Determination of Total Riboflavin in Cooked Sausages

Felicidad Valls,[†] M. Teresa Sancho,*[,‡] Miguel A. Fernández-Muiño,[‡] and Martín A. Checa[†]

Campofrío Alimentación, S.A., Fundación Sonsoles Ballvé s/n, 09007 Burgos (Castilla y León), Spain, and Departamento de Biotecnología y Ciencia de los Alimentos, Area de Nutrición y Bromatología, Universidad de Burgos, Plaza de Misael Bañuelos García s/n, 09001 Burgos (Castilla y León), Spain

A simple and rapid method for determining riboflavin content in cooked sausages by ion-pair reversed-phase liquid chromatography has been set up. Samples were subjected to acid and enzymatic hydrolysis. Sample extracts were directly chromatographed, avoiding purification and concentration treatment. Final determination was performed by high-performance liquid chromatography with fluorescence detector (excitation, 227 nm; emission, 520 nm), on a 25 cm × 4 mm i.d. Spherisorb ODS-2 cartridge using a mixture of 5 mM heptanesulfonic acid adjusted to pH 2.7 with phosphoric acid and acetonitrile (75:25, v/v) as mobile phase. Precision of the method was 1.3% (within a day) and 2.6% (between days). The detection limit was 0.015 mg/100 g. The recovery was >95%.

Keywords: *Riboflavin; cooked sausages; ion-pair HPLC*

INTRODUCTION

The water-soluble vitamin riboflavin structurally is composed of an isoalloxazine ring with a ribityl side chain at the nitrogen at position 10. This vitamin functions metabolically as the essential component of two flavin coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which act as intermediaries in transfers of electrons in biological oxidation−reduction reactions. Animal protein sources such as meats are good sources of riboflavin (Basu and Dickerson, 1996).

Acidification and enzyme hydrolytic treatment guarantee the complete hydrolysis of FAD and FMN to riboflavin. One of the most important problems of riboflavin analysis is the lability of flavins to light and alkaline conditions, so all analytical procedures have to be performed avoiding both factors (Basu and Dickerson, 1996).

Even though numerous methods have been reported for the determination of riboflavin content in foods, reversed-phase liquid chromatography has been recommended, employing an ultraviolet detector (Toma and Tabekhia, 1979; Ashoor et al., 1985; Suhara et al., 1985; Vidal-Valverde and Reche, 1990; Barna, 1991; Barna and Dworschak, 1994) or a fluorescence detector (Lumley and Wiggins, 1981; Fellman et al., 1982; Finglas and Faulks, 1984; Macrae et al., 1984; Brubacher et al., 1985; Johnsson and Branzell, 1987; Reyes et al., 1988; Ollilainen et al., 1990), because of the strong ultraviolet light absorption of flavins and their intense fluorescence.

In cooked sausages riboflavin analysis is difficult. On the one hand, riboflavin occurs as coenzyme forms complexed with proteins, which makes necessary two hydrolysis steps to yield the free vitamin (Basu and Dickerson, 1996). On the other hand, riboflavin analysis in these foodstuffs makes necessary attention to the reagents employed for removing the organic matter (Association of Vitamin Chemists, 1966) to avoid riboflavin decomposition.

The purpose of this paper has been to develop a simple, rapid, and reliable high-performance liquid chromatography (HPLC) method with fluorescence detection for the quantitation of total riboflavin and to apply this method to difficult matrices such as cooked sausages. Riboflavin separation from interferences has been researched with both reversed-phase and ion-pair reversed-phase chromatographic conditions. An additional purpose has been to study all of the steps of official analytical methods for improving or avoiding, if possible, some of them.

MATERIALS AND METHODS

Apparatus and Liquid Chromatography Conditions. A model HP1090 high-performance liquid chromatograph (Hewlett-Packard) equipped with an HP1046 fluorometric detector (Hewlett-Packard) was used. The chromatographic column was a 25 cm × 4 mm i.d. stainless steel cartridge (Teknokroma, Sant Cugat del Vallés, Barcelona, Spain) packed with Spherisorb ODS-2, 5 μm. A 10 μL volume of eluate was chromatographed, using a mixture of 75% heptanesulfonic acid solution (5 mM, pH 2.7) and 25% acetonitrile as mobile phase, isocratically pumped at a flow rate of 0.600 mL/min. The oven temperature was 35 °C. The riboflavin contents were determined at an excitation wavelength of 227 nm and an emission wavelength of 520 nm.

