Simultaneous Determination of Thiamin and Riboflavin in Mushrooms by Liquid Chromatography

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A simple, fast, inexpensive, and reliable method useful for the simultaneous, routine determination of thiamin and riboflavin in mushrooms is examined. It uses the extraction procedure, with slight modifications, proposed by the AOAC for the extraction of thiamin and riboflavin, followed by a liquid chromatographic separation on a reversed-phase Spherisorb ODS column with methanol/water as mobile phase gradient. Fluorometric detection is used at the following excitation and emission wavelengths, respectively, 360 and 425 nm in the case of thiamin and 422 and 515 nm for riboflavin. The analytical parameters of linearity, the precision of the method (RSD = 2.45 and 2.51% for thiamin and riboflavin, respectively), and the results of the comparison with the corresponding AOAC fluorometric methods show that the studied method is useful for the measurement of thiamin and riboflavin in fresh mushrooms.

Keywords: *Mushrooms; thiamin; riboflavin; liquid chromatographic separation*

INTRODUCTION

Fluorometric, microbiological (*1*−*4*), spectrophotometric (*5*, *6*), and polarographic (*7*) methods have been used to measure the thiamin and riboflavin content in foods. The microbiological methods show a high specificity and reliability but are time-consuming (*8*), whereas the specificity of the fluorometric methods is low (*9*, *10*). Therefore, both have been replaced by high-performance liquid chromatography (HPLC) methods, which are highly specific and sensitive and require short analysis times. Furthermore, HPLC methods allow simultaneous determination of different vitamins (*11*−*15*), and it is therefore possible to measure thiamin and riboflavin in one chromatographic run (*8*).

The determination of vitamins in foods is a two-step process, involving extraction and measurement. Thiamin and riboflavin must be freed from the proteic bindings by acid hydrolysis. Although hydrochloric acid is the agent most often used for this purpose (*8*, *14*, *16*−*22*), sulfuric acid has also been used (*23*, *24*). Acid hydrolysis is followed by enzymatic dephosphorylation with takadiastase, clarase, or amylase at pH 4−4.5 and at 45−50 °C for 30 min or at 37 °C for 12 h (*18*, *19*, *21*). Some authors (*5*, *25*−*29*) avoid this dephosphorylation step. Finally, 50% trichloroacetic acid at 100 °C is added to precipitate the proteins (*23*, *30*−*32*,). After that, further purification may be carried out.

Thiamin can be measured either by chromatography and UV detection (*33*, *34*) or by fluorometry detection. In the latter case the vitamin has to be oxidized to thiochrome with potassium ferrocyanate, before or after the chromatographic separation (*19*, *23*, *24*, *35*−*37*).

Riboflavin is usually measured directly by fluorometry (*5*, *27*, *31*, *38*) and UV spectrophotometry (*25*, *39a*,*b*, *40*). Riboflavin can be determined after conversion to lumiflavin by UV irradiation in an alkaline medium, before chromatographic separation and fluorometric detection (*41*). Hou and Wang (*42*) used electrochemical detection.

The simultaneous determination of thiamin and riboflavin is usually carried out by ion-pair reversed-phase chromatography, and different mobile phases and contraions have been used. The vitamins are detected either by UV spectrophotometry (*43*), by fluorometry (*17*, *18*, *28*−*30 43*−*47*), or by simultaneously using both techniques (*48*). The method most commonly used is fluorometric detection, with either single or dual detectors. The excitation and emission wavelengths must be changed depending on the vitamin when a single detector is used.

Although a variety of methods have been proposed to determine thiamin and riboflavin in foods, one that is simple, fast, inexpensive, and reliable and allows simultaneous routine determination of both vitamins in food is needed. Therefore, our purpose was to set up a method fulfilling these conditions that could be used to monitor the changes in the contents of these vitamins during the processing of vegetables.

MATERIALS AND METHODS

Apparatus. The liquid chromatographic (LC) system consisted of a series 1050 chromatograph with a quaternary pump system, a UV detector, an on-line degassing system, a 1046A fluorescence detector, and a ChemStation (series MS-DOS, 3365) integrator (Hewlett-Packard). A Spherisorb ODS-2 C_{18} column, 5 μm pore size, length 250 × 4.6 mm (Teknokroma, Barcelona, Spain), was used. The filters of 0.22 μm and 47 mm diameter were from Magna Nylon, MSI (Micron Separations). An RF-510 spectrofluorometer (Shimadzu) was used to detect the components.

