Simultaneous Determination of Thiamine and Riboflavin in Edible Marine Seaweeds by High-Performance Liquid Chromatography

D.I. Sánchez-Machado, J. López-Cervantes, J. López-Hernández*, and **P. Paseiro-Losada**

Departamento de Química Analítica, Nutrición y Bromatología, Facultad de Farmacia, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, La Coruña, Spain

Abstract

This study presents a high-performance liquid chromatography (HPLC) method for simultaneous determination of thiamine and riboflavin and the results of its application to a number of edible seaweeds that are sampled in dried form (*Himanthalia elongata, Laminaria ochroleuca, Undaria pinnatifida, Palmaria sp.,* and *Porphyra sp.*) or as canned food (*H. elongata* and *Saccorhiza polyschides*). Samples are prepared by acid and enzymatic hydrolysis. Optimized conditions for reversed-phase HPLC with fluorescence detection are as follow: column, Kromasil 100 C18; column temperature, 35°C; mobile phase, a 72:28 (v/v) mixture of 0.005M ammonium acetate (pH 6.7)–methanol; and flow rate, 1.35 mL/min. With these conditions, recovery is 95.52% for thiamine and 90.08% for riboflavin, and the method precision (relative standard deviation) is 2.66% for thiamine and 2.21% for riboflavin. On a dry weight basis, thiamine contents range from 0.14 µg/g in dried H. elongata to 2.02 µg/g in dried Porphyra and riboflavin contents from 0.31 µg/g in canned *H. elongata* to 6.15 µg/g in dried Porphyra.

Introduction

Thiamine (vitamin B_1) and riboflavin (vitamin B_2) are water-soluble members of the vitamin B complex (1). Thiamine pyrophosphate (TPP) and thiamine triphosphate (TTP) play essential roles in energy transformation, membrane transport, and nerve function. Riboflavin is a precursor of the coenzymes riboflavin mononucleotide and flavin adenine dinucleotide, which are also involved in energy metabolism (2).

The extraction of thiamine and riboflavin from foodstuff matrices for analytical purposes generally involves their separation from protein by acid hydrolysis (usually with hydrochloric acid) (3–12), followed by enzymatic dephosphorylation (3–6,9–13), although this latter process is sometimes omitted (7,8) and proteins are sometimes removed by precipitation with trichloroacetic acid (14). Both vitamins can be quantitated in foodstuffs by high-performance liquid chromatography (HPLC) with either UV detection (15) or fluorescence detection (3–13,16,17); in the latter case, riboflavin needs no derivatization because of its natural fluorescence, but thiamine requires prior oxidation to thiochrome by reaction with potassium hexacyanoferrate (III). Methods using fluorescence detection are generally regarded as faster, more sensitive, and more specific than those using UV detection.

Fresh or dried seaweed is a traditional food that is a significant component of the diet in many coastal areas, especially on the Pacific coasts of Asia and South America (18). Certain seaweeds are a source of protein, vitamins, and essential minerals (19–21), and their use by the food industry and consumption by the general public is increasing, especially in Europe. However, most of the specific nutritional information on edible seaweeds concerns traditional Japanese species (22), and even for these there are significant gaps in our knowledge. In particular, studies of the thiamine and riboflavin contents of algae have essentially concerned fresh microalgae of aquacultural interest, some of which have been reported to be rich in thiamine and relatively poor in riboflavin (23,24); for macroalgae, very little information is available in this area (25).

In this work we describe a rapid, reliable method for the simultaneous determination of thiamine and riboflavin in edible seaweeds, and we report both data validating the method and the results of its application to the determination of these vitamins in several specific edible seaweeds harvested on the northwest Iberian coast, which were sampled either in dried form (*Himanthalia elongata, Laminaria ochroleuca, Undaria pinnatifida, Palmaria sp.,* and *Porphyra sp.*) or as canned food (*H. elongata* and *Saccorhiza polyschides*).

Experimental

Reagents and standards

Thiamine chloride and riboflavin standards were purchased from Sigma (St. Louis, MO); HPLC-grade methanol, ammonium acetate, acetic acid, hydrochloric acid, ethanol, and sodium acetate from Merck (Darmstadt, Germany); clara-diastase and

* Author to whom correspondence should be addressed: email qnlhjul@usc.es.

potassium hexacyanoferrate (III) from Fluka (Buchs, Switzerland); and sodium hydroxide from Quimon (Barcelona, Spain). Doubly distilled water was obtained from a Milli-Q system (Waters, Bedford, MA).

