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Liquid Chromatographic Analysis of Riboflavin Vitamers in Foods Using Fluorescence Detection

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The analysis of free riboflavin (RF) and its two coenzymes, flavin mononucleotide (FMN) and flavin−adenine dinucleotide (FAD), is optimized using reversed phase liquid chromatography with fluorescence detection. The stationary phase was amide-based and endcapped with trimethylsilyl, and the isocratic mobile phase consisted of a 10:90 v/v acetonitrile/phosphate buffer (pH 5). Peaks were identified by the retention characteristics and fluorescence spectra. Detection limits were 0.03, 0.05, and 0.24 ng for RF, FMN, and FAD, respectively. The vitamins were extracted using acetonitrile and the phosphate buffer. The procedure was applied to the determination of B_2 vitamers in different types of food such as milk and soy-based infant formulas, beer, fruit juices, and honey of different types. Most B_2 vitamin appeared as RF, while the coenzymes were present in lower amounts. The method was validated using two certified reference materials, and results within the certified range were obtained.

KEYWORDS: Liquid chromatography; fluorescence; riboflavin vitamers; foods

INTRODUCTION

Vitamin B_2 can be found in nature as the free riboflavin (RF), but in most biological materials, it occurs predominantly in the form of two coenzymes, flavin mononucleotide (FMN) and flavin−adenine dinucleotide (FAD) (*1*), although there are other flavin derivatives present in nature (*2*). Most flavoprotein enzymes are involved in the complex respiratory processes that occur in the mitochondria of living cells, although some are involved in other aspects of metabolism. The Dietary Allowances Committee of the National Research Council recommends an intake of RF of 0.6 mg/1000 kcal, which is equivalent to 1.6 mg/day (*3*).

Several methods have been proposed for the determination of vitamin B_2 in food (*4*), usually involving the conversion of FMN and FAD to RF. The standard method for analysis of total RF in food is the AOAC fluorimetric method (*5*). There is growing interest in knowing not only total RF content but also flavin composition of food. Liquid chromatography (LC) using reversed phase columns has been applied to the determination of total RF (*6*−*12*) and also to the separation of the main flavins in foods, RF, FMN, and FAD (*4*, *13*−*22*). RF has a strong inherent fluorescence and can be detected very specifically with high sensitivity at its maximum fluorescence intensity at pH 6−7. The main sources of RF in foods are milk, eggs, meat products, and yeasts. RF is one of the most stable vitamins, and the alkaline conditions in which it is unstable are rarely encountered in foodstuffs, although it is sensitive to light. Extraction with hot dilute acids can split vitamin B_2−protein compounds, but the phosphoric acid esters of RF can only be hydrolyzed completely by means of enzymes (*23*).

In this study, a liquid chromatographic reversed phase procedure for the separation of free RF, FMN, and FAD is optimized using fluorescence detection. Application of the ion-pairing technique was prevented by using an amide-based stationary phase endcapped with trimethylsilyl (*24*−*26*), which also produced a considerable decrease in the peak widths by avoiding the interaction of the vitamers with the silanol groups of the stationary phase. The procedure was applied to the determination of the B_2 vitamers in different types of food, which are regular sources of this vitamin in the diet. These include baby food products (milk and soy-based infant formulas), beer (various types, with and without alcohol, in different containers), juices (fruits and fruits with milk), and honey from different origins (eucalyptus, thousand flowers, sugar cane, and chestnut). Previous LC methods for the separation of the B_2 vitamers do not analyze honey samples, and only one study (*18*) has been carried out in beer and fruit juices. Detection limits of the present study are lower than those of most published papers (*4*, *15*, *18*, *22*), and identification of the peaks using the fluorescence detector to continuously measure the spectrum while the solute passes through the flow cell was not carried out in previous studies. Additionally, the method is simple because the vitamers are isocratically eluted, while most published methods used gradient elution (*4*, *13*−*16*, *18*−*20*, *22*). The use of reference materials is invaluable for assessing method

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accuracy; however, only some recent papers have used these materials to prove the accuracy of the methods (*27*−*31*). The present study includes an original application of the use of two certified reference materials as a validation method for B_2 vitamers analysis.