Chemicals. Only analytical grade substances were used: acetic acid (d = 1.05), isobutanol, sodium acetate (anhydrous), hydrochloric acid (d = 1.18), hydrogen peroxide, potassium chloride, potassium ferrocyanate, sodium hydrosulfite, sodium hydroxide, trichloroacetic acid (Panreac Química, Barcelona, Spain); methanol (HPLC grade, Mallinckrodt Baker B.V., Deventer, Holland); and distilled−deionized water (Millipore-Milli Q water system, Millipore, Jafrey, MA). Thiamin hydrochloride and riboflavin were obtained from Sigma, Taufkirch-

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en, Germany. Takadiastase of *Aspergillus oryzae*, EC 3.2.1.1, was obtained from Fluka, Buchs, Switzerland.

All solvents used in LC determinations were filtered through 0.22 μm pore size filters.

Standard Solutions. Standard stock solutions of thiamin and riboflavin (100 μg/mL) were prepared in 0.01 M HCl and 0.02 M acetic acid. Working standard solutions (1 μg/mL) were made on the day of use from the stock solutions by suitable dilutions.

Sample. The method was tested on portabella mushrooms (*Agaricus bisporus*), the most extensively cultivated mushroom in the world, accounting for 38% of the world production of cultivated mushrooms.

Extraction. Fresh mushrooms were triturated and homogenized using a Polytron PT/2000 homogenizer (Kinematica AG, Littau, Switzerland). An aliquot of 2 g of the homogenate was weighed in an Erlenmeyer flask, and 60 mL of 0.1 M HCl was added. The mixture was then heated in a water bath (95−100 °C) for 30 min. It was allowed to cool, the pH value was adjusted to 4−4.5 with 2 M sodium acetate, and 5 mL of a freshly prepared 10% (w/v) takadiastase solution in water was added. After 3 h at 45−50 °C, 2 mL of 50% (w/v) trichloroacetic acid was added, and the mixture was then heated at 100 °C for 5 min. It was then allowed to cool to room temperature; after it had been brought to 100 mL of volume with water, it was filtered. This extract was used in the HPLC method using fluorometric detection for the simultaneous determination of thiamin and riboflavin.

RESULTS AND DISCUSSION

Different stationary phases have been used to measure thiamin and riboflavin. A Sperisorb ODS-2, 5 μm (250 × 4.6 mm), reversed-phase column was chosen because of its versatility and wide spectra of application.

A wavelength sweep with the chromatograph detector was done initially to select the excitation and emission wavelengths giving the maximum response for each vitamin. The wavelengths selected for excitation and emission were 360 and 425 nm, respectively, in the case of thiamin and 422 and 515 nm, respectively, for riboflavin. The excitation and emission wavelengths were changed after the elution of riboflavin to those for thiamin.

The second step was to determine the proper the mobile phase. The following were used: 0.04 M H_2SO_4 in water; an 0.2 M acetate buffer containing 0.005 M octanosulfonic acid in water, with acetonitrile at different ratios; methanol/water (50:50, v/v) and acetonitrile/water (50:50, v/v). The analysis was carried out under isocratic conditions, at a flow rate of 1 mL/min and at room temperature. The optimum results were obtained with methanol/water and with acetonitrile/water.

The retention times with the methanol/water (50:50, v/v) phase allowed separation of the peaks corresponding to thiamin and riboflavin from a standard solution ($\alpha = 1.4$, $R_s = 2.4$). When an extract of mushrooms was analyzed, a set of chromatographic peaks interfering in the riboflavin resolution ($\alpha = 1.1$, $R_s = 0.9$) appeared (see Figure 1). This made it necessary to apply a mobile phase gradient, and different gradients were studied. The best resolution and separation of chromatographic peaks corresponds to impurities; riboflavin ($\alpha = 1.3$, $R_s = 4$) and thiamin ($\alpha = 1.3$, $R_s = 2.9$) at the beginning of the analysis were obtained with gradient IV (Figure 2).