Reagents. Riboflavin was obtained from Merck (99%, article no. 107609, Darmstadt, Germany). Papain was obtained from Sigma Chemical Co. (article no. P4762, St. Louis, MO), pepsin was obtained from Merck (article no. 107190), and Clara-diastase (α-amylase, cellulase, invertase, peptidase, phosphatase, and sulfatase) was obtained from Fluka (article no. 27540, Buchs, Switzerland). Acetonitrile and methanol were of HPLC grade, and all other reagents were of analytical grade.

Samples. Six commercially purchased cooked sausages were analyzed: lunch, chopped pork, chopped beef, chopped turkey, vitamined chopped, and Sicilian mortadella. All of

* Author to whom correspondence should be addressed (fax +34947258813; e-mail mtsancho@ubu.es).

[†] Campofrío Alimentación, S.A.

[‡] Universidad de Burgos.

10.1021/jf9806006 CCC: $18.00 © 1999 American Chemical Society
Published on Web 02/05/1999

Table 1. Effect of Different Enzyme Preparations on Riboflavin Determination[a]

	sample (mg/100 g)		
enzyme[b]	lunch[c]	chopped beef[c]	chopped turkey[c]
papain	0.103 ± 0.005	0.082 ± 0.003	0.089 ± 0.002
pepsin	0.080 ± 0.003	0.062 ± 0.001	0.067 ± 0.003
Clara-diastase	0.186 ± 0.009	0.152 ± 0.005	0.171 ± 0.005
no enzyme	0.048 ± 0.002	0.042 ± 0.002	0.046 ± 0.002

[a] Riboflavin was determined on a fresh weight basis. [b] Enzyme treatment (6%, 50 °C, 3 h). [c] Data are expressed as the mean ± SD, $n = 6$.

them are composed of meats, fat, water, sugars, salt, different spices, and some additives such as preservatives. They mainly differ in meat composition (more or less quantities of pork, beef, and turkey meats, depending on the meat product) and grinding degree.

Sample Preparation. Riboflavin was extracted according to the Hägg (1994) method as modified for our purpose. Ten grams of finely ground samples was weighed in duplicate into 250 mL Erlenmeyer flasks. Sixty milliliters of 0.1 N HCl was added to the homogenized sample, and the mixtures were then stirred. The contents were autoclaved at 120 °C for 20 min. After the mixture had cooled to room temperature, the pH was adjusted to 4.0−4.5 with 2.5 M sodium acetate. 5 mL of freshly prepared aqueous enzyme solution [6% (w/v) Clara-diastase] was added, and the samples were incubated at 50 °C for 3 h. To precipitate the proteins, 2 mL of 50% (w/v) trichloroacetic acid was added and the samples were heated on a steam bath at 90 °C for 15 min. After the flasks were cooled to room temperature, the samples were brought to 100 mL with distilled water and then filtered through an Albet No. 1305 filter paper (Albet Co., Barcelona, Spain). Filtered samples were directly injected into the column.

Procedure with Standard Solutions. Stock solution of 50 μg/mL riboflavin in 0.02 N acetic acid solution was prepared and stored in darkness at 4 °C. Working standard solutions (10, 20, and 25 μg/mL) were prepared on the day of use by suitable dilutions. Aliquots of these solutions were treated as samples. The resulting peak areas were plotted against concentration (from 10 to 100 μg) for the calibration curve. The riboflavin content of the sample extracts was obtained by interpolation on the standard curve.

RESULTS AND DISCUSSION

For riboflavin extraction, three enzymes (papain, pepsin, and Clara-diastase), three different concentrations [3, 6, and 9% (all w/v)] and three incubation times (2, 3, and 4 h at 50 °C) have been tested. On the basis of the above results, samples were incubated with 6% (w/v) Clara-diastase at 50 °C for 3 h. Table 1 shows the effect of different enzyme preparations on riboflavin determination for various representative samples with optimum conditions [6% (w/v) Clara-diastase at 50 °C for 3 h]. Results were constant and independent of the sample studied. Papain gave a result that was 53.8% and pepsin 41.0% of that obtained with Clara-diastase.

