JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of Thiamine and Its Esters in Beers and Raw Materials Used for Their Manufacture by Liquid Chromatography with Postcolumn Derivatization

PILAR VIÑAS, CARMEN LÓPEZ-ERROZ, NURIA BALSALOBRE, AND MANUEL HERNÁNDEZ-CÓRDOBA*

Department of Analytical Chemistry, Faculty of Chemistry, University of Murcia, E-30071 Murcia, Spain

Thiamine and its mono- and pyrophosphate esters were determined in beer and the raw materials used for its manufacture (brewer's yeast, malt, raw grain, and hops) after separation using reversed-phase liquid chromatography. The method used fluorescence detection and a new amide-based stationary phase, which avoids the need to form ion pairs, leading to narrower peaks and a simpler mobile phase. Analyses were performed by isocratic elution with a phosphate buffer mobile phase and using a postcolumn derivatization system based on the oxidation of thiamine to fluorescent thiochrome with potassium ferricyanide in alkaline solution. Only thiamine was found in the beers and raw products, especially in brewer's yeast and malt. A stability study pointed to a faster decrease in the thiamine content of samples stored at room temperature and in sunlight.

KEYWORDS: Liquid chromatography; fluorescence; thiamine; thiamine esters; thiochrome; beer

INTRODUCTION

Vitamins are complex organic substances that occur in the biological materials we consume as food. Thiamine (vitamin B_1) occurs in foodstuffs in its free form or as its mono- or pyrophosphate esters complexed with protein. Although it is extremely widespread in small amounts, only a few foodstuffs, generally those that are rich in carbohydrate, can be regarded as good sources. Examples are legume seeds and the germ of cereal grains, cereal products, vegetables, meat, and milk products (*1*). Thiamine is very labile in neutral to basic solutions but not in acidic solutions. Loss of thiamine may occur for mildly acidic to neutral of basic foods on heat treatment, but more acidic foods would not experience this loss. The greatest losses during domestic cooking as well as in commercial food processing occur when the vitamin is leached into the cooking water.

Beer is a natural beverage manufactured from malted barley, water, yeast, and hops. Vitamins are found among the nonvolatile components of beer, which contain all of the important vitamins of the B group. They come from malt, increase during barley germination, and remain during toasting. The mean concentration of thiamine in beer (*2*) has been established as 29 μg/L. The thiamine content of beer is important for nutritional information.

Thiamine cannot be distinguised from its esters by the usual analytical techniques such as the fluorimetric method proposed by the AOAC (*3*), and no distinction is made in tables of food composition. It is important to know the speciation of thiamine in beer because there are different activities for the thiamine esters and also different stabilities. Then, when it is necessary to distinguish between all of the naturally occurring species of thiamine, liquid chromatography (LC) is usually the method of choice (*4*). Procedures for the determination of thiamine in foods using reversed-phase (*5*−*10*) or ion pair chromatography (*11*−*25*) have been proposed, and several reviews have also been published (*26*−*29*). UV spectrophotometry (*11*, *13*−*15*, *18*, *21*, *24*, *25*) has been used for samples that contain sufficient amounts of thiamine, while a fluorescence derivatization reaction based on the oxidation of thiamine to thiochrome, which shows strong fluorescence (*5*−*7*, *10*, *12*, *16*−*20*, *23*, *26*, *28*), is used for small concentrations. An alkaline solution of potassium hexacyanoferrate(III) is generally used as the oxidizing agent. However, no papers have been found concerning the determination of thiamine and its esters in beers and their raw materials.

In the present study, the separation of thiamine (T), thiamine monophosphate (TP), and thiamine pyrophosphate (TPP) is optimized by the reversed-phase technique without ion pair formation using a new amide-based stationary phase. Detection was performed by postcolumn fluorescence derivatization using a system involving the oxidation of the vitamins to the corresponding highly fluorescent thiochromes. The procedure could be applied to the determination of thiamine and its esters in beer and the raw materials used in its manufacture. Separation using the amide-based column is advantageous with respect to other existing methods (*11*−*25*) because the peaks are much narrower and column life is longer due to the simplicity of the mobile phase. Moreover, the use of the fluorescence detector

* To whom correspondence should be addressed. Fax: +34968364148. E-mail: hcordoba@um.es.