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 an Agilent FLD 1100 (Agilent Technologies, Waldbronn, Germany) operating with two channels at wavelengths of 270/516 and 452/516 nm (excitation and emission). The optimal excitation wavelength giving maximum fluorescence for the compounds was 270 nm, while the 425 nm wavelength was used to prevent interferences from the matrix. Aliquots of 100 μL were injected manually using a Rheodyne model 7125-075 injection valve (Rheodyne, Berkeley, CA). The analytical column (150 mm × 4.6 mm) used for the reversed phase technique was packed with Discovery RP-AmideC_{16} with a particle size of 5 μm (Supelco). A guard column packed with the same stationary phase was also used. An IKA KS 130 basic vibratory stirrer (IKA, Germany), an EBA 20 centrifuge (Hettich, Germany), and a P-Selecta ultrasonic bath of variable power were used for extraction of the vitamins from samples.

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 10 mM potassium dihydrogenphosphate solution of pH 5 was prepared daily from the commercial product (Panreac, Barcelona, Spain), and the pH was adjusted by adding dropwise 10 M potassium hydroxide (Panreac). Stock solution (1000 μg/mL) of RF was prepared by dissolving 10 mg of the commercial product (Sigma, St. Louis, MO), without previous purification, in 3 mL of 6 M phosphoric acid and diluting up to 10 mL with water; this solution was submitted to ultrasounds for 2 min. Stock solutions (1000 μg/mL) of FMN (Fluka, Buchs, Switzerland) and FAD (Sigma) were prepared by dissolving 10 mg of the commercial products, without previous purification, in 10 mL of water. All stock solutions were kept in dark bottles at 4 °C. Working standard solutions were prepared by dilution with 10 mM phosphate buffer solution (pH 5) on the same day of use. The molecular weights of the vitamers are as follows: RF, 376.4; FMN, 514.4; and FAD, 865.6.

Samples. The baby food products were milk and soy-based infant formulas. Other samples were beer of different types (with and without alcohol) packed in bottles or cans, juices (fruits and fruits with milk), and honey from different origins (eucalyptus, thousand flowers, sugar cane, and chestnut). The method was validated using two reference materials, milk powder CRM 421 and pig's liver CRM 487, which were supplied by the Community Bureau of Reference, BCR (Belgium).

Procedure. All operations were performed in subdued light. For the solid samples (powder milk and honey), amounts of 1−2 g were weighed into polypropylene tubes and 10 mL of ACN was added. The sample was homogenized by using a vibratory automatic stirrer at 3000*g* for 10 min. Then, 10 mL of the 10 mM phosphate buffer (pH 5) was added. After homogenization and centrifugation at 6000 rpm for 5 min, the supernatant was diluted to 25 mL in a calibrated flask using the phosphate buffer. Aliquots were filtered through 0.45 μm nylon Millipore chromatographic filters and injected into the chromatograph. The RF, FMN, and FAD concentrations were corrected for the impurity of the commercial standards using the labeled purity factors. The certified reference samples were analyzed in the same way. The liquid samples of juices and beer were only filtered through 0.45 μm nylon filters, diluted if necessary, and injected into the chromatograph.

RESULTS AND DISCUSSION

Optimization of the Chromatographic Separation. Separation was performed using reversed phase chromatography. To prevent the interaction of vitamers with the silanol groups of the stationary phase, an RP-AmideC_{16} phase with trimethylsilyl endcapping was selected. This phase is an ideal choice for basic compounds because it avoids the interaction of the vitamins with the silanol groups due to the endcapping of trimethylsilyl, which permitted the residual silanol groups to react more strongly.

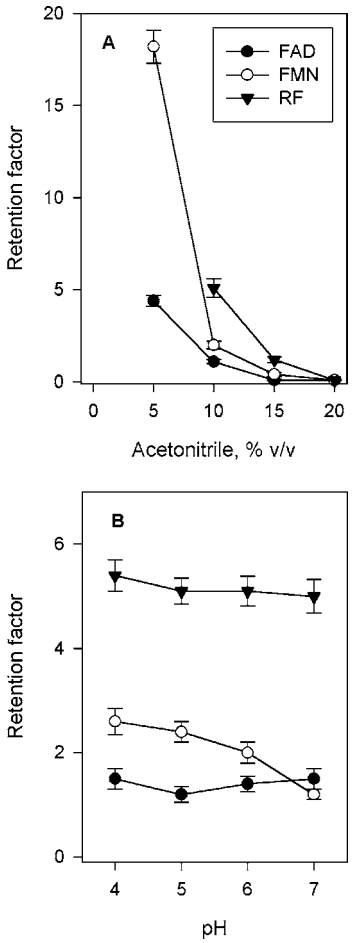

Figure 1. Variation of the retention factors of B_2 vitamers obtained by changing (**A**) the percentage of ACN, using a 10 mM KH_2PO_4 (pH 6) buffer, and (**B**) the pH of a 10 mM KH_2PO_4 buffer, in the presence of 10% v/v ACN. Error bars indicate the SD values for $n = 3$.

