Simultaneous Determination of Eight Water-Soluble Vitamins in Supplemented Foods by Liquid Chromatography

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A fast, simple, and reliable method for the isolation and determination of the vitamins thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid in food samples is proposed. The most relevant advantages of the proposed method are the simultaneous determination of the eight more common vitamins in enriched food products and a reduction of the time required for quantitative extraction, because the method consists merely of the addition of a precipitation solution and centrifugation of the sample. Furthermore, this method saves a substantial amount of reagents as compared with official methods, and minimal sample manipulation is achieved due to the few steps required. The chromatographic separation is carried out on a reverse phase C18 column, and the vitamins are detected at different wavelengths by either fluorescence or UV–visible detection. The proposed method was applied to the determination of water-soluble vitamins in supplemented milk, infant nutrition products, and milk powder certified reference material (CRM 421, BCR) with recoveries ranging from 90 to 100%.

KEYWORDS: Water-soluble vitamins; food labeling; supplemented foods; milk; liquid chromatography

INTRODUCTION

Vitamins are crucial for maintaining good health in humans; lack of a sufficient amount of any of them can cause serious diseases (*1*). The human diet does not always contain the amount of vitamins needed for normal development and maintenance of body functions. For this reason, certain food products are supplemented with vitamins, especially those directed to infant nutrition. Moreover, food processing and long periods of food storage may also lead to loss of vitamins. Thus, vitamin fortification allows the nutritional requirements of infant formulas and other baby foods to be met. A rapid and reliable analytical determination of the water-soluble vitamin content in food is needed for food laboratories, manufacturers, and regulatory authorities to confirm the percentage of the recommended dietary allowance (RDA) present in the final food products.

Current official methods (*2*−*12*) for the determination of water-soluble vitamins are based on spectroscopic, chromatographic, chemical, or microbiological techniques that are tedious and time-consuming. Different acid treatments followed by enzymatic digestion before microbiological assay or HPLC procedures have been reported, such as sulfuric acid and amylase (*13*), perchloric acid (*14*), trichloroacetic acid (*15*), or hydrochloric acid and taka-diastase (*16*) treatments. It is important to note that either official methods or HPLC multivitamer

methods are not suitable for the simultaneous determination of all the typically supplemented water-soluble vitamins in food.

Separation of water-soluble vitamins has been carried out using reverse-phase liquid chromatography (RP-LC) without ion-pair reagents (*16*−*18*) and also RP-LC with ion-pair chromatography (*15*, *19*, *20*).

Specific HPLC methods have been developed to quantify vitamin C in different foods (*21*−*26*), but only a few HPLC methods simultaneously determining vitamin C and other water-soluble vitamins have been found in the literature (*27*). Similarly, HPLC methods for the determination of pantothenic acid in several food matrices have been reported (*28*−*30*), and attempts to overcome problems of absorbance at low wavelength have utilized postcolumn derivatization (*31*) or mass spectrometry detection with electrospray ionization (*32*). To our knowledge, vitamin C and pantothenic acid have not been included previously in a simultaneous determination of water-soluble vitamins in infant food or vitamin-enriched food products.

In the present work, we describe a simple, fast, and reliable sample treatment procedure previous to the HPLC determination of eight water-soluble vitamins that are usually present in several supplemented food products (infant formulas, infant milk, and vitamin-enriched milks). The vitamins included are thiamin, riboflavin, niacin, pantothenic acid, pyridoxine, folic acid, cyanocobalamin, and ascorbic acid.

MATERIALS AND METHODS

Instrumentation. A Megafuge 1.0 centrifuge (Heraeus, Hanau, Germany) was used for sample treatment, and the chromatographic

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4532 *J. Agric. Food Chem.,* Vol. 54, No. 13, 2006

Zafra-Gómez et al.

separation was carried out in a 2695 Alliance chromatograph (Waters, Milford, MA) equipped with a 2475 fluorescence detector, a 2996 photodiode array detector, a 20−60 °C thermostated column oven, and an automatic injector. Millenium 4.0 software was used for data treatment.