Simultaneous determination of thiamin and riboflavin by LC with fluorometric detection has the disadvantage of requiring the oxidation of thiamin to thiochrome with potassium ferrocyanate in an alkaline medium, and in this medium, riboflavin degraded to a nonfluorescent compound (*46*). The adequate conditions for oxidizing thiamin to thiochrome, without affecting riboflavin, were studied. Different volumes and concentrations of potassium ferrocyanate and sodium hydroxide were assayed for this purpose. The optimized conditions were as follows: 300 μL of 1% (w/v) potassium ferrocyanate in 15% (w/v) NaOH was added to 5 mL of extract, and the mixture was shaken for 10 s and allowed to stand for 10 min. This reaction must be carried out in dark tubes because of the degradation of thiochrome by light. Acid must be added to neutralize and prevent the silica of the column from deteriorating due to hydrolysis. Orthophosphoric acid (17%, w/v) added at a volume of 100 μL to the sample gave a pH value of 7. An aliquot was filtered through a Teflon filter with a 0.5 μm pore size and then injected into the chromatograph. The riboflavin and thiamin retention times, respectively, were 3.7 and 4.8 min. Figure 3 shows the chromatograms of a reagent blank and a mushroom sample.

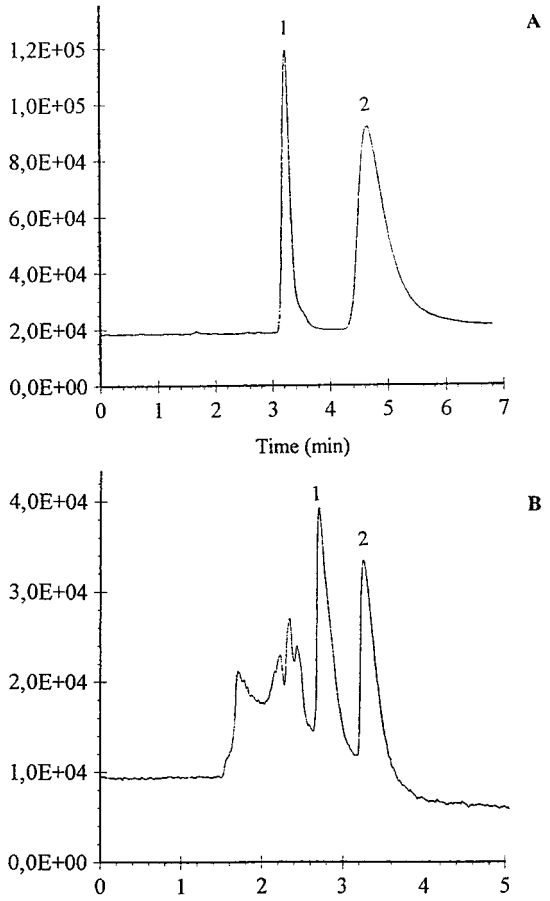

Figure 1. Chromatogram of (A) standard solution of (1) riboflavin ($t_r = 3.3$ min) and (2) thiamin ($t_r = 4.7$ min) and (B) extract of mushrooms (1, riboflavin, $t_r = 2.7$ min; 2, thiamin, $t_r = 3.3$ min) with methanol/water (50:50, v/v) as mobile phase.

To verify the quality and usefulness of the method, the analytical parameters linearity, sensitivity, precision, and percentage of recovery were determined.

Linearity and Limit of Detection (LOD). The LOD was calculated by applying the method proposed by Knoll (*49*); the largest noise fluctuation is measured in a chart interval that is a multiple of the analyte's chromatographic peak width at half peak height. The LOD is estimated using the formula $C_{LOD} = K_{LOD} h_n C_s / h_s$, where C_{LOD} is the LOD quantity, h_s/C_s is the analyte peak height/unit amount of analyte, h_n is the largest