The mobile phase was optimized using ACN/phosphate buffer mixtures at a flow rate of 1 mL/min. A detailed study on the influence of the ACN percentage, pH, and dihydrogenphosphate concentration was carried out. The influence of the ACN proportion was studied using mixtures of a 10 mM KH_2PO_4 (pH 6) solution and ACN at different proportions. **Figure 1A** shows the variation of the vitamer retention factors when the organic solvent was varied between 0 and 20% v/v. As can be seen, retention strongly decreased for higher ACN concentrations and flavins eluted together at the void time when using a 20% v/v mixture. Thus, separation can be achieved using a 10% v/v ACN proportion. The pH study was carried out using a mobile phase containing 10 mM potassium dihydrogenphosphate in the presence of 10% v/v ACN (**Figure 1B**). The pH values were varied between 4 and 7 by adding phosphoric acid or potassium hydroxide. When the pH increased, the retention of both RF and FAD was hardly modified but slightly decreased for FMN. On the other hand, the peak width decreased for higher pH values in the case of FAD and FMN but remained practically constant for RF. As can be appreciated, the best separation was achieved at pH 5, which was selected. When the influence of the dihydrogenphosphate concentration on the vitamer retention

Table 1. Calibration Characteristics of B_2 Vitamers

vitamer	$\lambda_{ex}/\lambda_{em}$ (nm)	intercept	slope (mL/ng)	correlation coefficient	linearity (ng/mL)	DL (ng/mL)	QL (ng/mL)	RSD (%)
RF	270/516	–0.9963 ± 0.14	1.5240 ± 0.01	0.9998	1–150	0.3	0.9	2.3
	452/516	–0.4913 ± 0.36	0.8890 ± 0.01	0.9999	10–150	1.2	4.0	2.7
FMN	270/516	–0.1937 ± 0.19	1.1922 ± 0.01	0.9999	1–100	0.5	1.6	1.5
	452/516	–0.4912 ± 0.31	0.7181 ± 0.01	0.9998	5–100	1.3	4.3	3.0
FAD	270/516	–0.1617 ± 0.11	0.1369 ± 0.001	0.9999	10–500	2.4	8.0	3.2
	452/516	0.1921 ± 0.12	0.0743 ± 0.001	0.9997	20–500	4.8	16.1	2.9

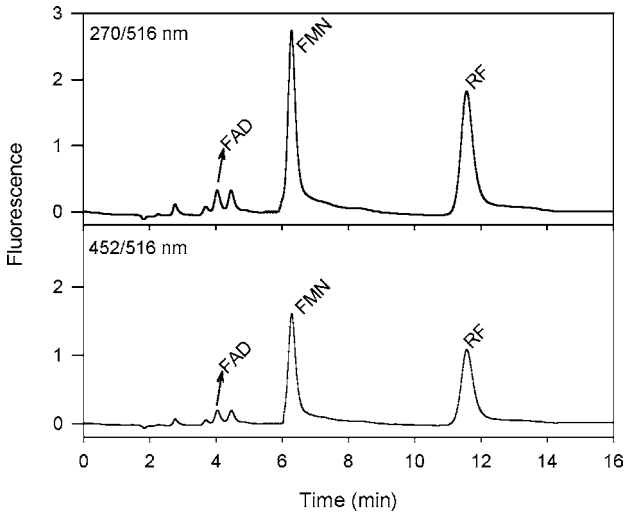

Figure 2. Chromatographic profile using isocratic elution. Flow rate, 1 mL/min; injected sample, 100 μL (containing 50 ng/mL each vitamer).

factors was studied, retention remained practically constant when the phosphate concentration increased and a 10 mM optimum concentration was selected. Furthermore, with this mobile phase, best separation was achieved with the impurities peaks contained in the standard solution of FMN, which overlapped the FAD peak when higher buffer concentrations were used. Thus, impurities of the commercial flavins (*15*, *19*) eluted at different times of those of standards, avoiding overlapping and possible interferences.

