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ARTICLES

Direct Fluorometric Determination of Fluorescent Substances in Powders: The Case of Riboflavin in Cereal Flours

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Front-face emission spectra of powders can be recorded with a commercial spectrofluorometer. By combining the emissions of a scatterer powder and of a wheat flour sample, the scattering contribution to the front-face emission spectra of flour is removed, and the fluorescence of the flour is isolated. The fluorescence depends on the concentration of the fluorophores. By choosing convenient measurement parameters and by measuring the emission spectra of flour samples suitably enriched with riboflavin, the fluorescence of riboflavin could be isolated from that of other substances present in flours and the concentration of vitamin B_2 in native substrates could be determined. This method is particularly apt for the measurement of vitamin B_2 in low riboflavin-containing powders such as wheat flours, which are usually analyzed through complex chemical and microbiological methods. The method is essentially phenomenological, in view of the interpretation difficulties connected to the origin of the fluorescence resulting from the absorption of multiply scattered photons.

KEYWORDS: Front-face fluorescence; scattering; powders; riboflavin; wheat flours; cereal flours

INTRODUCTION

Fluorescence from solids and powders, except for epifluorescence, has had a scarce development after the pioneering work of Wrighton et al. (*1*) and Ramasamy et al. (*2*). Recently, emission spectra of cereal powders have been obtained (*3*), and a qualitative interpretation of them was presented. In particular, it was shown that at least three different types of fluorophores cause the emission and reabsorption phenomena observed when the aromatic residues of the embodied proteins are excited with near-UV light (275 nm). According to this interpretation, part of the emission of the aromatic residues, centered at 335 nm, is reabsorbed by some chromophore in the flour and emitted as a band centered at 430 nm. In fact, in correspondence to this emission, an excitation band centered at 335 nm is present in practically all of the cereal flours investigated. Then, the 430 nm emission is partly absorbed by other chromophores and emitted at longer wavelengths. Nontrivial energy transfer mechanisms (*4*) could also be operative. However, no experiment was performed to ascertain the chemical nature of the hypothesized chromofluorophores, in particular of the fluorophores involved in the longer wavelength emissions. In addition, the large contribution of scattering to the measured emissions was not eliminated. Consequently, it was only possible to present and qualitatively discuss emission spectra and not pure fluorescence spectra from which a quantitative evaluation of the fluorophores contained could have been made.

It will be shown that using a 470 nm excitation wavelength, most of the emission of wheat flours at $\lambda > 500$ nm is due to the RBF fluorophore, **Figure 1**, and that it is possible to determine the concentration of the important vitamin B_2 (*5*) from the fluorescence, obtained after removal of the scattering contribution to the emission spectra with a suitable procedure described here.

More precisely, we could measure the overall content of the isoalloxazine fluorophore in flours from the edge fluorescence of the flour, i.e., from the fluorescence obtained when the longest wavelength in the lowest energy absorption band of RBF is irradiated. For this purpose, it is also necessary to measure the fluorescence of samples of the same flour artificially enriched with RBF.

A variety of chemical and microbiological methods are used to determine the vitamin B_2 in foods with variable results (*6*−*9*). Because of the different forms in which RBF is present in foods (flavoproteins, RBF, RBF mononucleotides, and RBF dinucleotides) (*10*), a multistep procedure is generally followed.

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In most cases, the first two steps are a drastic acid treatment of the sample and a biochemical treatment to reduce every form to free RBF. Then, high-performance liquid chromatography (HPLC) or microbiological analyses are performed on the extracts. When applied to food containing low amounts of RBF (i.e., in the order of 1−2 μg/g), these methods are affected by high relative errors (*7*, *8*) mostly derived from the two first steps. The complexity of these methods results in high costs in terms of materials, time, and money.

MATERIALS AND METHODS

Materials. Seeds of bread wheat (*Triticum aestivum* cv. Idra) and of durum wheat (*Triticum durum* cv. Grazia) were harvested in 2000 from northern Tuscany fields and stored in sealed glass containers at 10 °C in the dark. Seeds were ground in a break roller mill (Labormill 4 RB, Italy), and flours were stored at −18 °C. The flours obtained were very similar to the commercial ones.

