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Biochemical Fluorometric Method for the Determination of Riboflavin in Milk

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Methods of analysis of vitamin B_2 in foods generally consist of the extraction of the sample, followed by enzymatic hydrolysis and quantitative measurement of the analyte, typically through RP-HPLC. The scope of our work here is to present a soft method to measure the free riboflavin content of a nontransparent and nonhomogeneous matrix such as milk, avoiding any extraction and separation of phases that are required in any published method for determination of the free RBF content in foods. We combine the front-face (FF) measurement of the light emission of milk with the ability of the apo-form of the riboflavin-binding protein (RBP) from chicken egg white to quench the riboflavin fluorescence. Thus, we titrate the RBF present in milk by gradually adding a solution of RBP to the milk sample and measuring, upon each addition, the FF residual emission due to uncomplexed RBF. The RBP binding capability has been measured in the same matrix of the analyte. Our results indicate a concentration of free RBF practically co-incident with the certified value for total B_2 vitamin content in reference milk CRM 421.

KEYWORDS: Front-face fluorescence; riboflavin; apo-riboflavin-binding protein; milk fluorescence

INTRODUCTION

The relationship between the light emission of yogurt, milk, and other milk derivatives and their content of riboflavin, tryptophan, or vitamin A has been chemiometrically shown, together with the analytical potential of front-face (FF) emission by such diffusive means (*1*, *2*). The use of FF techniques is due, obviously, to the high opacity of such food samples which inhibits the use of the common right angle (RA) technique. However, many difficulties in the use of FF fluorescence spectroscopy remain, due to the strong light scattering which overlaps with the fluorescence.

We present here a biochemical fluorometric method to determine the concentration of riboflavin (RBF) in milk. We combine the FF measurement of the emission of milk with the ability of the apo-form of the riboflavin-binding protein (RBP) from chicken egg white to quench the riboflavin fluorescence.

To date, three facts are firmly established: the exceptionally high association constant of RBF to RBP in water solutions, pH 6−9, on the order of 7.7×10^8 M^{-1}, the one-to-one stoichiometry, and the complete quenching of the fluorescence of RBF when complexed to RBP (*3*). From the crystal structure of RBP (*4*) it emerges how the binding site can form tight complexes with RBF (*5*, *6*). Fluorescence quenching has been ascribed to the stacking of the isoalloxazine ring of RBF with the Trp 156 and Tyr 75 aromatic rings of RBP, strategically present in the site together with four additional tryptophans clustering in the vicinity of the site (*6*).

Here we exploit the characteristics of the complex RBF−RBP mentioned above, and we titrate the RBF present in milk by gradually adding a solution of RBP to the milk sample and measuring, upon each addition, the FF residual emission due to uncomplexed RBF. It is worth noticing that this method is completely different from the official (*7*) or semiofficial (*8*, *9*) methods. It is also different from the published methods of determination of free RBF in milk, and, in particular, the more or less drastic procedures of extraction (*9*−*14*), present in all of them, are avoided here. Finally, we introduce a procedure which also differs from the two examples of use of the RBP to titrate RBF reported in literature (*15*, *16*), where the titration is performed in *quasi-transparent* liquids such as dilute urine and dilute beer and RA-fluorescence is measured. We note that RA-fluorescence can be measured only with quasi-transparent foods, i.e., which have low light absorbance and low light scattering.

MATERIALS AND METHODS

Doubly distilled or HPLC Baker water was used to prepare solutions, buffers, and HPLC mobile phases. Certified reference material, milk powder CRM 421 (*8*, *17*), was supplied by the Community Bureau of Reference-BCR, Brussels, Belgium. We used water solutions of CRM 421, ca. 110 mg/mL, obtained by dissolution of dry milk powder in

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water with about 6 h of slow stirring. Solutions of RBF (Sigma R9504, > 99% pure), ca. 0.042 mg/mL, were prepared by dissolving about 10 mg of the commercial product in a convenient volume of water.

The concentration of RBF in water was determined from the light absorption at 445 nm, using the molar extinction coefficient of 12200 M^{-1} cm^{-1} (*18*). Riboflavin binding protein, RBP, from chicken egg white (apo-form, Sigma R8628) was dissolved in distilled water.