Chemicals and Reagents. All reagents were of analytical grade. Ascorbic acid (vitamin C), thiamin chloride hydrochloride, riboflavin, niacin, pantothenic acid, pyridoxine hydrochloride, folic acid, cyanocobalamin, and L-methionine were purchased from Sigma (Barcelona, Spain). HPLC-grade methanol was supplied by Panreac (Barcelona, Spain). Solvent and aqueous solutions were filtered through 0.2 μm membranes (Millipore, Bedford, MA). Triethylamine, glacial acetic acid (99%), orthophosphoric acid (85%), and potassium phosphate monobasic were obtained from Panreac. Zinc acetate hydrated, 1-octanesulfonic acid, phosphotungstic acid polyhydrated, and sodium hydroxide were obtained from Sigma. Water was purified using a Milli-Q system from Millipore.

The *precipitation solution* was prepared once a week by dissolving 9.10 g of zinc acetate, 5.46 g of phosphotungstic polyhydrated, and 5.8 mL of glacial acetic acid in 100 mL final volume of Milli-Q water. The *aqueous mobile phase buffer* (pH 2.95) was prepared daily by dissolving 6.8 g of potassium phosphate monobasic, 1.1 g of 1-octanesulfonic acid sodium salt, and 5 mL of triethylamine in 1 L of water. After that, the pH was adjusted by the addition of orthophosphoric acid.

Standards. A multistandard solution of ascorbic acid (1.0 g/L), thiamin (200 mg/L), riboflavin (30 mg/L), niacin (100 mg/L), pantothenic acid (100 mg/L), pyridoxine (40 mg/L), and cyanocobalamin (20 mg/L) was prepared in 2.4% (v/v) acetic acid (*solution 1*). Folic acid standard (50 mg/L) was prepared by dilution in sodium hydroxide (0.1 M) (*solution 2*). Solutions were kept in dark bottles at −20 °C to avoid vitamin degradation. Working standard were prepared by mixing both solutions 1:1 (v:v) and diluting with Milli-Q water just before use.

Samples. Vitamin-enriched milk and infant nutrition products were obtained from different supermarkets in Granada (Spain). Enriched milk 1 is a multivitamin ultrahigh-temperature (UHT) dairy drink for children, enriched milk 2 is a multivitamin UHT dairy drink for children over 1 year of age, and the infant milk is a supplemented milk powder. The proposed method was validated using a CRM 421 milk powder certified reference material (Community Bureau of Reference-BCR, Brussels, Belgium).

Sample Treatment. All operations were performed in subdued light. For solid samples (e.g., infant formula), 0.5 g of sample was placed in a glass or plastic tube (15 mL) and 4.5 g of heated water (40 °C) was added. The mixture was homogenized by vortex-stirring for 1 min, sonicated for 5 min, and kept for 60 min at room temperature and in darkness. For liquid samples, 5.0 g of homogenized sample was accurately weighed into tubes.

Then, 1.0 g of the precipitation solution was added into the sample tube, and the mixture was vortexed for 1 min at room temperature. After 15 min under darkness, tubes were centrifuged at 3500g for 5 min, and the supernatants were filtered through 0.22 μm nylon Millipore filters before injection into the HPLC column.

Chromatographic Conditions. The HPLC procedure is based on conditions described by Albalá-Hurtado et al. (*10*) with several modifications in order to be able to obtain a proper separation of the eight water-soluble vitamins. The separation was carried out in a Waters Alliance 2695 separation module using a gradient elution on a C18 Waters Spherisorb ODS-2 column (25 cm × 4.6 mm, 3 μm) set at 40 °C.

The mobile phase was a modified buffer phosphate (solvent A) and methanol (solvent B), and the initial values were 99.4% of A and 0.6% B followed by a 0.5 min hold; then four linear gradients were established to reach, first, a 94.0% A and 6.0% B composition at 4 min, a 70.0% A and 30.0% B composition at 12 min, and a 60.0% A and 40.0% B composition at 17 min followed by a return to initial conditions at 22 min and a hold for 8 min to get column conditioning. The flow rate was 1.0 mL/min during the entire run. The injection volume was 50 μL, and the autosampler temperature was set at 5 °C to avoid degradation.