The chromatographic profile obtained by isocratic elution with a mobile phase of 10:90 v/v ACN/10 mM KH_2PO_4 (pH 5) at a flow rate of 1 mL/min is shown in **Figure 2**. The elution order and the retention characteristics for the B_2 vitamers, expressed as retention time (t_R) and retention factor (k), were as follows: 1, FAD ($t_R = 4.02$; $k = 1.2$); 2, FMN ($t_R = 6.17$; $k = 2.4$); and 3, RF ($t_R = 11.24$; $k = 5.2$). Values for the separation factor (α) and resolution (R_S) were as follows: FAD/FMN ($\alpha = 2.0$; $R_S = 2.9$) and FMN/RF ($\alpha = 2.2$; $R_S = 3.4$).

Calibration, Repeatability, and Detection Limits. Calibration graphs were performed by the external standard technique following linear regression analysis by plotting concentration (ng/mL) against peak area. Standards were injected by triplicate. **Table 1** shows the equations obtained for the calibration graphs and the regression coefficients for the three compounds at the two excitation wavelengths selected for fluorescence measurement. The optimal wavelength giving maximum fluorescence for the compounds was 270 nm, while the 425 nm wavelength was used to prevent interferences from the matrix. The precision of the method was demonstrated by repetitive analyses, calculating the average relative standard deviation (RSD) for 10 replicate injections of the same sample at 100 ng/mL for FAD and 25 ng/mL for both RF and FMN. The detection limits were calculated on the basis of 3σ (σ being the residual SD around the regression line) and the quantitation limits on the basis of

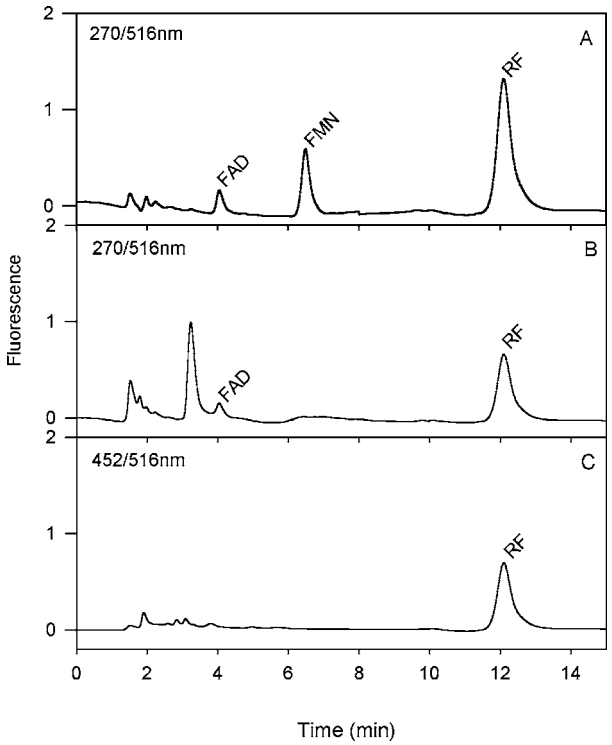

Figure 3. Elution profiles obtained for a milk infant formula (**A**), a beer (**B**), and a honey sample (**C**). Flow rate, 1 mL/min; injected sample, 100 μL.

10σ, using the regression lines for the standards. Values are also given in **Table 1**.

Optimization of the Extraction Procedure. Recovery Study. The simultaneous determination of RF and its vitamers required an appropriate extraction process so as not to disturb the flavin structures. Because flavins are sensitive to light, alkaline or extremely acid media, and phosphatase enzymes, a mild extraction process to prevent coenzyme hydrolysis must be performed. Usually, extractions are carried out using water, buffer solutions, or organic solvents such as methanol, phenol, ACN, glacial acetic acid, formic acid, or mixtures (*23*). Consequently, we assayed different solvents for extracting the flavins from the food samples and best results were obtained using an extraction process with an organic solvent, ACN, and further addition of the buffer solution, as indicated in the Experimental Procedures.