The concentrations of solutions other than standards were as follow: hydrochloric acid, 0.1N and 3.75N; ammonium acetate, 0.005M; sodium acetate, 2.5M; clara-diastase, 6% in water; and potassium hexacyanoferrate (III), 1% in 15% NaOH [potassium hexacyanoferrate (III) solutions were always prepared on the day of use, and until used were stored in amber flasks in a refrigerator].

Stock standard solutions of thiamine chloride were prepared by dissolution in 20% ethanol and stock standard solutions of riboflavin by dissolution in 0.02N acetic acid; both stock solutions were protected from the light and stored in a refrigerator pending use. Working calibration standards were made up by dilution of the stock standards to obtain thiamine hydrochloride solutions of 0.23–69.12 ng/mL and riboflavin solutions of 1.0–125.8 ng/mL.

Seaweeds

Dried seaweeds

Between July 2001 and April 2002, *Himanthalia elongata, Laminaria ochroleuca, Undaria pinnatifida, Palmaria sp.*, and *Porphyra sp.* were manually harvested at sites on the northwestern Iberian coast and were transported, in plastic mesh bags and at ambient temperature, to the seaweed processing plant of Algamar S.A. (Redondela, Pontevedra, Spain), where they were successively dried at 45ºC for 24 h, stored at room temperature for 3 days, and sealed in 100-g lots in polypropylene bags.

Canned seaweeds

In August 2001, *Himanthalia elongata* and *Saccorhiza polyschides* were harvested at sites in or on the Atlantic coast adjoining the Ría de Arousa (A Coruña, Spain) and were transported to the processing plant of Conservas y Ahumados Lou (Ribeira, A Coruña, Spain), where they were canned together in equal proportions by a process involving sterilization at 112°C for 40 min.

Sample preparation

All samples were dried in a vacuum oven at 62°C for 4 h (canned seaweeds were previously drained and predried in a conventional oven at 48°C for 48 h), and thiamine and riboflavin contents were expressed on a dried weight basis.

To extract thiamine and riboflavin, we performed acid hydrolysis as per Sims et al. (7) and used the methods of Valls et al. (9) for enzymatic hydrolysis and the derivatization and cleanup of thiamine. Specifically, 1 g of finely ground oven-dried sample was placed in an amber-colored flask, 15 mL of 0.1N HCl was added, and the mixture was heated in a water bath at 100°C for 30 min, allowed to cool to room temperature, brought to pH 4.3–4.7 with 2.5M sodium acetate, treated with 1.25 mL of a 6% aqueous solution of clara-diastase, incubated in an oven at 50°C for 3 h, filtered through Whatman No. 41 paper (Maidstone, England), and diluted to 25 mL in a volumetric flask with Milli-Q water. The resulting solutions remained stable during overnight storage in the refrigerator.

To derivatize its thiamine content, 2.5 mL of the solution prepared as mentioned was treated with 1.25 mL of a 1% solution of potassium hexacyanoferrate (III) in cold 15% aqueous NaOH, stirred in a vortex mixer for 15 s, left standing for 45 s, and finally mixed with 0.25 mL of 3.75N hydrochloric acid in the vortex mixer.

Prior to HPLC, samples were cleaned by passage through a Waters C_{18} Sep-Pak column. Following activation of the column by successive passage of 5 mL of methanol (followed by 5 mL of 0.005M ammonium acetate), 5 mL of sample solution was loaded, interferences were removed by passage of 5 mL of 0.005M ammonium acetate, vitamins were eluted with 5 mL of the HPLC mobile phase [a 72:28 (v/v) mixture of 0.005M ammonium acetate and methanol], and the eluate was passed through a Millipore 0.45-µm cellulose filter.

Equipment

High-pressure liquid chromatography was performed using an HP1000 quaternary pump and an HP1000 fluorescence detector from Hewlett-Packard (Waldbronn, Germany) together with a 20-µL injection loop from Rheodyne (Rohnert Park, CA), all controlled by Hewlett-Packard HPCHEM software. A 25- × 0.4-cm i.d. Kromasil 100 C_{18} column packed with 5-µm particles (Teknokroma, Barcelona, Spain) was thermostatted at 35ºC by means of a Mod. 8792 column thermostatting system from Spectra-Physics (San Jose, CA). The mobile phase was a 72:28 (v/v) mixture of 0.005M ammonium acetate–methanol, and the flow rate was 1.35 mL/min. Fluorescence was recorded at the optimal wavelength for thiamine for 6.2 min, followed by the optimal wavelength for riboflavin for another 3.8 min.