The official AOAC (1995) fluorometric method for determining riboflavin requires the organic matter oxidation in the sample extract with 4% $KMnO_4$ solution followed by the oxidant agent elimination with 3% H_2O_2 solution. To optimize the method, the conditions proposed in the official method have been compared with direct extract analysis avoiding the oxidation treatment. Table 2 shows data obtained in the study. The direct analysis gave 7.5% higher results, which shows the destructive influence of the $KMnO_4$ on the riboflavin.

For the HPLC conditions, the preferred mode has been reversed-phase C_{18} (Finglas and Faulks, 1984; Macrae et al., 1984; Ashoor et al., 1985; Brubacher et al., 1985; Suhara et al., 1985; Johnsson and Branzell, 1987; Ollilainen et al., 1990; Barna, 1991), C_8 (Fellman et al., 1982; Macrae et al., 1984), and C_{22} (Lumley and Wiggins, 1981). Some researchers (Toma and Tabekhia, 1979; Reyes et al., 1988; Vidal-Valverde and Reche, 1990; Barna and Dworschak, 1994) used ion-pair reversed phase. We found the latter technique better than the former, in which the rapid elution of riboflavin makes difficult its separation from interferences. Riboflavin, because of its structure, does not occur in ionic form, and therefore the effect of an ion-pair reagent on its retention time is not likely, but the presence of an ion-pair reagent can alter the retention times of some disturbing components, improving the separation. Proper separation was achieved with a low-cost Spherisorb ODS-2 cartridge stationary phase using a mixture of acetonitrile and 5 mM heptanesulfonic acid adjusted to pH 2.7 with phosphoric acid [25:75 (v/v)] as the mobile phase. The amount of acetonitrile and pH were regulated to find an adequate retention time for the vitamin. It was eluted in 4.032 min with a flow rate of 0.600 mL/min. Optimum fluorescence wavelengths were determined by scanning excitation and emission wavelengths of the riboflavin in the mobile phase, because the excitation and emission wavelengths proposed by several researchers (Hollman et al., 1993; Arella et al., 1996; van den Berg et al., 1996) gave us a low signal. The determination of riboflavin was finally performed at an excitation wavelength of 227 nm and an emission wavelength of 520 nm. Figures 1 and 2 show chromatograms of riboflavin in the standard and in one representative cooked sausage (chopped beef). The impurities next to the peak at 4.032 min appear in the blank.

The quantitation of riboflavin was evaluated on the basis of the standard curve prepared daily (12 times over 3 months). Response was linear from 10 to 100 μg, which is adequate for the concentration range in the

Table 2. Effect of Oxidation Treatment on Riboflavin Determination[a]

	sample extract[b] (mg/100 g)	
sample	oxidation[c]	direct analysis
Sicilian mortadella	0.109 ± 0.003	0.118 ± 0.005
vitamined chopped	0.615 ± 0.023	0.664 ± 0.027

[a] Riboflavin was determined on a fresh weight basis. [b] Data are expressed as the mean ± SD, $n = 10$. [c] Oxidation was made according to the official AOAC fluorometric method.

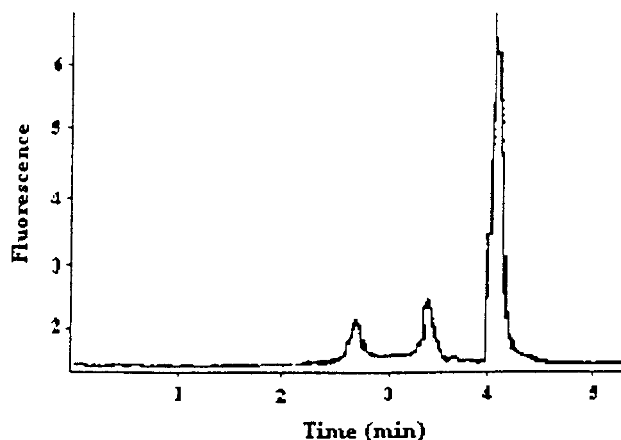

Figure 1. Chromatogram of riboflavin standard (20 μg; 4.032 min). See text for chromatographic conditions.

