposed by enzymatic hydrolysis with takadiastase according to the AOAC (1984) method (11), then centrifuged at 3 000 x *g* for 20 min. This extract supernatant was used for conventional analysis.

Determination of thiamine

HPLC procedure A mixture of 0.01 M sodium dihydrogen phosphate and 0.5 M sodium perchlorate solution (adjusted to pH 2.5 with 3 M perchloric acid) was pumped at a flow rate of 0.4 ml/min as the mobile phase. Then a 0.1% potassium hexacyanoferrate(III) -15% sodium hydroxide solution was provided at a flow rate of 0.4 ml/min by a proportioning pump and mixed with the column eluate to convert thiamine to thiochrome. The thiochrome was measured with a fluorescence detector.

Conventional procedure This was accomplished by the manual AOAC method (11).

RESULTS AND DISCUSSION

Determination of chromatographic conditions

To obtain the desired separation of thiamine, we used a Zorbax TMS column and systematically studied the different factors which control the retention of thiamine, using the brown rice (Akihihikari cultivar) extract prepared by enzymatic hydrolysis with takadiastase (9).

The mobile phase was the combination solution of sodium dihydrogen phosphate and sodium perchlorate as described in the experimental procedure. Figure 1 shows the relation between the retention time of thiamine and sodium perchlorate concentration range studied (0-0.5 M), at the concentration of sodium

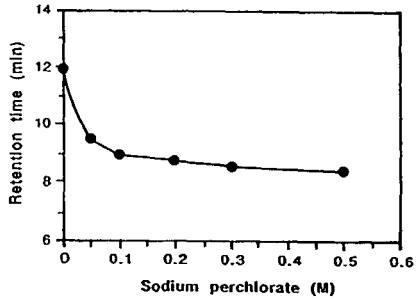

FIGURE 1. Effect of concentration of sodium perchlorate in the mobile phase on retention time of thiamine. Mobile phase is a mixture of 0.01 M sodium dihydrogen phosphate and 0-0.5 M sodium perchlorate solution adjusted to pH 2.2 with 3 M perchlorate. For other conditions, see text.

dihydrogen phosphate of 0.01 M, pH 2.2 and column oven temperature of 55°C. The retention time of thiamine gradually decreased with the increase in sodium perchlorate concentration. The use of 0.01 M sodium dihydrogen phosphate and 0.5 M sodium perchlorate solution as mobile phase gave the shortest retention time of thiamine and this concentration was used for the following studies.

The pH of the mobile phase was adjusted with 3 M perchloric acid. Figure 2 (a) illustrates the relation between the retention time of thiamine and pH range of 2.2-4.5 of the mobile phase. The retention behavior of the thiamine peak was strongly affected by the pH of the mobile phase, as expected. The retention time of thiamine markedly decreased with decreasing pH. This was probably due to the protonation of the amino group. It is obvious from Figure 2 (a) that, at pH 2.2 - 2.7, the retention times are convenient for rapid analysis. Therefore, we selected pH 2.5.

The efficacy of Zorbax TMS column is well known to increase with temperature. Figure 2 (b) shows the variation of the retention time with temperature between 40 and 60°C. The retention behavior of thiamine depended on the temperature. The retention time of thiamine decreased moderately as the temperature increased. A temperature of 55°C which gave a shorter retention time was selected.

Figure 3 shows the chromatograms obtained with fluorescence detection using the chromatographic conditions previously defined. Fluorescent peaks were observed at 4.5 and 8.5 min, which indicates that the second peak