10.1021/jf021071f CCC: $25.00
Published on Web 04/22/2003

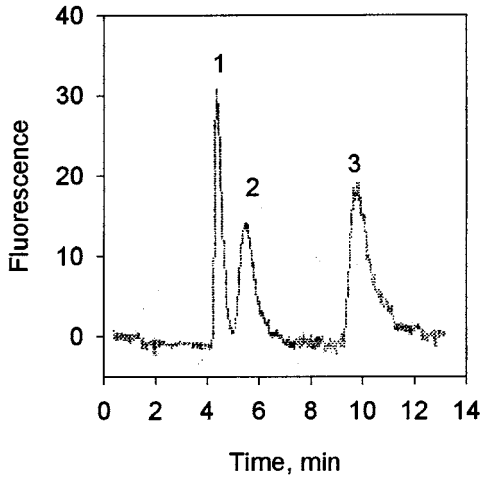

Figure 1. Chromatographic profile using the amide-based column. Flow rate, 1 mL/min; injected sample (containing 50 ng/mL of each compound), 100 μL. The peaks correspond to 1, TPP; 2, TP; and 3, T.

allowed lower detection limits than those obtained using the UV spectrophotometer (*30*).

EXPERIMENTAL PROCEDURES

Instrumentation. The LC system consisted of a Shimadzu LC-10ADvp (Shimadzu, Kyoto, Japan) liquid chromatograph operating at room temperature with a flow rate of 1 mL/min. The solvents were degassed using a membrane system Shimadzu DGU-14A. The fluorescence detector was a Kontron SFM 25 (Kontron, Zürich, Switzerland) operating at wavelengths of 375 and 465 nm (excitation and emission). The software was a Kontron integration pack. Aliquots of 100 μL were injected manually using a Rheodyne model 7125-075 injection valve (Rheodyne, Berkeley, CA). The analytical column used for the reversed-phase technique was packed with RP−AmideC_{16} with a particle size of 5 μm (Supelco). A guard column packed with the same stationary phase was also used. The postcolumn flow injection system consisted of a Gilson Minipuls HP4 peristaltic pump (Gilson, Villiers-Bel, France), a Hellma 176.052-QS fluorimetric flow cell with a dead volume of 25 μL, 0.8 mm i.d., PTFE tubing, and various end fittings and connectors (Omnifit, Cambridge, U.K.).

Reagents. Doubly distilled water was purified using a Milli-Q system (Millipore, Bedford, MA). Acetonitrile (ACN, Romil, Loughborough, U.K.) was of liquid chromatographic grade. The 25 mM potassium dihydrogen phosphate solution of pH 7 was prepared from the commercial product (Panreac, Barcelona, Spain). A derivatization solution was prepared by dissolving 5 g/L potassium hexacyanoferrate-(III) (Panreac) in 150 g/L sodium hydroxide (Riedel-de Haën, Seelze, Germany). Trichloroacetic acid (Sigma, St. Louis, MO) was also used. Stock solutions (1000 μg/mL) of T, TP, and TPP were prepared by dissolving 10 mg of the commercial products (Sigma), without previous purification, in 10 mL of water. They were kept in dark bottles at 4 °C. Working standard solutions were prepared by dilution with 25 mM phosphate buffer solution (pH 7) on the same day of use.

Samples. The liquid samples were different types of beer (with and without alcohol) packed in bottles or cans, filtered beer, beer in guard, fermenting beer, and must. The solid samples were brewer's and freeze-dried yeast, malt, raw grain, and hops.

Procedure. Separation was carried out with an isocratic mobile phase of 25 mM phosphate buffer (pH 7). The flow rate was 1 mL/min. The postcolumn flow manifold consisted of a T-piece in which the separated vitamins are mixed with the derivatization reagent. The resulting solution was then passed through a reaction coil (0.8 m × 0.8 mm i.d.) into the flow cell for fluorescence recording. The reagent was pumped at 0.4 mL/min with a peristaltic pump. Under these conditions, calibration graphs for the analytes diluted in 25 mM phosphate buffer solution (pH 7) were obtained.