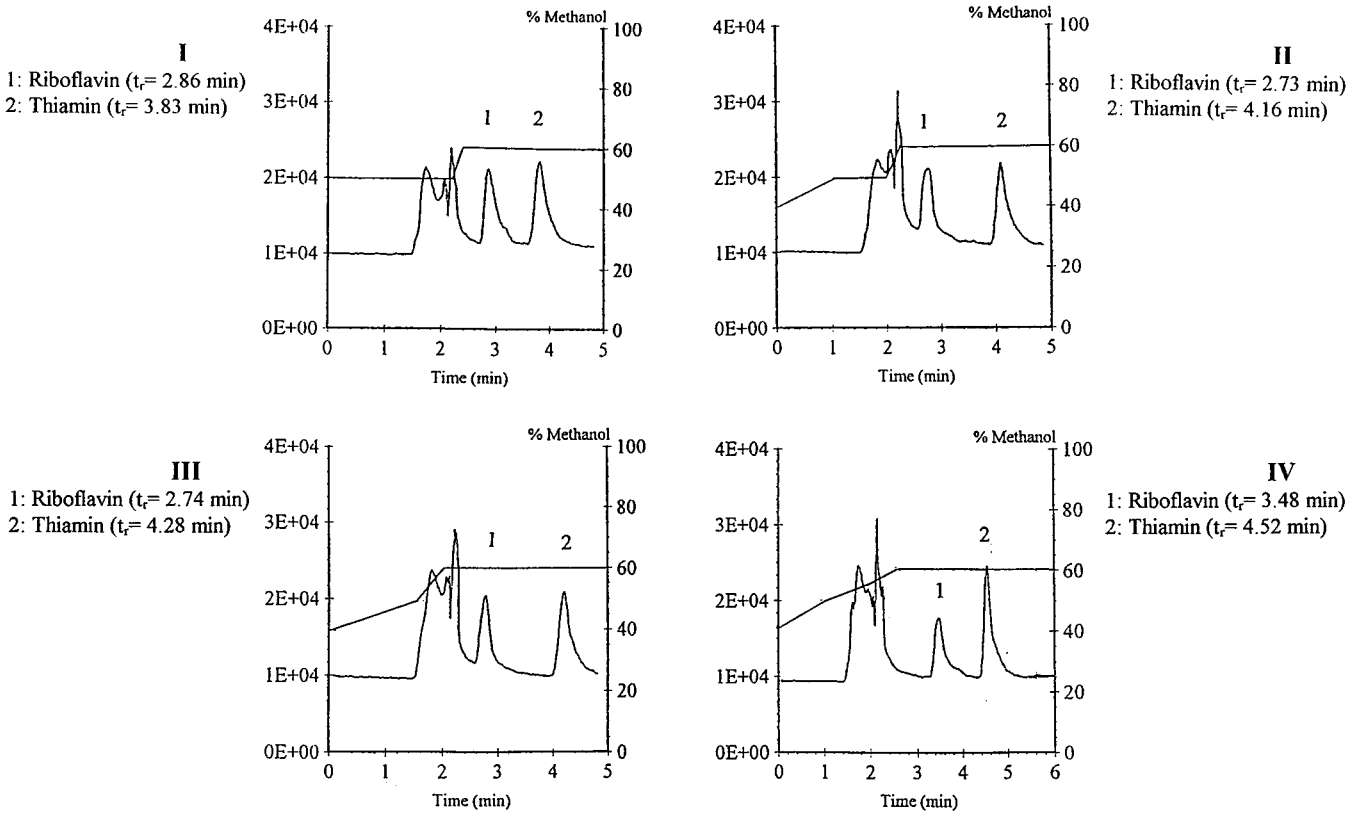

Figure 2. Chromatograms of thiamin and riboflavin in mushrooms at different mobile phase gradients.

noise fluctuation observed in the noise measurement interval, and K_{LOD} is a constant, determined for the measurement interval employed. The LODs calculated in this study were 0.0013 and 0.0086 μg/mL for thiamin and riboflavin, respectively. The responses were linear in the following ranges: 0.0013−0.573 μg/mL (y = 707590x + 16434, r = 0.999) and 0.0086−1.030 μg/mL (y = 6005111x + 55067, r = 0.995) for thiamin and riboflavin, respectively, where y is the peak area of the standard and x the standard content (μg/mL).

Precision. Instrumental precision was checked from six consecutive injections of a mushroom extract solution; the relative standard deviations (RSDs) obtained were 2.45% (1.133 ± 0.028 μg/mL) and 2.51% (5.861 ± 0.147 μg/mL) for thiamin and riboflavin, respectively. When the mushroom extract solution was prepared and measured on alternate days, the RSD values were 3.30% (1.020 ± 0.034 μg/mL) and 9.60% (6.062 ± 0.582 μg/mL) for thiamin and riboflavin, respectively. The repeatabilities of the method (RSDs) were 2.1% (1.008 ± 0.021 μg/mL, n = 6) and 6.8% (6.241 ± 0.424 μg/mL, n = 6), respectively.