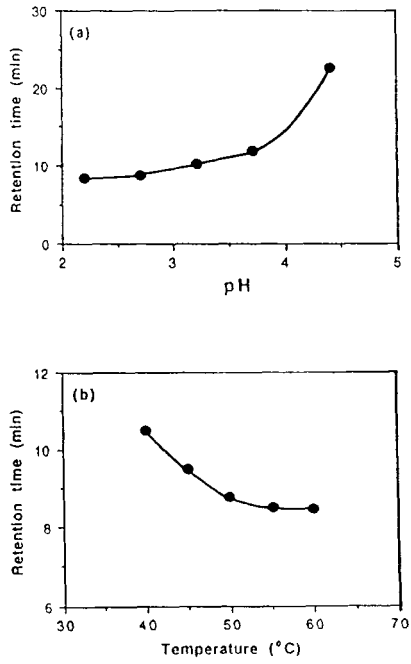

FIGURE 2. Effects of pH in mobile phase (a) and column oven temperature (b) on retention time of thiamine. The pH in the mobile phase was adjusted with 3 M perchloric acid. For other conditions, see text.

corresponded to the thiamine standard. Therefore, we precluded the unknown peak of interfering compounds present in the rice flour sample.

These findings indicated that the best chromatographic conditions were a mixture (pH 2.5) of 0.01 M sodium dihydrogen phosphate and 0.5 M sodium perchlorate solution, at a flow rate of 0.4 ml/min and a temperature of 55°C.

In order to check the linearity of the relationship between the amount of thiamine and peak height using the above chromatographic system, suitable amount of thiamine was weighed and mixed in a measuring flask, dissolved and suitably diluted with distilled water to serve as a standard solution. Various amounts of the standard solution were injected and chromatographed. The graphs exhibited good linearity and obeyed Beer's law in the thiamine concentration range of 0 - 35 ng, as shown in Figure 4. The linear regression

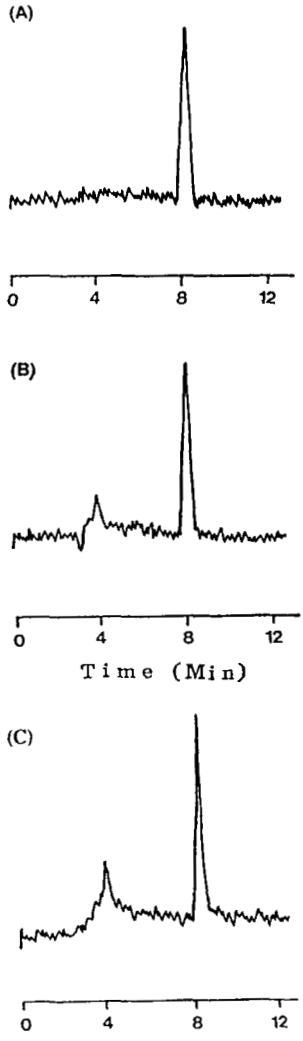

FIGURE 3. Chromatograms of thiamine standard at 30 mg/ml (A), and thiamine extracts from 90% polished rice(B) and brown rice (C) of Akihikari cultivar. Sample size is 5 µl. For other conditions, see text.

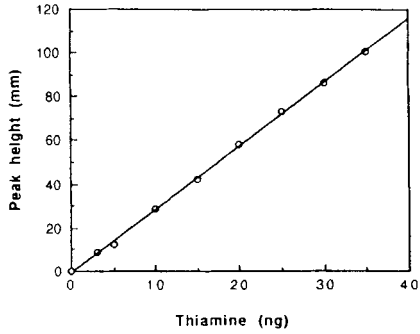

FIGURE 4. Thiamine calibration curve (peak height (mm) versus nanograms of thiamine injected). Linear regression line is y = 2.9404x - 0.9066 with a 0.999 correlation coefficient. For conditions, see text.