The concentration of the RBP protein solutions was determined from the weight of the RBP dissolved and from the volume of the solution as well as from the absorbance value at 281 nm, taking the specific absorption coefficient in water $E^{1\%} = 17$ from the literature (*3*). We used concentrations ranging from 3 to 4.5 mg/mL. The stock solutions of CRM 421 and of RBP were kept in dark bottles at 4 °C and used within 2 days from the preparation.

Absorbance spectra were measured with a Jasco V-550 spectrophotometer using quartz cells with 2, 5 or 10 mm optical path in order to measure absorbance values smaller than 1.5 au. Fluorescence spectra were recorded using a ISA Fluoromax II photon-counting spectrofluorometer accessorized for front-face measurements with a cell holder designed to set the incidence angle of the excitation beam at 31°, in order to minimize the radiation which was reflected and scattered by the window and the sample. The focal point of the spectrometers was just under the illuminated window of the cell containing the sample. Spectra were measured with ≥2 mL of sample, in a quartz cell (1 × 1 cm cross section), stirred by a Teflon-coated magnetic bar. The excitation and the emission slits were 0.5 and 1 nm, respectively; the integration time constant was 0.5 s, and the wavelength increment during spectral scanning was 1 nm. The excitation narrow spectral bandwidth was chosen in order to avoid any photolysis during the scanning of the many fluorescence spectra (emission, excitation, and synchronous) we performed on each sample. Fluorescence emission was measured up to 800 nm. The intensity of the spectra was determined as the ratio of the emission signal, *S* (counts per second, cps), to the intensity of light from the excitation monochromator, *R* (μA), measured by means of a photomultiplier and a photodiode, respectively. No further correction of the spectra was performed. In order to reduce the noise/signal ratio to a negligible value, we used the fluorescence intensity integrated between 520 and 600 nm, i.e., in the fluorescence highest intensity spectral range.

The RBF titration experiments were performed by adding aliquots of 15−30 μL of the RBP titrant to the sample in order to gradually quench its fluorescence. After each addition, the cell containing the sample was slowly agitated by hand for half minute and then the residual emission was measured. The magnetic bar was stirred during the measurement, i.e., for about 180 s. Between 7 and 12 additions were necessary in our titration experiments. The CRM 421 milk was prone to deposit on the windows of the cuvette, and therefore, after each scanning of CRM 421 samples, we verified any anomalous deposition of milk material on the window exposed to the excitation light by visual inspection of the cuvette. In the case of such anomalous deposition, usually the deposit could be removed thanks to a further gentle manual agitation of the cuvette. At least two consistent spectra were recorded, each time repositioning the cell in the cell holder.

An HPLC Jasco 880-Pu coupled to a spectrophotometric detector Shimadzu SP-10 was employed in the measurement of free RBF and FMN in milk. The method of Viñas et al. (*11*) for extraction of flavins from milk powder was adopted, while the HPLC procedure reported in (*11*) was modified in order to adapt it to our hardware. In particular, the mobile phase was a 35/65 (v/v) mixture of methanol and 10 mM phosphate buffer, pH = 5, the column was a Waters Spherisorb ODS2, 5 μm, RPC18 250 × 4.6 mm with a RPC18 precolumn of 10 mm, and spectrophotometric revelation was used. For maximum selectivity, the wavelength used in revelation was 450 nm. With a flow of 0.5 mL/min the retention times of RBF and of FMN were 18.5 and 7 min, respectively. FAD, with a retention time of 5.1 min, was poorly resolved from other substances contained in the extract of CRM 421 which absorb at 450 nm and have retention times between 5 and 6 min.