Sample Preparation. Liquid samples of beer were submitted to a 1:1 dilution using the 25 mM phosphate buffer solution (pH 7), filtered through a 0.45 μm nylon Millipore chromatographic filter, and directly injected for analysis. Solid samples were previously powdered and submitted to acid hydrolysis. The procedure consisted of weighing sample amounts of between 0.02 and 1 g (depending on the sample) and adding 5 mL of a 20 g/L trichloroacetic acid solution. The mixture was sonicated for 15 min and then centrifuged at 3000*g* for 15 min. The supernatant was recovered, and the residue was reextracted twice with successive 2 mL aliquots of 20 g/L trichloroacetic acid. The extracts were combined, neutralized with 2 M potassium hydroxide, and diluted up to 25 mL with the 25 mM phosphate buffer solution (pH 7). An aliquot was filtered through a 0.45 μm nylon Millipore chromatographic filter and analyzed. Incomplete elution of the sample components contributes to the steady deterioration of the chromatographic column, and to prevent this, the column was washed first with water and then with acetonitrile at the end of each day and stored in acetonitrile.

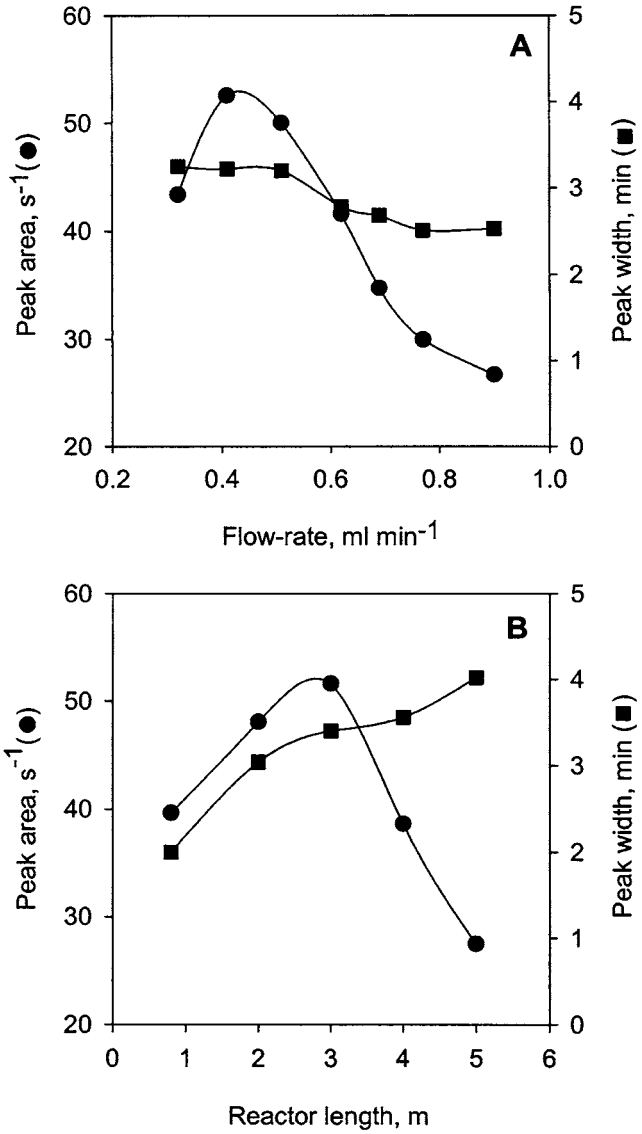

Figure 2. Influence of the flow rate of the derivatizing stream and the reaction coil length on the fluorescence signals. Thiamine injected, 100 ng/mL.

RESULTS AND DISCUSSION

Optimization of the Separation of the Thiamine Esters Using an Amide-Based Column. Reversed-phase separation was preferred to ion pair chromatography because it is more straightforward. However, thiamine and its esters are of an ionic