Accuracy. Accuracy was estimated through recovery assays. A portion of a mushroom sample to which a known amount of standard thiamin and riboflavin (2.269 and 10.228 μg/g, respectively, approximately twice thiamin and riboflavin concentrations) was added was subjected to entire extraction and determination process. The recovery percentages obtained were 91.6 and 96.7% for thiamin and riboflavin, respectively.

To evaluate the ruggedness of thiamin and riboflavin determination, the precision and accuracy of the proposed gradient HPLC method was compared with that corresponding to the AOAC fluorometic method (*1*).

In the comparison of precision, equal batches of mushrooms were triturated for both the LC determi-

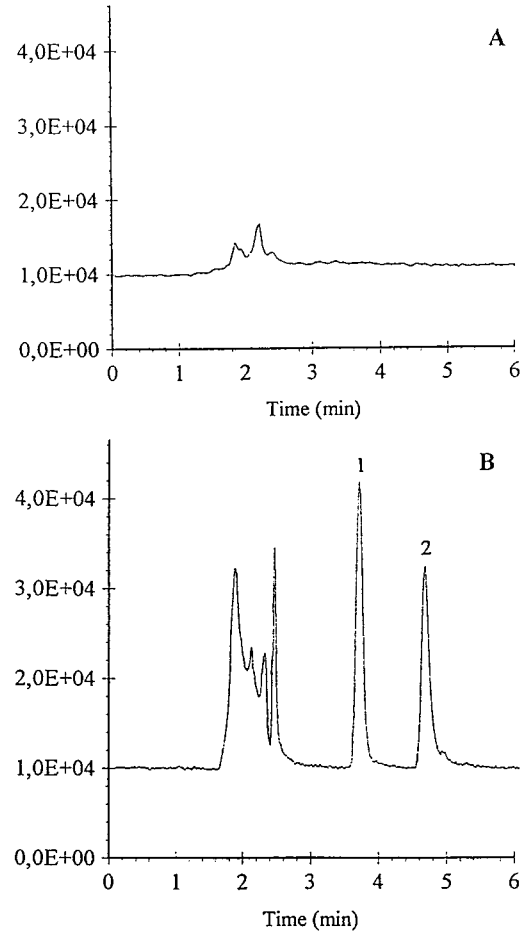

Figure 3. Chromatogram of thiamin and riboflavin in (A) blank of reactives and (B) mushroom sample (1, riboflavin, t_r = 3.7 min; 2, thiamin, t_r = 4.8 min).

Table 1. *F* Test for Comparison of the Precision of the Fluorometric Method and the Liquid Chromatographic Method for the Determination of Thiamin and Riboflavin[a]

	LC method		AOAC method		
	N	S'^2	*N*	S'^2	*F*
thiamin	6	7.38	6	1.90	3.88
riboflavin	6	0.41	6	1.15	2.80

[a] *N*, number of replicates. S'^2, corrected sample variance. $F = S_1'^2/S_2'^2$. Tabulated *F* values: $F_{0.05(5,5)} = 5.05$ ($p = 0.05$), $F_{0.01(5,5)} = 10.97$ ($p = 0.01$).

Table 2. Comparison of Thiamin and Riboflavin Contents (Micrograms per Gram) Obtained by the AOAC and Liquid Chromatographic Methods[a]

	thiamin		riboflavin	
sample	AOAC	LC	AOAC	LC
1	0.761	1.028	6.43	6.69
2	0.733	0.995	5.43	5.61
3	0.774	1.021	5.89	5.93
4	0.741	0.972	6.10	6.18
5	0.777	1.008	6.29	6.66
6	0.791	1.021	5.81	6.37
$X \pm$ SD	0.763 ± 0.022	1.008 ± 0.021	5.99 ± 0.36	6.24 ± 0.42
d	0.240	0.248		
s	0.013	0.194		
t	46.315	3.131		

[a] *SD*, deviation standard; *d*, mean of the differences; *s*, standard deviation of the differences; $t^{0.05/2}{}_5 = 3.163$ ($p = 0.05$); $t^{0.01/2}{}_5 = 4.770$ ($p = 0.05$).

nation and the AOAC fluorometric method. A comparison of variance showed that the two methods were similar in precision ($p > 0.05$, Table 1).