RESULTS AND DISCUSSION

Titration of CRM 421 Solutions. The RBF contained in a solution prepared from the certified material CRM 421 shows an emission spectrum with a maximum at about 525 nm. **Figure 1A** reports the sequence of the emission spectra of CRM 421 with $\lambda_{exc} = 460$ nm during a typical titration with 15 μL aliquots of a solution of riboflavin binding protein, RBP. The emission intensity decrease is proportional to the amount of RBP added to the solution. The emission tends to be constant near the equivalence point: the limiting spectrum corresponds to the scattering intensity together with the residual, small fluorescence of unquenchable fluorophores present in the sample. This spectrum was subtracted from the emission at each of the titration steps, and the spectra so obtained are represented in **Figure 1B**. Finally, **Figure 2** reports the integral of the fluorescence between 520 and 600 nm, as a function of the volume of RBP solution added, taking into account the effects of the progressive dilution of the samples during the titration on both scattering and fluorescence. The effect on the scattering was determined in an experiment where we added a sufficient amount of *solid* RBP to a sample of milk CRM 421 in order to obtain an immediate disappearance of the fluorescence (data not shown). In this experiment, *which avoids any dilution*, the scattering was identical to that measured at the end of the titrations described above, where a dilution was associated with each titration step, showing that the scattering remains constant, at least with our moderate dilutions which are up to ca. 10%.

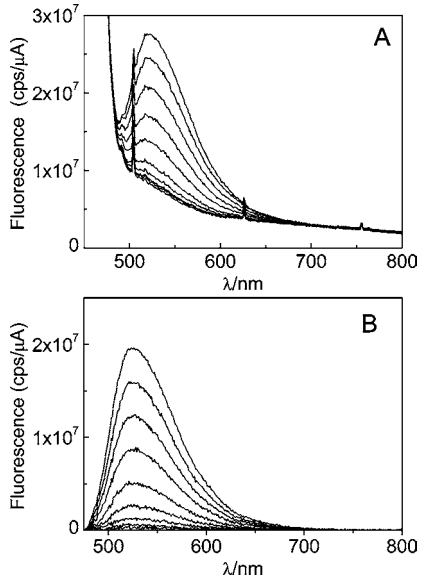

Figure 1. Decrease of the intensity of the emission fluorescence spectra, with 460 nm excitation, of a sample of CRM 421 milk, on stepwise addition of 15 μL of an aqueous solution of RBP. (**A**) Experimental emission spectra. (**B**) Fluorescence spectra calculated by subtraction of the scattering from the emission spectra of **Figure 1A**.

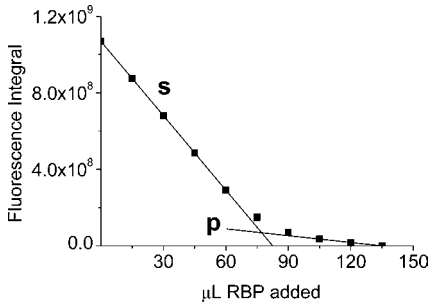

Figure 2. Titration of CRM 421 solutions with RBP. The fluorescence, integrated in the 520–600 nm spectral range, as a function of the added volume of RBP. The cross point of the straight lines s and p locates the titration end-point.

Next, we determined the effects of dilution on the fluorescence by diluting the milk with an increasing amount of water (data not shown). The decrease of the emission was proportional to the reduction of the concentration of fluorophores due to the dilution. Since we have seen that the scattering remains constant upon dilution, the measured decrease of the emission could be ascribed to a decrease of the fluorescence, and the dependence of the fluorescence on the sample dilution during the titration could be determined.

Figure 2 shows that the quenching of the RBF fluorescence appears remarkably linear with the volume of the RBP solution added, in agreement with the very high value of the binding constant. The volume of RBP solution that completely quenches the RBF fluorescence was 77.2 μL. This value is the *x*-coordinate of the cross-point of the two straight lines (s and p) presented in **Figure 2**. Line s represents the fluorescence of free RBF, in rapid and linear decrease with the added apoprotein, while p represents the fluorescence slowly decreasing after the end-point of the RBF titration, when the excess of RBP begins to titrate chemical fluorescent species different from RBF. The slope of line p is 1 order of magnitude smaller than that of line s.

We could determine the RBF content of the sample of CRM 421 by using the Sigma nominal equivalence of RBP to RBF, 1 mg to 8.5 μg, and taking into account the weight of RBP dissolved in the titrating solution, the titrating volume, and the weight of dry CRM 421 dissolved in 2 mL of aqueous solution. We calculated the RBF content in CRM 421 as 13.3 mg/kg.