Flours were sieved by means of an analytical sieve shaker (AS 200 basic Retsch GmbH & Co. KG, Haan, Germany) to perform a partial determination of the size of flour particles. Thus, 33% (in weight) of Idra flour grains resulted with >106 μm maximum size, 37% were between 106 and 63 μm, and 30% resulted with <63 μm maximum diameter. For Grazia, a durum variety, largely different percentages were obtained as follows: 58, 38, and 4%, respectively.

Starch was obtained after manual extraction of gluten, which was discarded from Idra flour. After the washing water was centrifuged, the starch was collected and purified from proteins with 0.1 M Tris-HCl buffer, pH 7.8, containing 4% Triton X-114 and subsequent washings with distilled water until the detergent was removed.

All solvents, of HPLC grade, were from Carlo Erba, Italy. RBF (98% purity) and FAD (90% purity) were from Sigma-Aldrich Co. (Milano, Italy). RBF solutions were made by dissolving weighted amounts of RBF in methanol or ethanol. $BaSO_4$ and $BaCO_3$ were obtained from Solvay Bario e Derivati SpA, Italy.

Fluorophore Addition to Powders. Accurately known amounts of RBF were added to weighted samples of powder (typically 500 mg) by adding suitable volumes of a methanol or ethanol solution with known concentration of RBF. Care was taken in order to get well-wetted samples, which were manually mixed to homogeneity and dryness. Samples so prepared were immediately used or kept at −18 °C, in the dark, before measurements.

Spectroscopic Measurements. A Cary 219 spectrophotometer was used for absorbance measurements. Emission spectra were recorded with an ISA Fluoromax II spectrofluorometer. The instrument was equipped with a cell holder designed for a 1 mm optical path absorption cell oriented for frontal spectroscopy measurements (35° incidence of excitation light beam). Thus, direct reflection of the excitation light into the emission monochromator was avoided. The cell, in Suprasil, was open at the top to be easily filled up with powders. Powders were packed until uniformity by means of a few gentle shocks of the cell upon a wood surface.

Spectral bandwidths of 0.25 and 6 nm were employed for the excitation and the emission slits, respectively. The integration time was 0.5 s, and the wavelength increment in measurements was 5 nm. In these experimental conditions, spectra with satisfactory intensity, resolution, and signal-to-noise ratios were recorded, meanwhile the substrate photolysis (*10*) result was negligible. On the contrary, a significant (>10%) reduction of the emission intensity was observed after recording 11 emission spectra (excitations at 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, and 500 nm); the emission was measured each nanometer up to 800 nm. It is useful to remember that photo-reduced forms of RBF are nonfluorescent (*10*).

Spectra were determined as the ratio *S*/*R* of the emission signal, *S* (counts per second), to the intensity of light from the excitation monochromator, *R* (μA), measured by means of a photomultiplier and a photodiode, respectively. No correction to the spectra was applied.

RESULTS AND DISCUSSION

Light in Powdered Samples. When the excitation light impinges on a powder sample, it is subjected to reflection, due to the different refraction index of the material with respect to air, to diffraction, to the granular form of the material or to disomogeneities inside each granule, to refraction at each boundary, and finally to absorption inside the granule. Reflection, refraction, and diffraction are embodied in the general term scattering; in our case, we are essentially concerned with multiple Mie scattering (*11*, *12*). Scattered light has the same wavelength as the incident light. However, stray light photons with different wavelengths are also present mostly because of imperfect rejection of these photons, coming from the lamp, by the excitation monochromator.

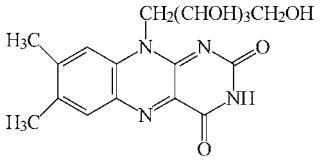

Figure 1. RBF.