The fluorescence detection was set at 290/410 nm (excitation/emission) for pyridoxine, held for 10 min, and changed to 400/520 nm (excitation/emission) for riboflavin determination. The optimal wavelengths in UV−vis detection were set at 245 nm (thiamin), 261 nm (niacin), 195 nm (pantothenic acid), 282 nm (folic acid and vitamin C), and 370 nm (cyanocobalamin).

RESULTS AND DISCUSSION

Sample Treatment. Determination of water-soluble vitamins by official methods based on chemical or microbiological techniques is laborious and time-consuming. Available fast methods for enriched food products, such as protein precipitation with trichloroacetic acid (*15*), have shown, in our hands, poor extraction capacity for some vitamins, such as niacin or ascorbic acid. At the same time, we have noticed the appearance of impurities that interfere with the chromatographic determination of pantothenic acid and folic acid, possibly due to the use of strong acids, such as sulfuric acid (*33*), hydrochloric acid (*16*), or trichloroacetic acid (*15*), for protein precipitation.

Investigation of possible sample treatments of milk-related products turned us to a precipitation solution containing zinc and wolframium salts in an acid medium. This solution is currently used for milk treatment in the analysis of lactulose as described in the method by the International Dairy Federation (*34*). We decided to evaluate similar treatment conditions to extract water-soluble vitamins by precipitation of proteins and fat in vitamin-enriched milks. By using the chromatographic conditions described below, we were able to verify that all eight supplemented vitamins could be isolated, separated, and quantified. Next, the ratio of precipitation solution and sample was optimized for maximum recovery. The volumes were decreased to minimize final residues on the analysis because heavy metals are present in the precipitation solution. The final conditions for sample treatment are simple and fast and can be extended to other products such as infant formulas, infant milk, and enriched fruit juices.

Chromatographic Separation. Separation is based on methodology described by Albalá-Hurtado et al. (*15*) using ion-pair liquid chromatography, where 1-octanesulfonic acid sodium salt was added to the aqueous mobile phase. Several modifications were introduced in order to be able to obtain a proper separation of the eight water-soluble vitamins. Triethylamine was also added to improve peak symmetry. The total mobile phase was optimized using mixtures of the aqueous mobile phase and methanol. Acetonitrile was also assayed without good results. The pH was an extremely critical factor for the separations of vitamins, as has been previously reported (*15, 35*). pH values from 2.5 to 4.0 were tested by the addition of orthophosphoric acid to the mobile phase, and it was concluded that the pH should be lower than 3.0 to resolve the critical vitamin pairs niacin/pyridoxine and riboflavin/cyanocobalamin. The initial methanol concentration in the mobile phase must be lower than 1% to obtain good separation of all vitamins under study but, nevertheless, present in order to avoid an excessive retention time for thiamine. Therefore, pH 2.95 and 0.6% of initial methanol concentration were selected.

Analytical Characteristics of the HPLC Method. Scan analysis of standard vitamins was performed to check the optimum conditions for detection. Wavelengths for UV−visible and fluorescence detection were selected according to the elution time of each vitamin (see Materials and Methods). To obtain adequate linear range to measure vitamin C in selected samples, 282 nm was selected as the wavelength instead of the maximum at 244 nm. Calibration graphs for samples treated according to