On the other hand, the concentration of RBP could also be calculated as 3.29 mg/mL from the measured absorbance of RPB solution at 281 nm and the value $E^{1\%} = 17.0$, providing a quite different value for the RBF content of CRM 421, namely 10.2 mg/kg. The above contrasting results forced us to experimentally establish the quenching capacity in milk of the RBP actually used. Therefore, we added 75 μL of the solution of RBF, equivalent to 0.00357 mg of RBF, to a volume of 2 mL of the same CRM 421 milk and carried out a titration with RBP of this RBF-enriched milk.

The extra volume of RBP required to titrate the RBF-enriched milk, 92.9 μL, allows us to determine the content of RBF in this RBF-enriched CRM 421 as well as the RBF fluorescence quenching capability of RBP in milk. Thus, we calculated that 1 mg of RBP is equivalent to 8.97 μg of RBF in CRM milk, i.e., 5.5% higher than the nominal value from Sigma and considerably lower than the stoichiometric value 12.5 μg/mg. The calculated content of RBF in CRM 421, assuming the value 1 mg for 8.97 μg as the RBF fluorescence quenching capability of RBP, is 14.0 mg/kg. Upon repeating the titration experiments four times, we obtained an average value of 14.5 $\pm$ 0.5 mg/kg.

Analysis of the Titration Curves. In order to single out the precise end-point in the titration experiments, we calculated the expected titration curve on the basis of (a) the sole complexation reaction of RBF to RBP with the association constant reported for the reaction in water (*3*), $K_a = 7.7 \times 10^8$ M^{-1}, (b) the concentration of the RBP used to titrate the milk and its measured complexing capability, and (c) the value of the RBF concentration found in the CRM milk, as described above. In **Figure 3** we compare this theoretical curve, B, to the experimental titration curve, A, already reported in **Figure 2**. We note the following facts: (1) the experimental and theoretical curves are close in the linear zone (for added volumes up to ca. 60 μL), (2) the theoretical curve approaches the *x*-axis with a curvature radius much smaller than the experimental curve, thus better defining the titration end-point, (3) such a titration end-point or, in fact, the intersection of the extended linear part of the theoretical curve (q) with the *x*-axis at 77.7 μL, is close to the *x*-value of the crossing point at 77.2 μL of the linear part of the experimental curve (s) with the line passing through the last three points of the titration curve (p). In all our titrations we observed that the titration end-point determined as we have previously defined was consistently within 1% of the end-point of the theoretical curve.

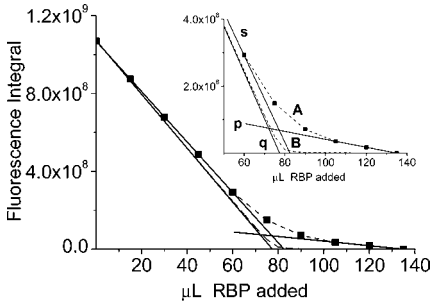

Figure 3. Titration data (■) already shown in **Figure 2** are here reported on the dotted curve A**.** The dotted curve B denotes the theoretical titration curve (see text). The inset reports an enlarged view of the plot. Straight lines s and p represent the linear portions of curve A, while line q represents the linear portion of curve B.

In **Figure 3** one can see also that the experimental curve, at ca. 80 μL of added RBP, considerably diverges from linearity and from the theoretical curve. This fact claims for the presence in the milk of at least another fluorescent substance, which is different from RBF, has a much lower affinity for RBP, and is mostly titrated after the end-point of the titration of RBF. In common milks, other fluorescent molecules such as flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) are present, and a significative presence of the above molecules in CRM 421 has also been reported (*11*). In addition to RBF, FAD, and FMN, less known flavin derivatives such as 7α-hydroxyriboflavin, 10-hydroxyethylflavin (10-HEF), 10-formylmethylflavin have been found and quantified in bovine and human milks (*13*, *19*, *20*). Finally, other flavins derived from degradation of riboflavin or other B2 vitamers can be present, particularly in highly degraded milks (*13*).