Results and Discussion

Presence of thiamine and riboflavin in the seaweeds

In preliminary experiments, samples prepared from seaweeds as described previously showed the same retention time (5.40 ± 0.09 min for thiamine and 6.96 ± 0.15 min for riboflavin) and very similar excitation and emission as mixtures of the stock riboflavin and derivatized stock thiamine solutions when run on a 250- × 4.6-cm i.d. Spherisorb ODS2 column with 5-µm particles using a 1.4 mL/min flow of a 3:7 mixture of methanol–0.005M ammonium acetate of unadjusted pH. In particular, when run using the fluorescence excitation and emission wavelengths that afforded the best response for the individually run standards (l_{ex} = 370 nm and l_{em} = 435 nm for thiamine and l_{ex} = 370 and l_{em} = 520 nm for riboflavin), they showed single peaks that had the same retention times as the standards and, when in scan mode, afforded fluorescence spectra that were identical with those of the standards. These findings confirmed the presence of thiamine and riboflavin in these foodstuffs and validated the choices of wavelengths for quantitation.

Optimization of HPLC conditions

The simultaneous analysis of thiamine and riboflavin by HPLC has been historically problematic. It has been difficult to achieve adequate peak separation for B_1 and B_2 without interfering peaks (6,7,16). In view of the problems observed by researchers, most of the HPLC methods for determination of thiamine and riboflavin in food are based on the same principle, with modifications only in the extraction, cleanup, and chromatographic conditions

(4,7,12,14,16). In the present study, different chromatographic conditions have been tested.

With a mobile phase of 28:72 methanol–0.005M ammonium acetate of pH 5 (7), the thiamine peak eluted first and showed considerable tailing, resulting in its overlapping the more symmetric riboflavin peak. This problem was largely solved using a mobile phase of pH 6.7, thiamine being ionic but not riboflavin (16). The symmetry of both peaks was improved by replacing the Spherisorb ODS2 column mentioned with a 250- × 4.6-cm i.d. 5-µm particle Kromasil 100 C_{18} column. In trials of three mobile phase compositions [25:75, 28:72, and 30:70 (v/v) mixtures of methanol and 0.005M aqueous ammonium acetate] and three mobile phase flow rates (1.30, 1.35, and 1.47 mL/min), the best peak resolution was achieved with the 28:72 mixture and a 1.35 mL/min flow rate. These conditions were then used in trials of four different column temperatures (26ºC, 28ºC, 30ºC, and 35°C), in which best results were obtained at 35°C. All of the stated optimization experiments were performed using 1:1 mixtures of thiamine and riboflavin standards. Chromatograms of this mixture and of a prepared sample of *Porphyra sp.*, both obtained under the optimized conditions, are shown in Figures 1A and 1B, respectively. The estimated *k'* values (4.84 ± 0.25 for thiamine and 6.52 ± 0.35 for riboflavin, for an average of 18 injections) fell in the range of typical chromatographic separation.

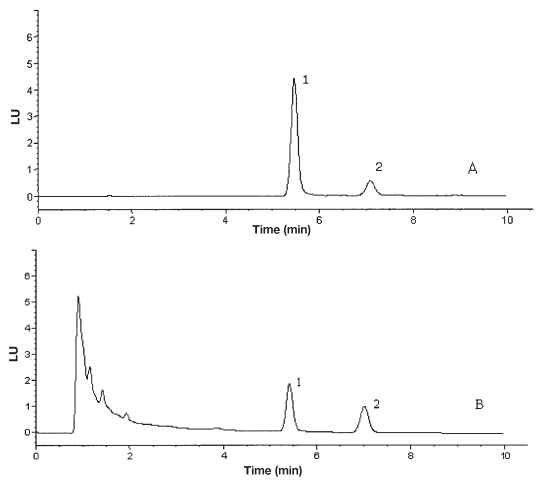

Figure 1. Chromatograms of a 1:1 mixture of thiamine and riboflavin standards (A) and a prepared sample of *Porphyra sp.* (B). Peaks identified: thiamine (1) and riboflavin (2).