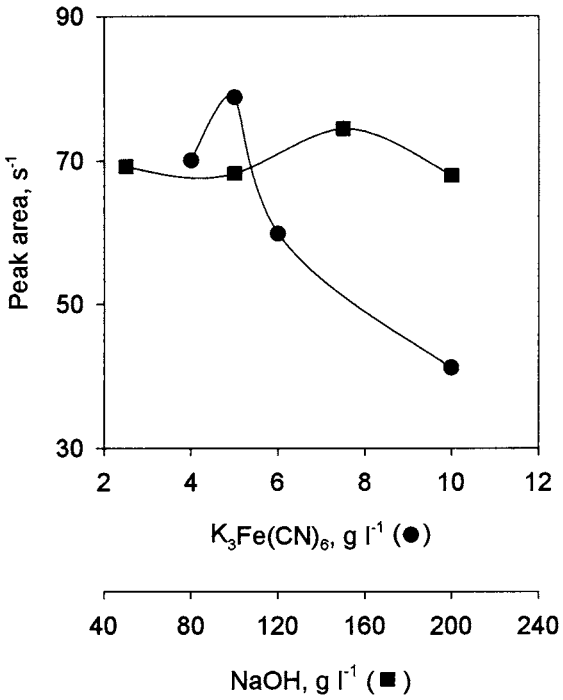

Figure 3. Influence of the potassium hexacyanoferrate(III) and the sodium hydroxide concentrations on the fluorescence signals. Thiamine injected, 100 ng/mL.

character, and reversed-phase separation using a conventional ODS column provided poor results due to the interaction of the thiamine compounds (of a basic nature) with the silanol groups of the silica-based columns. Thus, a stationary phase for basic compounds involving a ligand with amide groups (RP−AmideC_{16}) and the endcapping of trimethylsilyl was selected. This endcapping process permitted the residual silanol groups to react more strongly and gave good results for the determination of thiamine derivatives in pharmaceutical products (*30*).

Table 1. Chromatographic Parameters Using the RP–AmideC_{16} Column

ACN (%)	k'			α		R_s	
	TPP	TP	T	TPP/TP	TP/T	TPP/TP	TP/T
0	0.83	1.43	3.17	1.72	2.22	1.17	2.42
2	0.52	0.57	1.30	1.10	2.28	0.06	0.77
5	0.30	0.35	0.87	1.17	2.49	0.06	0.60

The optimal composition of the mobile phase using the RP−AmideC_{16} column was studied using several phosphate buffers at pH values ranging between 4 and 8 (values recommended by the manufacturer) and different phosphate concentrations. The best results were obtained using a 25 mM potassium dihydrogen phosphate buffer at pH 7. The flow rate was 1 mL/min. The addition of acetonitrile (ACN) at a low proportion was tried, and **Table 1** shows the variation in retention (calculated as the capacity factor, k') with ACN percentages between 0 and 5% v/v for thiamine and its esters. As expected, retention decreased for all of the vitamins when the proportion of organic solvent was increased. **Table 1** also shows the values of the separation factor (α) and the resolution (R_s). As can be seen, the best separation between the three compounds was achieved using a 100% phosphate buffer as the mobile phase because the α and R_s values were maximum in these conditions and total separation only took 11 min. The chromatographic profile obtained using this mobile phase (25 mM KH_2PO_4, pH