Titration of FMN and FAD Present in RBF Solutions. To clarify the effect of the presence of FMN or FAD during the titration of RBF, we titrated with RBP (a) a solution of RBF and FMN in water having comparable concentrations and (b) a solution of *pure* RBF having the identical concentration of RBF in solution a. In these titrations, the low absorbance and the almost zero-scattering of the solutions allowed us to use the right angle technique to measure the fluorescence. The resulting titration curves are reported in **Figure 4**. The linear part of curve p**,** referring to solution a, has a slope 1.675×10^6 cps/(μA·μL) close to the slope of the titration curve of the pure RBF solution b, 1.699×10^6, curve s. In addition, the linear parts of both curves have equal extension. Both facts show that RBF and FMN are titrated sequentially. We repeated the same experiment with FAD instead of FMN. The resulting titration curve (data not shown) is much flatter than the one of FMN in **Figure 4**. This behavior is due to the binding constant of FAD to RBP, which is 10000 times smaller than that of RBF to RBP (the binding constant of FMN is ca. 1000 times smaller than that of RBF), and to the FAD fluorescence quantum yield, which is much smaller than those of RBF and FMN. We conclude that during the titration of RBF with RBP in water, the titration of

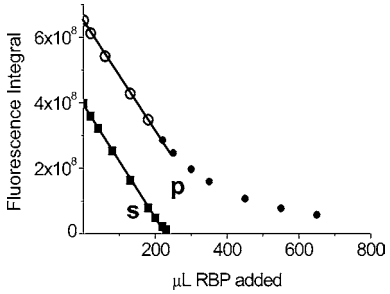

Figure 4. Titration of RBF in water (square points) and of a mixture of RBF and FMN (open and filled circle points). Plot of the fluorescence, integrated in the 520–600 nm spectral range, as a function of the added volume of RBP. Straight lines s and p represent the linear portions of the two curves.

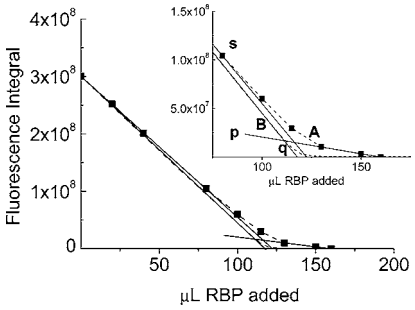

Figure 5. Titration of an aqueous solution of RBF with RBP. The fluorescence, integrated in the 520–600 nm spectral range, as a function of the added volume of RBP is reported as curve A, while the theoretical titration is described by the dotted curve B (see text). The inset reports an enlarged view of the plot. Straight lines s and p represent the linear portions of curve A, while line q represents the linear portion of curve B.

flavins such as FMN and FAD, both with low complexation constants to RBP and in concentration lower than RBF, cannot effectively interfere with the titration of RBF.

Comparison of the Titration of Pure RBF in Water and in CRM421: Flavins Present in the Titrant RBP. In a further experiment we titrated an aqueous solution of pure RBF with a RBP solution. The concentration of RBF was similar to the one in common milks. As before, we used the RA fluorimetry obtaining a RA fluorescence intensity, roughly four times lower than the FF fluorescence measured in CRM milk. The titration curve, A, is reported in **Figure 5** together with the corresponding "theoretical" curve, B. Also in this case the complexation constant $7.7 \times 10^8\ M^{-1}$ has been used to calculate the theoretical curve. The experimental titration curve is close to the theoretical one except in the final titration region where it becomes similar to the curve in the CRM 421 titration, **Figure 3**. This fact is puzzling since the RBF was more than 99% pure and one can exclude that it contains other flavins. A possible explanation is that some flavins partially occupy the binding site of the apo-RBP used in the titration and that these molecules become free and fluorescent during the titration of the much more strongly bonding RBF.

This hypothesis is supported by the titration with RBF of a water solution of RBP shown in **Figure 6**. In the figure we observe a slow growth of the RA fluorescence of the apo-RBP solution up to 70 μL of RBF solution added, near the complete titration of the protein (about 77 μL). After this point a much more rapid, 44-fold, increase follows, because of the fluorescence of the RBF added in excess. The slow increase of the

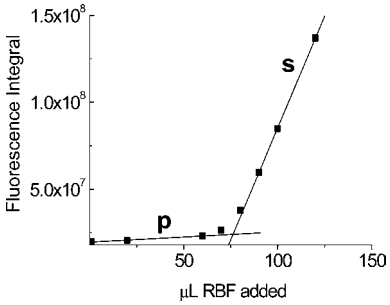

Figure 6. Titration of 2.1 mL of a RBP aqueous solution with RBF. Plot of the RA fluorescence, integrated in the 520–600 nm spectral range, as a function of the added volume of RBF aqueous solution. The cross-point of the straight lines s and p locates the titration end-point.

fluorescence in the 0−70 μL zone is caused by the fluorescent flavins which are gradually shifted out the binding site of RBP by RBF.