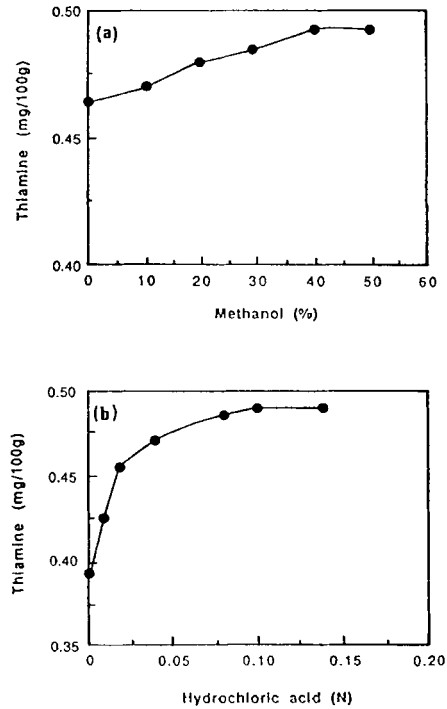

FIGURE 5. Effects of methanol (a) and hydrochloric acid (b) concentration in extracting solvent on recovery of thiamine from brown rice of Akihikari cultivar. Extraction solvent consisted of a mixture of hydrochloric acid (0-0.14 M) and methanol (0-50%). For other conditions, see text.

TABLE 1

Influences of incubation temperature (a) and incubation time (b) on thiamine extracted from brown rice flour of Akihikari cultivar

Incubation temperature* (°C)	Thiamine extracted*** (mg/100g)
20	0.463
40	0.470
50	0.485
60	0.492
70	0.491
Incubation time ** (min)	**Thiamine extracted*** (mg/100g)**
10	0.472
20	0.480
30	0.492
40	0.491
50	0.492
60	0.491

*This experiments were performed under the incubation time of 1 hr.
**These experiments were carried out under the incubation temperature of 60 °C.
***Average of duplicate analyses.

between the peak height (y) and the thiamine content (x) was expressed as y= 2.9404x- 0.9066 (r = 0.999). This indicated the high sensitivity of this method.

Optimization of thiamine extraction conditions

Instead of using the enzymatic hydrolysis procedure for thiamine extraction, we performed the extraction procedure by hydrolysis with a mixture of hydrochloride and methanol (HCl-MeOH), using brown rice (Akihihikari cultivar) to develop a simple and rapid method of extracting thiamine from rice.

A mixture of HCl-MeOH was used as the extracting solvent. The MeOH concentration for extracting the thiamine from rice flour was examined for efficiency. Portions of 3.00 g of rice flour were weighed and extracted at 60°C for

TABLE 2

Thiamine content of rice determined by the HPLC and the AOAC methods

Rice Cultivar	Sample	Thiamine (mg/100g)	
		HCl-MeOH & HPLC*	AOAC**
Koshihikari	Polished rice (90%)	0.22 ± 0.01	0.25
	Polished rice (93%)	0.35 ± 0.01	0.34
	Brown rice	0.53 ± 0.03	0.51
Sasanishiki	Polished rice (90%)	0.20 ± 0.01	0.18
	Polished rice (93%)	0.32 ± 0.02	0.33
	Brown rice	0.46 ± 0.02	0.47
Toyonishiki	Polished rice (90%)	0.13 ± 0.01	0.15
	Polished rice (93%)	0.24 ± 0.01	0.28
	Brown rice	0.41 ± 0.01	0.36
Kiyonishiki	Polished rice (90%)	0.18 ± 0.01	0.17
	Polished rice (93%)	0.33 ± 0.01	0.31
	Brown rice	0.47 ± 0.01	0.45
Akihikari	Polished rice (90%)	0.25 ± 0.02	0.23
	Polished rice (93%)	0.40 ± 0.01	0.37
	Brown rice	0.49 ± 0.02	0.49
Sasanishiki	Polished rice (90%)	0.21 ± 0.01	0.19
	Polished rice (93%)	0.36 ± 0.02	0.39
	Brown rice	0.46 ± 0.02	0.43

For the HPLC conditions, see text.
* The combination of extraction with 0.1 M HCl-40% MeOH and dertermination by HPLC. The values are average ± standard deviation for six replicate analyses.
**AOAC method. The values are average of duplicate analyses.

1 hr with a mixture of 0.1 M HCl and MeOH at a concentration ranging from 0 to 50%. Figure 5 (a) presents the relation between thiamine content detected and MeOH concentration, and indicates that 40% MeOH gave a maximum thiamine content.