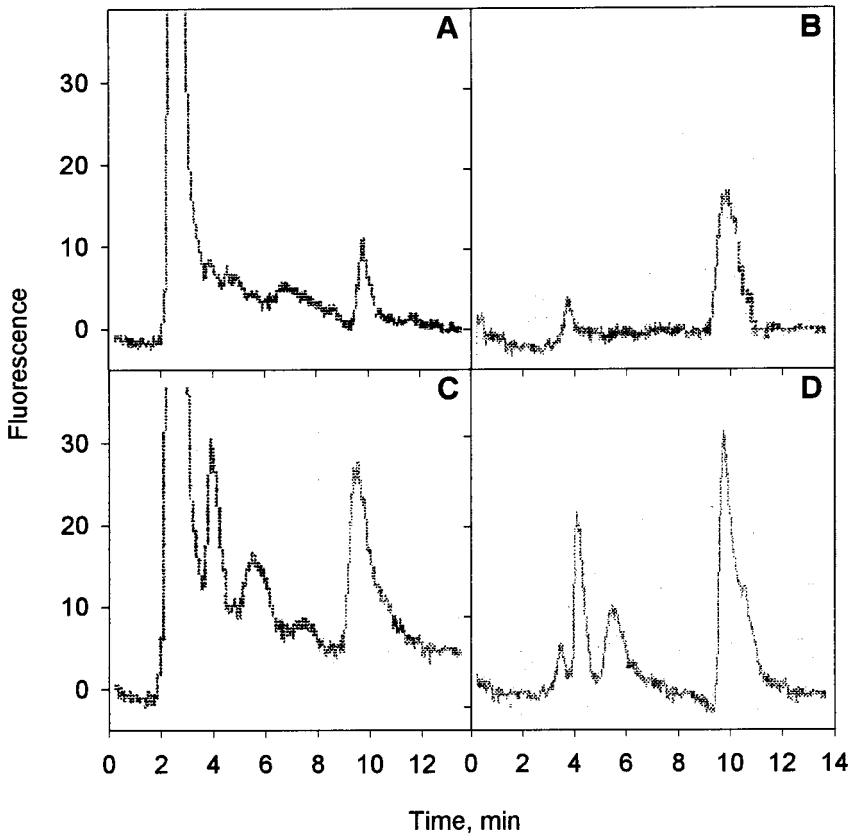

Figure 4. Chromatograms for different samples of beer (**A**); crude grain (**B**); beer spiked with 50 ng/mL of T, TP, and TPP (**C**); and spiked crude grain (**D**) using the AmideC_{16} column.

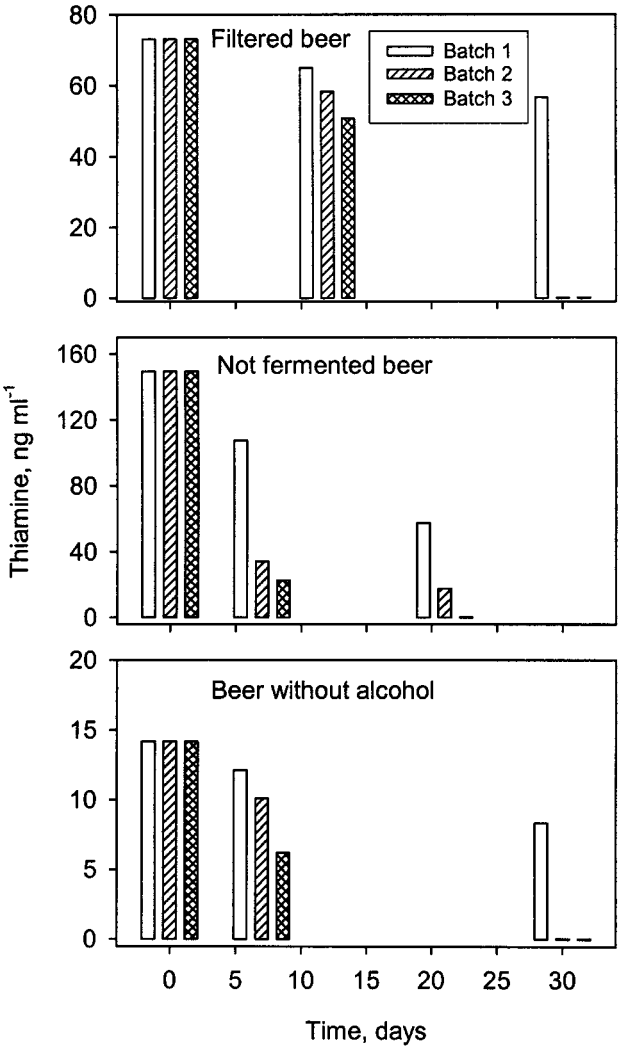

Figure 5. Influence of external conditions on the stability of thiamine. Batch 1, beer stored at 4 °C; batch 2, beer stored at room temperature and protected from light; and batch 3, beer stored at room temperature and not protected from sunlight.

7) is shown in **Figure 1**. The elution order and the retention characteristics were as follows: 1, TPP (t_R = 4.2 min; k' = 0.8); 2, TP (t_R = 5.6 min; k' = 1.4); and 3, T (t_R = 9.6 min; k' = 3.2).