Water-Soluble Vitamins in Foods

J. Agric. Food Chem., Vol. 54, No. 13, 2006 **4533**

Table 1. Analytical Characteristics of the HPLC Method

parameter[a]	vitamin C	thiamin	riboflavin	niacin	panthotenic acid	pyridoxine	folic acid	cyanocobalamin
k	9	10	9	10	9	9	8	9
a	1.25×10^4	2.35×10^3	7.39×10^4	1.12×10^3	2.68×10^3	-6.47×10^4	4.05×10^3	-1.08×10^2
s_a	1.22×10^4	3.30×10^3	1.28×10^5	2.13×10^3	4.34×10^2	6.99×10^4	6.52×10^2	2.12×10^2
b (kg mg^{-1})	4.89×10^4	6.09×10^5	9.73×10^6	1.01×10^5	8.26×10^4	6.51×10^6	1.50×10^5	5.05×10^4
s_b (kg mg^{-1})	2.02×10^2	2.22×10^2	7.74×10^4	2.62×10^2	5.05×10^2	3.05×10^4	2.31×10^3	1.26×10^2
LDR (mg kg^{-1})	2.5–150.0	0.06–25.0	0.01–5.00	0.03–25.0	0.05–25.0	0.04–6.00	0.04–7.50	0.02–5.00
R^2 (%)	99.98	99.99	99.95	99.99	99.97	99.98	99.84	99.99
S_{RC}	2.89×10^4	8.13×10^3	2.97×10^5	5.21×10^3	9.68×10^3	1.63×10^5	1.37×10^4	4.73×10^2
CC$_\alpha$ (mg kg^{-1})	0.580	0.01	0.003	0.006	0.01	0.03	0.01	0.01
CC$_\beta$ (mg kg^{-1})	0.950	0.02	0.005	0.010	0.02	0.04	0.02	0.02
LOQ (mg kg^{-1})	2.49	0.06	0.01	0.030	0.05	0.11	0.04	0.04
RSD (%)	2.4	0.8	0.9	0.6	0.9	3.7	1.3	0.5
P_{lof} (%)	36.9	54.5	50.2	52.7	62.6	48.8	15.2	36.4

[a] k, calibration levels; a, intercept; s_a, intercept standard deviation; b, slope; s_b, slope standard deviation; R^2, determination coefficient; LDR, linear dynamic range; CC$_{\alpha,0.05}$, decision limit; CC$_{\beta,0.05}$, detection capability; RSD, relative standard deviation; S_{RC}, regression standard deviation; P_{lof}, P value for lack of fit test.

Table 2. Determination of Vitamins in Reference Material

	milk powder CRM 421				
	certified	found[a]	t_{calcd}	t_{tab}[b]	conclusion[a]
ascorbic acid	769.0 ± 42.0	744.0 ± 35.0	1.727	2.120	H_o
thiamin	6.51 ± 0.48	6.16 ± 0.50	1.529	2.074	H_o
riboflavin	14.5 ± 1.0	13.1 ± 2.0	2.172	2.179	H_o
niacin	68.0 ± 2.4	65.2 ± 4.5	1.979	2.131	H_o
pantothenic acid	not certified	61.2 ± 3.0			
pyridoxine	6.66 ± 0.85[c]	6.08 ± 0.95	1.626	2.111	H_o
folic acid	1.40 ± 0.2[d]	1.35 ± 0.20	0.275	2.074	H_o
cyanocobalamin	0.034 ± 0.005	<LD			

[a] Mean of 10 determinations ± standard deviation (mg kg^{-1}). [b] t_{tab} (α = 0.05; $n_1 + n_2 - 2$ fd). [c] Certified material includes pyridoxal, pyridoxamine, and pyridoxine. [d] Certified material includes total folates.

Table 3. Recovery Assay for B5 and B12

	recovery assay for pantothenic acid and cyanocobalamin		
	added (mg kg^{-1})	found[a] (mg kg^{-1})	% recovery
pantothenic acid	0.100	0.095	95.0
	1.000	0.980	98.0
	5.000	4.850	97.0
cyanocobalamin	0.050	0.047	95.3
	1.000	1.040	104.0
	5.000	5.120	102.4

[a] Mean of 10 determinations.

the analytical procedure described above were carried out. The calibration graphs are linear for the concentration range shown in **Table 1**. Linearity of the calibration graphs was tested according to the recommendation of the Analytical Methods Committee (*36*); the lack-of-fit (lof) test was applied to two replicates and three injections of each standard. The results for the intercept (*a*), slope (*b*), correlation coefficient (*R²*), and probability level of the lof test [P_{lof} (%)] are also summarized

in **Table 1**. Thus, the data yield good linearity within the stated range. The precision, as repeatability, determined as relative standard deviation (RSD) of 10 independent determinations of the same sample, was measured for a concentration of 75.0 mg kg^{-1} for vitamin C; 12.5 mg kg^{-1} for thiamine, niacin, and pantothenic acid; 2.5 mg kg^{-1} for riboflavin, pyridoxine, and cyanocobalamin; and 3.5 mg kg^{-1} for folic acid. The RSD is below 4% in all cases (**Table 1**).