Typical chromatographic profiles obtained by application of the procedure to the analysis of foods are shown in **Figure 3** for a milk infant formula (**A**), a beer (**B**), and a honey sample (**C**). Similar chromatograms were obtained for the other food samples. The profiles demonstrated the absence of interfering peaks for quantitation of the B_2 vitamers. FMN in beer and FAD and FMN in honey were not present above the detection limits. The standard additions method was used to investigate the possibility of interference by the matrix. Slopes of the standard

Table 2. Recovery of B_2 Vitamers from Foods

sample	$\lambda_{ex}/\lambda_{em}$ (nm)	vitamer	added (ng/g)	recovery (%) (RSD, $n = 6$)
infant formula (starting milk)	270/516	RF	100	98.4 (1.6)
			200	99.2 (1.4)
		FMN	200	98.3 (2.2)
			400	100.3 (1.8)
		FAD	200	102.5 (3.7)
			400	98.7 (2.4)
canned multifruits juice	270/516	RF	100	97.3 (2.8)
			200	97.7 (2.1)
		FMN	200	99.7 (4.3)
			400	98.8 (2.7)
		FAD	200	98.3 (3.1)
			400	101.0 (2.4)
barrel beer	270/516	RF	100	100.2 (2.0)
			200	98.7 (2.1)
		FMN	200	97.6 (2.7)
			400	98.6 (2.4)
		FAD	200	96.8 (3.9)
			400	98.0 (2.5)
eucalyptus honey	452/516	RF	100	99.1 (3.9)
			200	98.3 (2.8)
		FMN	200	96.9 (4.2)
			400	97.4 (3.2)
		FAD	200	97.1 (6.3)
			400	98.0 (4.7)

additions calibration graphs for the food samples were similar to those of aqueous standards, confirming that the matrix did not interfere and that calibration can be carried out using aqueous standards.

The efficiency of the extraction method was confirmed by performing a recovery study. The samples were spiked at the beginning of the extraction procedure at two levels of 100 and 200 ng/g for RF and 200 and 400 ng/g for FMN and FAD, and then, spiked and unspiked samples were treated as described in the Experimental Procedures and analyzed. Recovery data are shown in **Table 2**, and the values indicate that recovery was essentially quantitative and that coenzyme degradation was minimal.

Analysis of Food Samples. The B_2 vitamers were quantified in the different food samples. The RF, FMN, and FAD concentrations were corrected for the impurity of the commercial standards using the labeled purity factors. **Table 3** summarizes the results obtained using the proposed method as well as the mean labeled contents for some of the samples indicated by the manufacturers. In the infant formula samples, the vitamin was mostly in the form of RF, while FAD and FMN were also present in lower amounts. In the fruits juices, vitamin B_2 was present as RF and FAD in similar amounts and lower levels of FMN. Conversely, the vitamin in beer is predominantly in the free form RF, with only a small amount of FAD, while FMN was not detected in any beer above the detection limit. In the honey samples, only the free form RF was detected, being determined at 425 nm to avoid interferences from the honey matrix. The total RF content was calculated from the different amounts obtained for the vitamers (μg/g), corrected by the RF molecular weight to obtain the total RF content.

The chromatographic peaks were identified by (i) comparing the retention data obtained for the sample, the standards, and the sample spiked with the standards under identical conditions and (ii) using the fluorescence detector to continuously measure the spectrum while the solute passed through the flow cell. Good

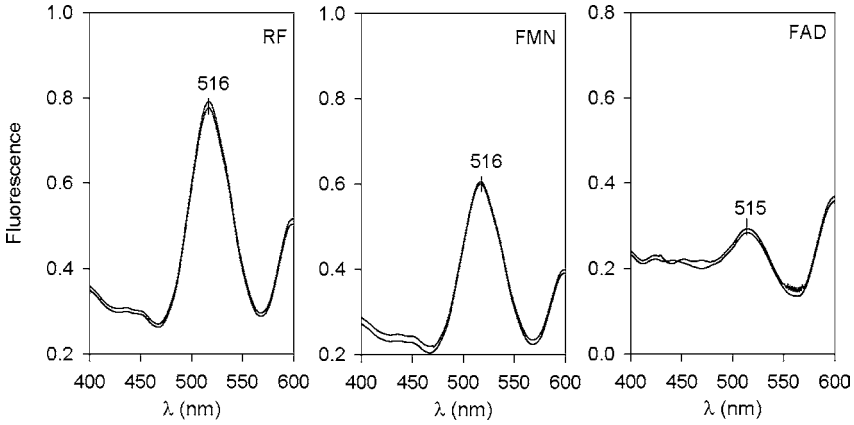

Figure 4. Fluorescence spectra of the three standards and the spectra of the peaks found in an infant formula milk sample.