Optimization of Postcolumn Derivatization. The experimental parameters were optimized to obtain maximum fluorescence intensity when injecting 100 ng/mL of thiamine. **Figure 2** shows the results obtained when varying the flow rate of the derivatizing stream (**A**) and the reactor length (**B**). When the flow rate was varied between 0.3 and 0.9 mL/min, the maximum fluorescence was obtained at 0.4 mL/min; higher rates led to a rapid decrease in fluorescence because the time was insufficient for the derivatization reaction to proceed. The width of the peaks slightly decreased at high flow rates. The length of the reactor was varied in the 0.8−5 m range, and maximum sensitivity was obtained using a 3 m reactor; however, the peak width considerably increased when this length was used, resulting in overlapping peaks. Consequently, a reactor length of 0.8 m was selected as a compromise between sensitivity and good resolution of the chromatographic peaks. The same conditions for the derivatization system were also checked for the thiamine esters, and similar results were obtained.

Table 2. Calibration Graphs for the Vitamins

parameter	TPP	TP	T
intercept	−0.0943	0.0795	−0.0765
slope (mL/ng)	0.1291	0.1285	0.5261
correlation coefficient	0.9998	0.9998	0.9999
linearity (ng/mL)	5–110	5–110	2–250
detection limit (ng/mL)	0.75	1.0	0.06
quantitation limit (ng/mL)	2.5	3.4	0.2
precision (RSD, %)	3.6	3.1	2.0

The optimal concentrations of the chemical reagents were also studied, and the results are shown in **Figure 3**. Variation of the potassium hexacyanoferrate(III) concentration in the 4−10 g/L range showed maximum fluorescence at a concentration of 5 g/L and then a decrease. As the sodium hydroxide concentration was increased above 50 g/L, the peak area slightly increased until a plateau was reached near 150 g/L; this concentration was used in all further work. Again, similar results were obtained for the thiamine and its esters. Heating the reactor coil between 25 and 60 °C decreased the fluorescence probably due to the thermal decomposition of thiamine. Consequently, room temperature was selected.

Calibration, Detection Limits and Repeatability. Table 2 shows the equations obtained for the calibration graphs of the vitamins by plotting concentration (ng/mL) against peak area. The precision of the method calculated from the relative standard deviation (RSD) for 10 replicate determinations of the same sample at 50 ng/mL is also included. The detection limits were calculated on the basis of 3σ, and the quantitation limits were calculated on the basis of 10σ, using the regression lines for the standards. Values are also given in **Table 2**.

Recovery Study and Analysis of Samples. The procedure was applied to the analysis of beers and raw materials. **Figure 4** shows typical chromatographic profiles for a beer (**A**), a crude grain sample (**B**), and the corresponding spiked samples of beer (**C**) and grain (**D**).

The profiles demonstrated the absence of interfering peaks for the quantitation of thiamine and its esters. The standard additions method was used to investigate the possibility of interference by the matrix. Each graph was constructed from four points, and each point represented the mean of three injections. Slopes of the standard additions calibration graphs for beer samples were similar to those of aqueous standards, confirming that the beer matrix did not interfere and that calibration can be carried out with the simplest method using aqueous standards.

For the solid samples, an appropriate hydrolysis, which does not cause changes in the structures, was carried out prior to identification and quantification. An acid hydrolysis using trichloroacetic acid was chosen because such a procedure does not destroy the phosphate esters. An enzymatic hydrolysis procedure was not included to avoid dephosphorylization of the thiamine phosphate esters. The extraction procedure was optimized using a beer yeast sample, first checking the number of extractions necessary to quantitatively extract thiamine; the recovery percentages were 89, 97, and 98.6% for one, two, and three successive extractions, respectively, meaning that three successive extractions are adequate for extraction. Next, extraction using a homogenizer and an ultrasonic bath was compared, and best results were achieved with the latter. Finally, the addition of water or trichloroacetic acid was assayed. Recovery percentages of 74, 94, 97, and 79% were obtained for extractions performed with water and trichloroacetic acid concentrations

Table 3. Determination of Thiamine in Beer and Its Raw Materials

liquid samples	thiamine (ng/mL)[a]	recovery (%)[b]
filtered beer	73 ± 2.3	98.4
nonfermented beer	149 ± 9	99.1
beer in guard	44 ± 1.1	101.0
canned beer	58 ± 2.9	99.4
black beer	105 ± 6	99.5
canned beer without alcohol	14.2 ± 0.8	99.4
solid samples	**thiamine (μg/g)[a]**	**recovery (%)[b]**
brewer's yeast	5.1 ± 0.3	96.3
yeast (freeze-dried)	110 ± 4	101.4
hops	0.34 ± 0.01	97.9
malt	6.5 ± 0.3	98.9
raw grain	0.66 ± 0.04	97.7

[a] Mean ± standard deviation, $n = 3$. [b] 50 ng/mL of thiamine added.

of 10, 20, and 50 g/L, respectively. A 20 g/L concentration was selected. The extracts thus obtained were also submitted to standard additions, and again, no differences in the slopes with respect to aqueous slopes were obtained, confirming the suitability of calibration using aqueous standards.