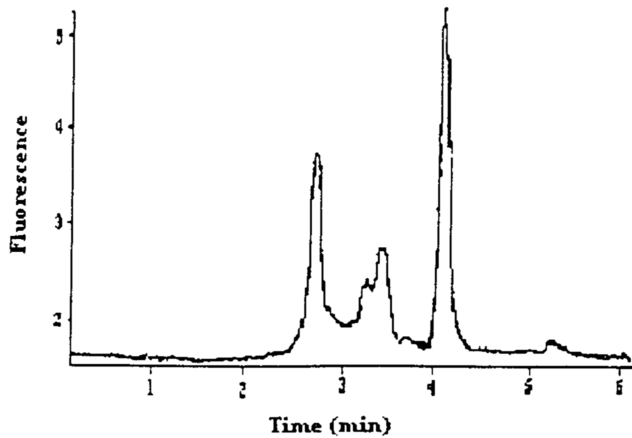

Figure 2. Chromatogram of riboflavin determination of a cooked sausage (0.152 mg/100 g; 4.039 min). See text for chromatographic conditions.

Table 3. Calibration Data for Riboflavin[a]

retention time (min)	amount (μg)	area
4.032	0.0	0.00
	10.0	18.07
	20.0	36.13
	40.0	73.07
	80.0	136.41
	100.0	178.70

[a] r^2 = 0.9993; linear regression = 0.656 + 1.754 (amount).

Table 4. Recoveries of Riboflavin Added to Meat Sample by HPLC Method

	before addition (mg/100 g)	amount added (mg/100 g)	amount found (mg/100 g)	recovery[a] (%)
chopped pork	0.156	0.100	0.253	96.0
	0.160	0.100	0.254	97.0
	0.154	0.100	0.254	97.0
	0.156	0.200	0.350	96.5
	0.158	0.200	0.351	97.0
	0.160	0.200	0.352	97.5
mean ± SD	0.157 ± 0.002			96.8 ± 0.5

[a] Percent recovery = (amount found − mean value)/amount added × 100.

products analyzed. Linear regression coefficients (around 0.9993) were extremely satisfactory. Table 3 shows the calibration data for riboflavin. The detection limit of riboflavin was found to be 0.015 (mg/100 g) with a signal-to-noise ratio of 3. Sensitivity is adequate to measure levels occurring in meat products. The precision was tested by determining riboflavin content in six aliquots of the same sample studied in parallel. The coefficient of variation within a day was 1.35, and the variation between days (10 samples analyzed in duplicate over 3 months) was 2.6% on average. The recovery test was made on a representative sample by adding two standard concentrations of riboflavin (Table 4), and results ranged from 96.0 to 97.5%.

In conclusion, the application of the present method to the analysis of riboflavin results in a rapid, sensitive, precise, and accurate procedure. It allows the quantification of the vitamin and avoids problems of sample stability. Elimination of the oxidation step minimizes the loss of riboflavin, increasing the sensitivity. However, more collaborative studies will be necessary in the future. Publication of this method might be a first step in the development of other interlaboratory studies.

ACKNOWLEDGMENT

We acknowledge Campofrío Alimentación, S.A., for the collaboration given.

LITERATURE CITED

Arella, F.; Lahely, S.; Bourguignon, J. B.; Hasselmann, C. Liquid chromatographic determination of vitamins B_1 and B_2 in foods. A collaborative study. *Food Chem.* **1996**, *56*, 81–86.

Ashoor, S.; Knox, M.; Olsen, J.; Deger, D. Improved liquid chromatographic determination of riboflavin in milk and dairy products. *J. Assoc. Off. Anal. Chem.* **1985**, *68* (4), 693–696.

Association of Official Analytical Chemists. *Official Methods of Analysis*, 15th ed.; AOAC: Washington, DC, 1995.

Association of Vitamin Chemists, Inc. *Methods of Vitamin Assay*, 3rd ed.; Interscience Publishers: New York, 1966.