A fundamental aspect that needs to be examined in the validation of any analytical method is its limit of detection in order to determine if an analyte is present in the sample. In this paper, a criterion for method performance has been used that includes the decision limit, CC$_\alpha$, and the detection capability, CC$_\beta$ (*37*). The decision limit is the limit from which it can be decided that a sample is contaminated with an error probability of α. The detection capability is the smallest content of the analyte that may be detected, identified, and/or quantified in a sample with an error probability of β. Decision limit and detection capacity, which are better adjusted to a statistical evaluation, are implemented. Thus, CC$_\alpha$ (α = 5%), CC$_\beta$ (β = 5%), and quantification limits were calculated, and the results obtained are also summarized in **Table 1**.

Validation of the Method Using Certified Reference Materials. The reliability of the proposed method for the determination of water-soluble vitamins in milk and infant formulas was confirmed by using a certified reference material milk powder, CRM 421. All vitamins are certified in CRM 421, except for pantothenic acid and cyanocobalamin. Ten independent determinations were carried out for the same certified material, and a mean was obtained. The results are shown in **Table 2**. A statistical test was carried out to compare obtained and certified values. The test includes a variance comparison (Snedecor F test) and means comparison based on Student's t test (*38*). In all cases, it can be concluded by H_o; therefore,

Table 4. Results[a] Obtained for Commercial Milk Products

		ascorbic acid	thiamin	riboflavin	niacin	pantothenic acid	pyridoxine	folic acid	cyanocobalamin
enriched milk 1	labeled	90	2.1	2.4	27.0	9.0	3.0	0.3	0.0015
	found	110.0	2.1	2.5	30.0	11.2	3.1	0.4	nd
enriched milk 2	labeled	100	0.8	2.0	14.0	3.0	1.1	0.15	4.5
	found	120.0	0.7	1.7	19.4	3.5	0.7	0.30	nd
infant milk	labeled	500	6	12	80	30	4	0.7	0.021
	found	490.0	7.3	14.8	67.8	35	5.0	0.75	nd
UHT milk	ref[b]	12.8	0.33	1.800	0.900	0.350	0.410	0.046	0.004
	found	0.0	0.33	1.679	0.780	0.356	0.376	0.042	nd