The procedure was applied to the determination of thiamine and its esters in beer and raw materials. The peaks were identified by comparing the retention data obtained for the sample, the standards, and the sample spiked with the standards under identical conditions. The thiamine esters were not detected in any of the beer or raw materials analyzed at the detection limit of the method. **Table 3** shows the results obtained for the thiamine content of different types of beer and in the solid samples used as raw materials.

To confirm the results, the efficiency of the extraction method was established by performing a recovery study. Absolute recoveries were evaluated by adding 50 ng/mL of thiamine to beers and solid samples. The samples were spiked at the beginning of the extraction procedure, and then, spiked and unspiked samples were treated as described in the experimental procedure and analyzed. Recovery data are shown in **Table 3**, and the values indicate that recovery was essentially quantitative.

Because thiamine has been described as a very labile vitamin, a stability study in several beer samples was carried out. For the study, three batches were used as follows: (i) beer stored at 4 °C; (ii) beer stored at room temperature and protected from light; and (iii) beer stored at room temperature and not protected from light. Thiamine was again found in all of the samples. **Figure 5** shows the results obtained for the thiamine content when samples of the three batches were stored for 30 days in the above-mentioned conditions before analysis. As can be seen, a sharper decrease in the thiamine content was observed in the samples stored at room temperature and under sunlight. Similar results were obtained for other beer samples.

CONCLUSION

The use of reversed phase LC with an AmideC_{16} column is highly suitable for determining thiamine and its esters of an ionic character using isocratic elution. The postcolumn fluorescence derivatization reaction proposed considerably lowers the detection limits of this group of vitamins. The procedure was sensitive enough to be applied to the determination of thiamine and its esters in beers and their raw products. The highest amounts of thiamine were detected in brewer's yeast and malt.

LITERATURE CITED

(1) Coultate, T. P. *Food. The Chemistry of Its Components*, 2nd ed.; Royal Society of Chemistry: London, 1989.

(2) Piendl, A. The role of beer in present-day nutrition. *Brauwelt Int*. **1990**, *3*, 174−176.

(3) Association of Official Analytical Chemists. In *Official Methods of Analysis*; Williams, S., Ed.; Association of Official Analytical Chemists: Arlington, VA, 1984; p 836.

(4) Van Niekerk, P. J. Determination of Vitamins. In *HPLC in Food Analysis*; Macrae, R., Ed.; Academic Press: London, 1988; p 156.

(5) Vanderslice, J. T.; Huang, M. H. A. Liquid-chromatographic analysis of thiamine and its phosphates in food products using amprolium as an internal standard. *J. Micronutr. Anal.* **1986**, *2*, 189−199.

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

(7) Ollilainen, V.; Vahteristo, L.; Uusi-Rauva, A.; Varo, P.; Koivistoinen, P.; Huttunen, J. HPLC determination of total thiamin (vitamin B_1) in foods. *J. Food Compos. Anal.* **1993**, *6*, 152−165.

(8) Alyabis, A. M.; Simpson, K. L. Comparison of reverse-phase C-18 open column with the Bio-Rex 70 column in the determination of thiamin. *J. Food Compos. Anal.* **1993**, *6*, 166−171.

(9) Olleta, F. J.; Llanos, E.; Barcos, R.; Ancin, 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*, 155−158.

(10) Ren, Y. P.; Chen, H. W.; Chen, Q. J.; Bao, L. B.; Huang, B. F.; He, Q. H. Determination of thiamine in foods by high-performance liquid chromatography with postcolumn photochemical derivatization and fluorescence detection. *Fenxi Huaxue* **2000**, *28*, 554−558.