In the comparison of accuracy, six batches of mushrooms were analyzed by using the two methods, twice and on different days. The mean contents were compared by a *t* test, and the results showed that there are no significant differences between the methods for riboflavin ($p > 0.05$). The means of thiamin for the LC method were higher than those obtained with the AOAC methods, and the difference was significant ($p < 0.05$, $p < 0.01$, Table 2). Similar results from Chase et al. (*50*) show that the LC results of thiamin exceeded the AOAC results and averaged 19% higher. This could be due to greater manipulation of the sample and interferences in the determination that exist.

There was only one reference material, lyophilized green beans BCR383, (Community Bureau of Reference, Commission of the European Communities), to analyze that provided an estimation of the thiamin content, but it gave no information on riboflavin. The thiamin content found was 0.210 ± 0.004 mg/100 g, and the reported one was 0.22 ± 0.04 mg/100 g. The comparison of the proposed method with the official method and the determination of a reference material shows us that the method described herein is an appropiate and exact method for thiamin and riboflavin determination.

The proposed method combines the use of methanol/water as the mobile phase and a C_{18} ODS column, with a high versatility. This makes it economical to use and gives an advantage over other methods (*5*, *18*, *28*−*30*, *43*, *44*, *46*, *48*). A short analysis time of 6 min together with the accuracy, sensitivity, and precision of the method makes it useful for the routine determination of thiamin and riboflavin.

LITERATURE CITED

(1) AOAC. *Official Methods of Analysis of the Association of Official Analytical Chemists*, 15th ed.; Helrich, K. Ed.; Association of Official Analytical Chemists: Arlington, VA, 1990; Vol. 2.

(2) Fox, J. B.; Ackerman, S. A.; Thayer, D. W. Fluorometric determination of thiamine vitaminers in chicken. *J. AOAC Int*. **1992**, *75* (2), 346−354.

(3) Fox, J. B.; Lakritz, L.; Kohout, K. M.; Thayer, D. W. Water concentration/activity and loss of vitamins B_1 and E in pork due to gamma-radiation. *J. Food Sci*. **1994**, *59* (6), 1291−1295.

(4) Fox, J. B.; Lakritz, L.; Hampson, J.; Richardson, R.; Ward, K.; Thayer, D. W. Gamma irradiation effects on thiamin and riboflavin in beef, lamb, pork, and turkey. *J. Food Sci*. **1995**, *60* (3), 596−598.

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

(6) Perez-Ruiz, T.; Martínez-Lozano, C.; Tomás, V.; Val, O. Photochemical spectrophotometric determination of riboflavin and riboflavin 5′-phosphate by manual and flow injection methods. *Analyst* **1994**, *119*, 1199−1203.

(7) González, H. M.; Marchante, P.; Alpízar, J.; Mañes, J. Determinación de riboflavina en formulaciones multivitamínicas por polarografía diferencial de impulsos. *An. Rel. Acad. Farm*. **1993**, *59*, 441−449.

(8) Hagg, 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.

(9) Finglas, P. M.; Faulks, R. M. Critical review of HPLC methods for the determination of thiamin, riboflavin and niacin in food. *J. Micronutr. Anal*. **1987**, *3*, 251−283.

(10) Macrae, R. HPLC determination of vitamins. *J. Micronutr. Anal*. **1990**, *7*, 247−260.

(11) Kothari, R. M.; Taylor, M. W. Simultaneous separation of water-soluble vitamins and coenzymes by reversed-phase high-performance liquid chromatography. *J. Chromatogr*. **1982**, *247* (1), 187−192.

(12) Lam, F.; Holcomb, I. J.; Fusari, S. A. Liquid chromatographic assay of ascorbic acid, niacinamide, piridoxine, thiamine and riboflavine in multivitamin-mineral preparations. *J. Assoc. Off. Anal. Chem*. **1984**, *67* (5), 1007−1011.

(13) Amint, M.; Reusch, J. High-performance liquid chromatographic of water-soluble vitamins. III. Simultaneous determination of vitamin B_1, B_2, B_6, B_{12} and C, nicotinamide and folic acid in capsule preparations by ion-par reversed-phase high-performance liquid chromatography. *Analyst* **1987**, *112* (7), 989−991.