[a] Mean of 10 determinations (mg kg^{-1}); nd, not detected. [b] Reference value from *Soucci* (*39*).
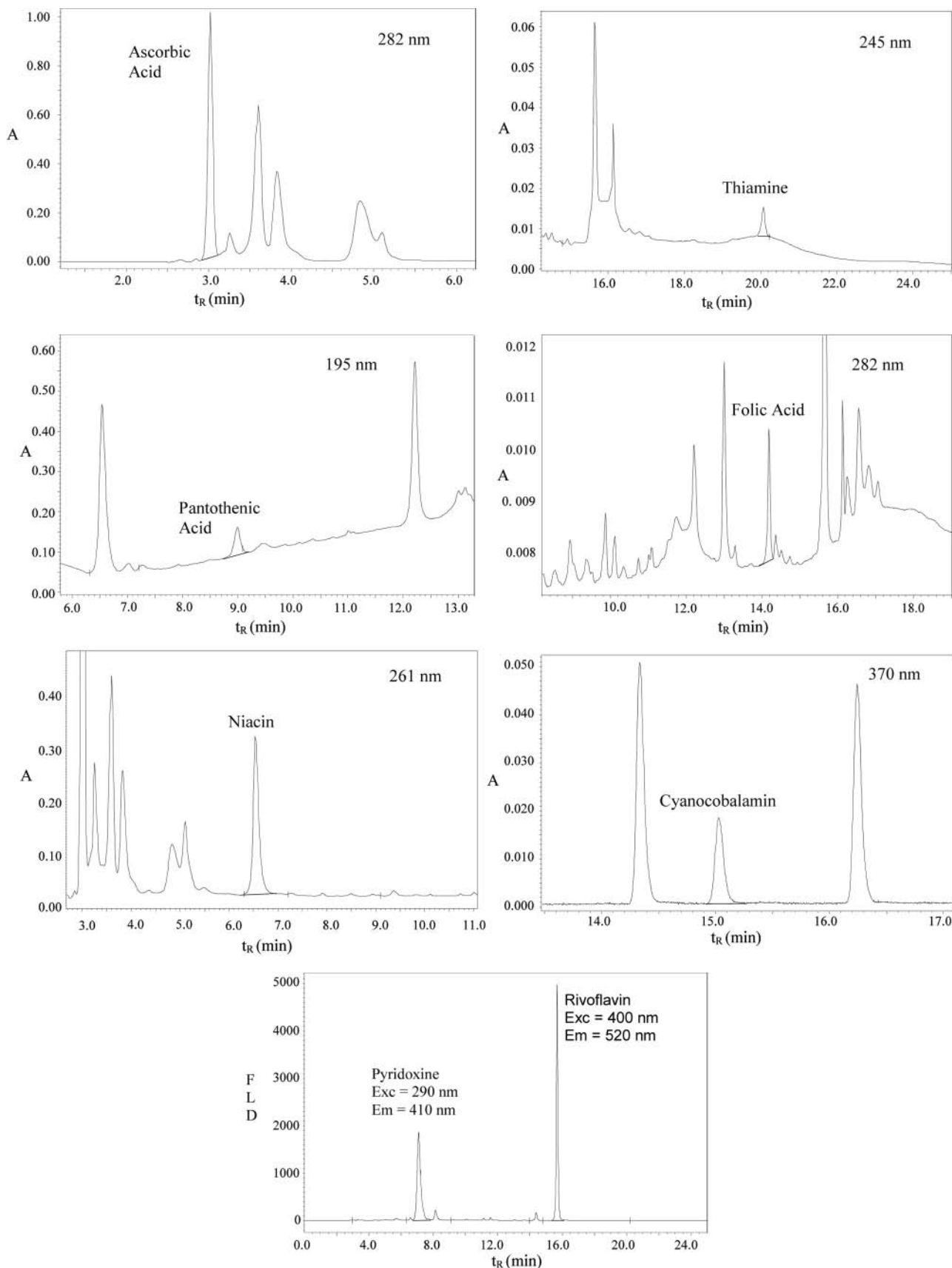
Figure 1. Representative chromatograms of a supplemented commercial milk sample.

there were no significant differences between the results obtained by the chromatographic method and the certified values (test performed at significance level of $\alpha < 0.05$). These data also confirm the efficiency of the sample treatment procedure for the recovery of all studied vitamins from the samples.

A recovery assay was carried out according to the standard addition procedure due to the absence of pantothenic acid and the low level of cyanocobalamin present in the certified material. Three addition levels (0.10, 1.00, and 5.00 mg kg^{-1} for pantothenic acid and 0.05, 1.00, and 5.00 mg kg^{-1} for

Water-Soluble Vitamins in Foods

J. Agric. Food Chem., Vol. 54, No. 13, 2006   **4535**

cyanocobalamin, respectively) were used for each vitamin. Ten determinations were carried out for each addition level. Mean recoveries obtained were in all cases higher than 95% for both compounds (**Table 3**).

Analysis of Food Samples. The water-soluble vitamins specified previously were quantified in different food samples. Several commercial products, including infant food, infant milk, and enriched milk products, were analyzed. The chromatographic peaks in the samples analyzed were identified by comparing spectra and retention data obtained for the standards and spiked samples under optimized method conditions. **Figure 1** shows an example of UV−vis at different wavelengths and a fluorescence chromatogram obtained for a commercial enriched milk sample. Values obtained for four specific food samples, three supplemented and a nonsupplemented milk, are shown in **Table 4**. Most of the results are slightly higher than the labeled amounts in the commercial products. Actually, most vitamin-enriched products are manufactured with a small overaddition of these vitamers, so that the labeled amounts are still present in the food at the expiration date. It is important to point out that the present methodology is adequate for the eight previously specified vitamin forms but not for other natural forms, such as nicotinic acid, pyridoxamine, and pyridoxal, which could be present in these samples.

In conclusion, the proposed method is a powerful tool to improve the throughput and reliability of any food laboratory for the determination of the typical eight water-soluble vitamins found in supplemented food samples. It is a simple, rapid, and economical procedure. It is important to remark that ascorbic and pantothenic acid can also be measured with the same analysis.

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