(11) Ayi, B. K.; Yuhas, D. A.; Moffett, K. S.; Joyce, D. M.; DeAngelis, N. J. Liquid-chromatographic determination of thiamine in infant formula products by using ultra-violet detection. *J. Assoc. Off. Anal. Chem.* **1985**, *68*, 1087−1092.

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

(13) Lavigne, C.; Zee, J. A.; Simard, R. E.; Gosselin, C. High-performance liquid-chromatographic diode-array determination of ascorbic acid, thiamine and riboflavine in goat's milk. *J. Chromatogr.* **1987**, *410*, 201−205.

(14) Nicolas, E. 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*, 792−798.

(15) Vidal-Valverde, C.; Reche, A. Improved high-performance liquid-chromatographic method for thiamine analysis in foods. *Z. Lebensm. Unters Forsch.* **1990**, *19*, 313−318.

(16) Otles, S. Comparative determination of vitamin B_1 (thiamine) and B_2 (riboflavine) in foods by using different enzyme preparations. *Z. Lebensm. Unters Forsch.* **1991**, *193*, 347−350.

(17) Bognar, A. Determination of vitamin B_1 in food by high-performance liquid chromatography and postcolumn derivatization. *Fresenius' J. Anal. Chem.* **1992**, *343*, 155−156.

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

(19) Ohta, H.; Maeda, M.; Nogata, Y.; Yoza, K. I.; Takeda, Y.; Osajima, Y. Simple determination of thiamine in rice by high-performance liquid chromatography with postcolumn derivatization. *J. Liq. Chromatogr.* **1993**, *16*, 2617−2629.

(20) Chase, G. W., Jr.; Landen, W. O., Jr.; Soliman, A. G. M.; Eitenmiller, R. R. Method modification for liquid-chromatographic determination of thiamine, riboflavin and pyridoxine in medical foods. *J. Assoc. Off. Anal. Chem. Int.* **1993**, *76*, 1276−1280.

(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.* **1994**, *668*, 359−363.

(22) Yamanaka, K.; Horimoto, S.; Matsuoka, M.; Banno, K. Analysis of thiamine in dried yeast by high-performance liquid chromatography and high-performance liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry. *Chromatographia* **1994**, *39*, 91−96.

(23) Gehring, T. A.; Cooper, W. M.; Holder, C. L.; Thompson, H. C., Jr. Liquid-chromatographic determination of thiamine in rodent feed by postcolumn derivatization and fluorescence detection. *J. Assoc. Off. Anal. Chem. Int.* **1995**, *78*, 307−309.

(24) Yamanaka, K.; Matsuoka, M.; Banno, K. Determination of thiamine in dried yeast by high-performance liquid chromatography using a clean up column of CM cellulose. *J. Chromatogr. A* **1996**, *726*, 237−240.

(25) Blanco, D.; Llaneza, M. B.; Gutierrez, M. D. A paired-ion liquid-chromatographic method for thiamine determination in selected foods. *J. Liq. Chromatogr. Relat. Technol.* **1996**, *19*, 2155−2164.

(26) Abdel-Kader, Z. M. Comparison of AOAC and high-performance liquid-chromatographic methods for thiamine determination in foods. *Food Chem.* **1992**, *43*, 393−397.

(27) Hollman, 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. II. Water-soluble vitamins. *Analyst* **1993**, *118*, 481−488.

(28) Analytical Methods Committee. Determination of thiamine and riboflavin in pet foods and animal feeding stuffs. *Analyst* **2000**, *125*, 353−360.

(29) Lynch, P. L. M.; Young, I. S. Determination of thiamine by high-performance liquid chromatography. *J. Chromatogr.* **2000**, *881*, 267−284.

(30) Viñas, P.; López-Erroz, C.; Balsalobre, N.; Hernández-Córdoba, M. Comparison of ion-pair and amide-based column reversed-phase liquid chromatography for the separation of thiamine-related compounds. *J. Chromatogr. B* **2001**, *757*, 301−308.

Received for review October 22, 2002. Revised manuscript received January 28, 2003. Accepted February 25, 2003. We are grateful to the Spanish Ministery of Science and Technology (Project BQU2000-0218) and INIA (Project CAL 01-25) for financial support.

JF021071F