(14) Dawson, K. R.; Unklesbay, N. F.; Hedrick, H. B. HPLC determination of riboflavin, niacin and thiamin in beef, pork and lamb after alternate heat-processing methods. *J. Agric. Food Chem*. **1988**, *36*, 1176−1179.

(15) Chase, G. W.; Landen, W. O.; Eilenmiller, R. R.; Soliman, A. M. Liquid chromatographic determination of thiamine, riboflavin and pyridoxine in infant formula. *J. Assoc. Off. Anal. Chem*. **1992**, *75* (3), 561−565.

(16) Hvass, A. determination of thiamine and riboflavine in meat products by high performance liquid chromatography. *Proc. Eur. Mtg. Meat Res. Workers* **1983**, *29* (2), 649−656.

(17) Wills, R. B. H.; Wimalasiri, P.; Greenfield, H. Comparative determination of thiamin and riboflavin in foods by high-performance liquid chromatography and fluorimetric methods. *J. Micronutr. Anal*. **1985**, *1* (1), 23−29.

(18) Hasselmann, C.; Franck, D.; Grimm, P.; Diop, P. A.; Soules, C. High-performance liquid chromatographic analysis of thiamin riboflavin in dietetic foods. *J. Micronutr. Anal*. **1989**, *5* (4), 269−279.