Figure 5 (b) shows the effect of HCl concentration on the efficiency of extracting thiamine from rice, under 40% MeOH. The extraction efficiency of thiamine depended on the HCl concentration. The best efficiency was observed

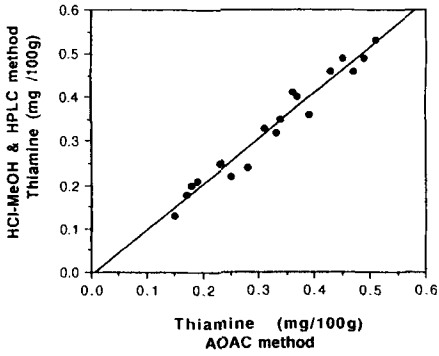

FIGURE 6. Relation between thiamine contents in the rices determined by hydrochloric acid-methanol (HCl-MeOH) extraction & HPLC method and AOAC method. A linear regression is obtained at y = 1.0417x-0.0054 with a correlation coefficient of 0.958. For conditions, see Experimental section.

in the HCl concentration range of 0.04-0.14 M. We selected 0.1 M HCl-40% MeOH as extracting solvent.

We examined the optimal incubation temperature and time for extracting thiamine from rice flour with 0.1 M HCl-40% MeOH. Table 1 shows the relationship between the amounts of thiamine and the incubation temperature (20-70°C) under the incubation time of 1 hr. The maximum relative amount was incubated at 60°C.

The incubation time affected thiamine extraction at the incubation temperature of 60°C as is also shown in Table 1. The highest thiamine content from rice flour was observed after the incubation time of 30 - 60 min.

These finding indicated that the best conditions for extracting thiamine from rice flour was to reflux 3.00 g of rice flour with 0.1 M HCl-40% MeOH for 30 min at 60°C, as mentioned in the experimental procedure.

Recovery of thiamine from rice

In order to determine the recovery of thiamine, we added suitable amounts of thiamine standard to brown rice flour with a known content before the HCl-MeOH hydrolysis. The amount of thiamine added to the brown rice flour was 0.2

mg per 100 g. In the case of Sasanishiki brown rice (0.46 mg /100 g as shown in Table 2), the total amount was determined to be 0.65 mg per 100 g, *i.e.*, a 98 % recovery. Repeated experiments gave recoveries in the range 94-101%, indicating a high accuracy.

Thiamine content of six rice cultivars and comparison of the two methods

The thiamine contents in brown, 93% and 90% polished rice of six rice cultivars were determined, in order to demonstrate the validity of this method. As shown in Table 2, the brown rice of Koshihikari had a higher thiamine content, followed by Kiyonishiki and Akihikari. As expected, the thiamine content of all rice cultivars investigated gradually decreased in the order of brown rice, 93% polished rice and 90% polished rice. This suggested that the bran had a high content of thiamine.

The two methods for analyzing the thiamine content in rice were compared for precision. Table 2 also shows the thiamine contents determined by the combination of HCl-MeOH extraction and HPLC described in the experimental procedure (HCl-MeOH & HPLC) and the AOAC method (11). The linear relationship between the thiamine contents (y) obtained by HCl-MeOH & HPLC method and those (x) by AOAC method method was expressed as y= 1.0417x - 0.0064. A high correlation coefficient (r=0.958) was obtained as shown in Figure 6, which indicates that the two methods give comparable results.

CONCLUSIONS

According to these results, the combination of HCl-MeOH extraction and HPLC with a fluorescent detection is convenient and highly sensitive in determining the thiamine in rice. It is also more rapid and simple than the conventional technique (11). In particular, HCl-MeOH extraction is a useful procedure to save time. Furthermore, there is no need to use enzymes for hydrolysis, or isobutanol as solvent for extracting thiamine. This simple extraction would also be applicable for recovery of thiamine from various plant materials because most of the thiamine in plants is present in a free form (1).

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid from the Ministry of Agriculture, Forestry and Fisheries of Japan. One of authors (Y.T) is indebted to the Science and Technology Agency of Japan for a National Institute Postdoctoral Fellowship.

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Received: December 23, 1992
Accepted: January 13, 1993