Barna, E. Comparison of data obtained by HPLC and microbiological determination of riboflavin in ready-to-eat foods. *Acta Aliment.* **1991**, *21* (1), 3–9.

Barna, E.; Dworschak, E. Determination of thiamine (vitamin B_1) and riboflavin (vitamin B_2) in meat and liver by high performance liquid chromatography. *J. Chromatogr.* **1994**, *668* (2), 359–363.

Basu, T. K.; Dickerson, J. W. T. *Vitamins in Human Health and Disease*; CAB International: Wallingford, U.K., 1996.

Brubacher, G.; Müller-Mulot, W.; Southgate, D. *Method for the Determination of Vitamins in Food*; Elsevier Science Publishers: London, U.K., 1985.

Fellman, J.; Artz, W.; Tassinari, P.; Cole, C.; Augustin, J. Simultaneous determination of thiamin and riboflavin in selected foods by high-performance liquid chromatography. *J. Food Sci.* **1982**, *47*, 2048–2050.

Finglas, P.; Faulks, R. The HPLC analysis of thiamin and riboflavin in potatoes. *Food Chem.* **1984**, *15*, 37–44.

Hägg, M. Effect of various commercially available enzymes in the liquid chromatographic determination with external standardization of thiamine and riboflavin in foods. *J. AOAC Int.* **1994**, *77* (3), 681–686.

Hollmann, P. C. H.; Slangen, J. H.; Wagstaffe, P. J.; Faure, U.; Southgate, D. A. T.; Finglas, P. M. Intercomparison of methods for the determination of vitamins in foods. Part 2. Water-soluble vitamins. *Analyst* **1993**, *118*, 481–488.

Johnsson, H.; Branzell, C. High performance liquid chromatographic determination of riboflavin in food. A comparison with a microbiological method. *Int. J. Vit. Nutr. Res.* **1987**, *57*, 53–58.

Lumley, I. D.; Wiggins, R. A. Determination of riboflavin and flavin mononucleotide in foodstuffs using high-performance liquid chromatography and a column-enrichment technique. *Analyst* **1981**, *106* (10), 1103–1108.

Macrae, R.; Nicolson, I. A.; Richardson, D. P.; Scott, K. J. Chromatographic determination of vitamins in dairy products. *Proceedings, Challenges in Dairy Analytical Techniques*; Royal Society of Chemistry: Reading, U.K., 1984; pp 155–166.

Ollilainen, V.; Mattila, P.; Varo, P.; Koivistoinen, P.; Huttunen, J. The HPLC determination of total riboflavin in foods. *J. Micronutr. Anal.* **1990**, *8*, 199–207.

Reyes, E.; Norris, K.; Taylor, C.; Potts, D. Comparison of paired-ion liquid chromatographic method with AOAC fluorometric and microbiological method for riboflavin determination in selected foods. *J. Assoc. Off. Anal. Chem.* **1988**, *71* (1), 16–19.

Suhara, T.; Torida, S.; Ito, K.; Fukumoto, Y. Determination of vitamin B_2 in feed premixes by high-performance liquid chromatography. *Shiryo Kenkyu Hokoku (Tokyo Hishiryo Kensasho)* **1985**, *10*, 152–163.

Toma, R.; Tabekhia, M. High performance liquid chromatographic analysis of B-vitamins in rice and rice products. *J. Food Sci.* **1979**, *44* (1), 263–268.

van den Berg, H.; van Schaik, F.; Finglas, P. M.; de Froidmont-Görtz, I. Third EU MAT intercomparison on methods for

the determination of vitamins B_1, B_2 and B_6 in food. *Food Chem.* **1996**, *57*, 101−108.

Vidal-Valverde, C.; Reche, A. Reliable system for the analysis of riboflavin in foods by high performance liquid chromatography and UV detection. *J. Liq. Chromatogr.* **1990**, *13* (10), 2089−2101.

Received for review June 5, 1998. Revised manuscript received November 6, 1998. Accepted December 2, 1998. We acknowledge the Spanish Ministry of Education and Science for the predoctoral study grant.

JF9806006