(19) Ollilainen, V.; Vahteristo, L.; Uusi-Rauva, A.; Varo, P. The HPLC determination of total thiamin in foods. *J. Food Compos. Anal*. **1993**, *6* (2), 152−165.
(20) Sims, A. Simultaneous liquid chromatographic determination of thiamine and riboflavin in selected foods. *J. AOAC Int*. **1993**, *76* (5), 1156−1160.
(21) 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. A* **1994**, *665*, 359−363.
(22) Hagg, M.; Kumpulainen, J. Thiamine and riboflavin contents in finnish pig, heifer, and cow livers and in pork loin. *J. Food Compos. Anal*. **1994**, *7* (4), 301−306.
(23) Abdel-Kader, Z. M. Comparison of AOAC and high-performance liquid chromatographic methods for thiamin determination in foods. *Food Chem*. **1992**, *43*, 393−397.
(24) Bognar, A. Determination of vitamin B_1 in food by high performance liquid chromatography and post-column derivatization. *Fresenius' J. Anal. Chem*. **1992**, *343*, 155−156.
(25) Ashoor, S. H.; Seperich, G. J.; Monte, W. C.; Welty, J. HPLC determination of riboflavin in eggs and dairy products. *J. Food Sci*. **1983**, *48* (1), 92−94, 110.
(26) Ashoor, S. H.; Knox, M. J.; Olsen, J. R.; Deger, D. A. Improved liquid chromatographic determination of riboflavin in milk and dairy products. *J. Assoc. Off. Anal. Chem*. **1985**, *68* (4), 693−696.
(27) Ribarova, F.; Shishkov, S.; Obretenova, N.; Metchkneva, L. Comparative determination of riboflavin in milk HPLC and lumiflavin methods. Die *Nahrung* **1987**, *31* (1), 77−79.
(28) Fernando, S. M.; Murphy, P. A. HPLC determination of thiamin and riboflavin in soybeans and tofu. *J. Agric. Food Chem*. **1990**, *38*, 163−167.
(29) Sims, A.; Shoemaker, D. Simultaneous liquid chromatographic determination of thiamine and riboflavin in selected foods. *J. Assoc. Off. Anal. Chem*. **1993**, *76* (5), 1156−1160.
(30) Fellman, J. K.; Artz, W. E.; Tassanari, P. D.; Cole, C. L.; Augustin, J. Simultaneous determination of thiamin and riboflavin in selected foods by high-performance liquid chromatography. *J. Food Sci*. **1982**, *47* (6), 2048−2050.
(31) Ollilainem, V.; Mattila, P.; Varo, P. The HPLC determination of total riboflavin in foods. *J. Micronutr. Anal*. **1990**, *8*, 199−207.
(32) Barna, E. Comparison of data obtained by HPLC and microbiological determination of riboflavin in ready-to-eat foods. *Acta Aliment*. **1992**, *21* (1), 3−9.
(33) Hilker, D. M.; Clifford, A. J. Thiamin analysis and separation of thiamin phosphate esters by high-performance liquid chromatography. *J. Chromatogr. Biomed. Appl*. **1982**, *231* (2), 433−438.
(34) Nicolas, C. C.; Pfender, K. A. Fast and simple liquid chromatographic determination of nonphosphorylated thiamine in infant formula, milk, and other foods. *J. Assoc. Off. Anal. Chem*. **1990**, *73* (5), 792−798.
(35) Bötticher, A. L.; Bötticher, D. Simple rapid determination of thiamin by a HPLC method in foods, body fluids, urine and faeces. *Int. J. Vit. Nutr. Res*. **1986**, *56* (2), 155−159.
(36) Bailey, A. L.; Finglas, P. M. A normal phase high performnace liquid chromatographic method for the determination of thiamine in blood and tissue samples. *J. Micronutr. Anal*. **1990**, *7* (2), 147−157.
(37) Laschi-Loquerie, A.; Vallas, S.; Viollet, J.; Leclercq, M.; Fayol, V. High performance liquid chromatographic determination of total thiamine in biological and food products. *Int. J. Vit. Nutr. Res*. **1992**, *62* (3), 248−251.
(38) Russell, L. F.; Vanderslice, J. T. Non-degradative extraction and simultaneous quantitation of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in foods by HPLC. *Food Chem*. **1992**, *43* (2), 151−162.
(39) (a) 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. B* **1990**, *13* (10), 2089−2101. (b) Vidal-Valverde, C.; Reche, A. An improved high performance liquid chromatographic method for thiamin analysis in foods. *Z. Lebensm. Unters. Fosrch*. **1990**, *19* (415), 313−318.
(40) Muñoz, A.; Ortiz, R.; Murcia, M. A. Determination by HPLC of changes in riboflavin levels in milk and non-dairy imitation milk during refrigerated storage. *Food Chem*. **1994**, *49*, 203−206.
(41) Ang, C. Y. W.; Mosely, F. A. Determination of thiamin and riboflavin in meat and meat products by high-pressure liquid chromatography. *J. Agric. Food Chem*. **1980**, *28*, 483−489.
(42) Hou, W.; Wang, E. Liquid chromatography with series dual-electrode electrochemical detection for riboflavin. *Analyst* **1990**, *115* (2), 139−141.
(43) Kamman, J. F.; Labuza, T. P.; Warthesen, J. J. Thiamin and riboflavin analysis by high performance liquid chromatography. *J. Food Sci*. **1980**, *45*, 1497−1499, 1504.
(44) Skurray, G. R. A rapid method for delectively determining small amounts of niacin, riboflavin and thiamine in foods. *Food Chem*. **1981** *7* (2), 77−80.
(45) Augustin, J. Simultaneous determination of thiamine and riboflavin in foods by liquid chromatography. *J. Assoc. Off. Anal. Chem*. **1984**, *67* (5), 1012−1015.
(46) Mauro, D. J.; Wetzel, D. L. Simultaneous determination of thiamine and riboflavin in enriched cereal based products by high performance liquid chromatography using selective detection. *J. Chromatogr*. **1984**, *299* (1), 281−287.
(47) Reyes, E. S. P.; Subryan, L. An improved method of simultaneous HPLC assay of riboflavine and thiamin in selected cereal products. *J. Food Compos. Anal*. **1989**, *2* (1), 41−47.
(48) Olleta, F. J.; Llanos, E.; Barcos, R.; Aucín, M. C.; Martin, O. Determination of vitamins B_1 (thiamine), B_2 (riboflavine), B_6 (pyridoxine) and C (ascorbic acid) in mushrooms and lentils by HPLC. Effect of canning and storage. *Quim. Anal*. **1993**, *12* (3), 155−158.
(49) Knoll, J. E. Estimation of the limit of detection in chromatography. *J. Chromatogr. Sci*. **1985**, *23*, 422−425.
(50) Chase, G. W.; Landen, W. O., Jr.; Soliman, A. M.; Eitenniller, R. R. Method modification for liquid chromatographic determination of thiamine, riboflavin, and pyridoxine in medical foods. *J. AOAC Int*. **1993**, *76* (6), 1276−1780.

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