The gradual release of fluorescent molecules from RBP should occur also during the titration of CRM with apo-RBP (**Figure 3**) and of aqueous solutions of RBF (**Figures 4** and **5**). In fact, the fluorescence due to these species, which increases on RBP addition, is also shown by the small but significant divergence of the experimental curve A with respect to the theoretical curve B (**Figures 3** and **5**).

From the data measured in the titration of RBF in water with RBP, we calculate the binding capability of RBP in water and compare it with that obtained in the CRM milk solution, obtaining the values 8.74 vs 8.97 μg /mg, respectively. We consider this difference (2.6%) within the experimental error. The equal binding capability and the close similarity between experimental and theoretical titration curves in milk and in water show that in our experimental conditions the binding of RBF to the apoprotein is not significantly altered on passing from water to milk mediums, as we implicitly assumed when we adopted the binding constant determined in water (*3*) to calculate the theoretical titration curve in milk (**Figure 3**).

Content of Free RBF in CRM 421. Comparison with the Certified Values. Finally it is worth discussing the agreement of our results with the certified value of the RBF concentration in dry CRM 421 milk. In principle, our method should give only the *free* RBF present in milk, since it does not involve any reactive stage. As already discussed, other species like the vitamers FMN and FAD, whose binding constants to RBP are very low (*3*), cannot be cotitrated with free RBF. On the other side, the titration of minor, actually unknown, flavins with binding constant closer to that of RBF, such as 10-HEF, cannot be excluded. The content of free RBF in CRM 421 that we determined is 14.5 ± 0.5 mg/kg, identical to the certified value. The latter, however, refers to the RBF present in the *final* mixture which results from a complex analytical process consisting of the HCl digestion with heat of milk powder followed by takadiastase treatment and analysis of the liquid phase separated from an heterogeneous mixture. We roughly evaluate the free RBF content of CRM 421 to be ca. 26 mg/kg in the case that no losses occurred in the CRM production processes. This evaluation is based on literature data regarding common milks and BCR information on CRM 421 and on CRM 380. In a native milk, free RBF is about 75% of total RBF (*11*, *13*), which is about 14 mg/kg of dry mass, as reported for the milk CRM 380 (*21*). In CRM 421 the free RBF should be 75% of 14 mg/kg, plus the RBF which was added to native milk, 16 mg/kg (*22*), giving about 26 mg/kg, thus largely exceeding the certified *total* value of RBF, only 14.5 mg/kg dry milk. The

massive losses (*22*) of vitamin B_2, were on the order of 10 mg/kg, and a partial transformation in some flavins, actually undetermined, is possible.

Content of Free RBF in CRM 421. Comparison with the Results from the Extraction and HPLC Measurement. Following one (*11*) of the mildest extraction procedures indicated in the literature (*9*−*14*) and an HPLC method adapted to our hardware (see the Materials and Methods), we determined the free RBF and the FMN quantities in CRM 421 as 13.4 ± 0.4 and 1.7 ± 0.3 mg/kg dry CRM powder, respectively. The RBF concentration from the HPLC analysis of extracts is slight smaller than the value determined with the RBP titration, 14.5 ± 0.5 mg/kg. We cannot establish if this difference could be due either to some losses during the extraction procedure or to the presence of some fluorescent flavin derivatives able to react with RBP during the titration of RBF in CRM 421.

In conclusion, we propose a method to measure the content of free RBF of a nontransparent and nonhomogeneous matrix as milk, which, as far as we know, is the only one avoiding any extraction and phase separation and the connected risks of degradation and/or losses. The method is also adaptable to other nontransparent and nonhomogeneous matrices such as yogurt, cream, soy milk, egg yolk, and other dairy products.

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