Table 3. Determination of B_2 Vitamers in Foods

sample	$\lambda_{ex}/\lambda_{em}$ (nm)	found[a] (μg/g) RF	FMN	FAD	total RF
infant formula (starting milk)	270/516	8.95 ± 0.42	0.510 ± 0.021	3.14 ± 0.09	10.69 ± 0.48 (11.0[b])
infant formula (follow-on milk)	270/516	6.51 ± 0.04	0.447 ± 0.012	2.36 ± 0.08	7.86 ± 0.09 (7.6[b])
infant formula (soy-based)	270/516	6.98 ± 0.14	0.144 ± 0.006	ND	7.09 ± 0.14 (7.9[b])
canned multifruits juice	270/516	0.092 ± 0.005	0.052 ± 0.002	0.150 ± 0.008	0.195 ± 0.010
orange juice	270/516	0.107 ± 0.001	0.011 ± 0.001	0.078 ± 0.001	0.149 ± 0.002
canned multifruits juice with milk	270/516	0.136 ± 0.002	0.017 ± 0.001	0.089 ± 0.002	0.187 ± 0.004
barrel beer	270/516	0.156 ± 0.004	ND	0.019 ± 0.001	0.164 ± 0.004
canned beer	270/516	0.278 ± 0.009	ND	0.053 ± 0.001	0.301 ± 0.009
canned beer without alcohol	270/516	0.169 ± 0.010	ND	0.014 ± 0.005	0.175 ± 0.012
eucalyptus honey	452/516	0.142 ± 0.003	ND	ND	0.142 ± 0.003
sugar cane honey	452/516	0.109 ± 0.006	ND	ND	0.109 ± 0.006
chestnut honey	452/516	0.739 ± 0.010	ND	ND	0.739 ± 0.010
thousand flowers honey	452/516	0.179 ± 0.003	ND	ND	0.179 ± 0.003

[a] Mean ± SD, $n = 6$. [b] Labeled contents. ND means not detected above the detection limit.

Table 4. Determination of Vitamin B_2 in Certified Reference Materials

	milk powder (CRM 421)		pig's liver (CRM 487)	
vitamer	found[a] (μg/g)	certified[b] (μg/g)	found[a] (μg/g)	certified[b] (μg/g)
RF	11.29 ± 0.22		47.29 ± 1.72	
FMN	2.11 ± 0.12		10.37 ± 0.42	
FAD	5.51 ± 0.30		106.99 ± 4.3	
total RF	15.23 ± 0.45	14.5 ± 0.6	101.40 ± 4.01	106.8 ± 5.6

[a] Mean ± SD, $n = 6$. [b] Total B_2 content was certified as RF.

agreement was found when the fluorescence spectra of the different peaks obtained for the standards, the samples, and the spiked samples were compared. **Figure 4** shows an example of this agreement between the fluorescence spectra of the three standards and the spectra of the vitamer peaks found in the infant milk sample. Similar agreements were obtained for the fluorescence spectra of the chromatographic peaks found in the rest of the food samples. The fluorescence efficiency (relative quantum yield) of flavins is dependent on pH, excitation wavelength, and slit width. FAD has about 20% lower fluorescence efficiency than FMN and RF (*13*, *16*, *18*). The absolute quantum yield of FMN fluorescence is 5−10 times higher than that of FAD at pH 7.

Validation of the Method Using Certified Reference Materials. The reliability of the proposed method for the determination of vitamin B_2 was confirmed by using two certified reference materials, milk powder (CRM 421) and pig's liver (CRM 487). The results obtained are shown in **Table 4**. As can be seen, the three vitamers appeared in the two certified reference materials. In the powder milk, RF was the predominant form followed by FAD and FMN in smaller amounts. Conversely, in the pig's liver, the predominant form was the coenzyme FAD with lower contents of RF and FMN. As only the total vitamin B_2 content was certified as RF, a statistical study was carried out to compare the results obtained using the proposed procedure (calculating the total vitamin B_2 in the form of RF) and the certified values, using the paired *t*-test as the normality test passed ($P > 0.200$). The value of the statistic *t* was −0.701 ($P = 0.611$), meaning that the change between treatments was not great enough to exclude the possibility that the difference is due to chance. Consequently, it can be concluded that there were no significant differences between the results obtained by the chromatographic method and the certified values. These data also confirm the efficiency of the extraction procedure for recovering both free supplemented and endogenous B_2 compounds in the samples.

CONCLUSION

Reliable separation of RF and the two coenzymes, FMN and FAD, was achieved using reversed phase LC with fluorescence detection. The procedure applied isocratic elution and a stationary phase involving a ligand with amide groups and trimethylsilyl endcapping. The method can be applied to the differentiation of the B_2 vitamers in different types of food. Validation of the method was performed using two certified reference materials with good results.

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