Table I. Analytical Quality Parameters of the Proposed Method for Simultaneous Determination of Thiamine and Riboflavin in Edible Seaweeds

Parameter	Thiamine	Riboflavin
Calibration line		
y-Intercept	0.4624	0.1205
Slope	0.6631	0.1247
r	0.9998	0.9996
Calibrated range (ng/mL)	0.23–69.12	1.0–125.8
Precision (*n* = 6)		
Mean (µg/g)	2.59	3.95
RSD (%)	2.66	2.21
LOD (ng/mL)	0.02	0.20
Recovery (%)	95.52	90.08

Method quality parameters

Calibration lines constructed using duplicate injections of five concentrations in the ranges 0.23–69.12 ng/mL for thiamine and 1.0–125.8 ng/mL for riboflavin had Pearson correlation coefficients of $r > 0.9996$ (Table I). Precision [relative standard deviation (RSD) expressed as a percentage], evaluated using duplicate injections of six *U. pinnatifida* extracts prepared on the same day, was 2.66% for thiamine and 2.21% for riboflavin, which is sufficient for routine analyses in quality control laboratories. The limits of detection (LODs), the concentrations affording a signal three times the background noise (26), were 0.02 ng/mL for thiamine and 0.20 ng/mL for riboflavin, which are smaller than those presented in other works. Fernando and Murphy (8) reported 5 and 2 ng/mL for thiamine and riboflavin, respectively, and Augustin has also reported (16) 0.5 and 1.0 ng/mL for thiamine and riboflavin, respectively. For evaluation of recovery, six samples of powdered *U. pinnatifida* were spiked with approximately their own contents of thiamine and riboflavin prior to extraction, derivatization, and quantitation; the values obtained (95.52% for thiamine and 90.08% for riboflavin) (Table I) are acceptable, considering the concentrations and type of both analytes (27).

Thiamine and riboflavin contents of seaweeds

The thiamine and riboflavin contents of the processed seaweeds were determined by separate preparation and analysis of 10 samples of each. In all cases, the riboflavin content was higher than thiamine content (Table II). Thiamine contents ranged from 0.14 µg/g in dried *H. elongata* to 2.02 µg/g in dried *Porphyra* and riboflavin contents from 0.31 µg/g in canned *H. elongata* to 6.15 µg/g in dried *Porphyra*. These values are, in many cases, approximately twice than those reported for cooked vegetables such as cauliflower, cabbage, beets, or spinach (25). Among the dried seaweeds, the rhodophytes (*Palmaria* and *Porphyra*) were richer in both thiamine and riboflavin than the phaeophytes (*H. elongata*, *L. ochroleuca*, and *U. pinnatifida*). The thiamine and riboflavin

Table II. Thiamine and Riboflavin Contents of Some Edible Marine Seaweeds

	Concentration (g/g d.w.)*	
Sample	Thiamine	Riboflavin
Dried seaweeds		
Himanthalia elongata	0.14 ± 0.02	1.14 ± 0.14
Laminaria ochroleuca	0.40 ± 0.13	0.98 ± 0.17
Undaria pinnatifida	1.18 ± 0.23	1.40 ± 0.13
Palmaria sp.	1.15 ± 0.22	4.27 ± 0.39
Porphyra sp.	2.02 ± 0.15	6.15 ± 0.49
Canned seaweeds		
Himanthalia elongata	0.26 ± 0.04	0.31 ± 0.05
Saccorhiza polyschides	0.55 ± 0.05	0.90 ± 0.12

* Means ± standard deviations (*n* = 10).

contents of dried *H. elongata* were, respectively, 52% and 366% of their values in canned *H. elongata*, probably because thiamine will have undergone thermal degradation during drying, whereas riboflavin, which is photolabile in aqueous solutions, will have been relatively more susceptible to prolonged precanning washing and the salinity of the canning solution (17,28). Finally, it should be pointed out that the thiamine and riboflavin contents of seaweeds doubtlessly depends not only on species but also, like other aspects of their nutritional composition, on geographical location, time of year, and water temperature (20).

Conclusion

In this work, we optimized HPLC conditions for simultaneous determination of the thiamine and riboflavin contents of dried or canned seaweeds following sample preparation by acid hydrolysis, enzymatic hydrolysis, and derivatization of thiamine. The method developed exhibits satisfactory accuracy, precision, LOD, and linearity. It is probably applicable to other vegetable species of similar chemical composition. Red seaweeds proved to have higher thiamine and riboflavin contents than brown seaweeds. Thiamine content was reduced more by drying than canning, and riboflavin content was reduced more by canning than drying.

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