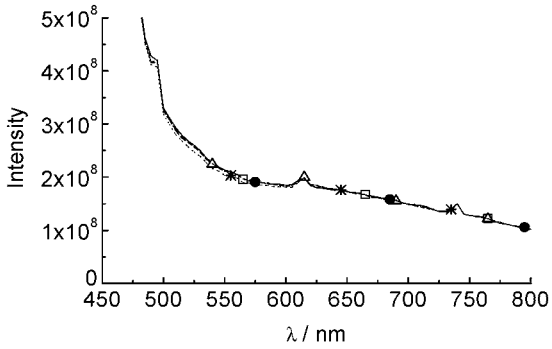

Figure 2. Emission spectra, under 450 nm light excitation, of some barium salts (grain size/μm): $BaCO_3$, □ (0.3); $BaSO_4$, △ (0.9); $BaCO_3$, ∗ (1.3); and $BaSO_4$, ● (1.8).

Absorption involves each chromophore with a band overlapping with the wavelength of excitation light; it may give rise to fluorescence, which, contrary to scattering, is substantial only at wavelengths longer than the exciting light. In the presence of fluorophores absorbing this light, absorption and reemission at even longer wavelengths may follow. This kind of event can recur, as was shown for flours (*3*), if the sources of light, the emitting fluorophores, and the sinks of light, the chromophores, are close to each other, and multiple scattering dominates.

To achieve information on the concentration of a given fluorophore, we have to face two problems: first, we have to single out the photons emitted by the target fluorophore from photons due to scattering or to the fluorescence of different fluorophores; and second, we need to find out the relationship between the concentration of the target fluorophore in the sample and its fluorescence. In the following, an experimental approach, resolving both problems, will be described.

Scattering and Fluorescence in Emission Spectra. When we record, in the experimental conditions described in the Materials and Methods section, the emission spectrum of a pure scatterer powder, i.e., a powder completely lacking in chromophores and fluorophores active in the spectral region of interest, results similar to those reported in **Figure 2** are obtained. Clearly, the intensity recorded at wavelengths different from the excitation one is due both to stray light of the excitation monochromator and to the incomplete rejection of the excitation photons by the emission monochromator. In fact, both of these kinds of photons are massively directed to the emission side of the fluorometer by the intense scattering processes occurring in the powder.

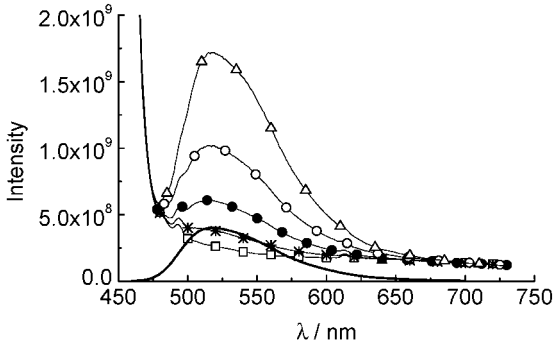

Figure 3. Emission spectra on 450 nm excitation of RBF (—) in EtOH solution (×30) and barium sulfate added with variable amounts of RBF. From bottom to top: 0 (□), 1.5 (*), 3.5 (●), 7.5 (○), and 14.5 (△) μg/g.

We experimentally proved that pure scatterers with different particle dimensions, packing densities, or chemical natures give emission spectra proportional to each other. As an example, **Figure 2** reports the emission spectra of four different scatterers, namely, $BaSO_4$ with 0.9 or 1.8 μM average particle diameter and $BaCO_3$ with 0.3 or 1.3 μM average particle diameter, obtained with an excitation wavelength of 450 nm. At each wavelength, the emission intensity of the four samples differs from their average by some 0.5%. Different scatterers, such as Na_2SO_4 and $CaCO_3$, give diffusion intensities proportional to those of Barium salts shown in **Figure 2**. Emission spectra proportional to barium ones have been observed also for organic scatterers, at least under excitation wavelengths in the violet-blue spectral region. For example, in the range 485−800 nm, under 470 nm excitation, the emission of purified starch was strictly proportional to the emission of $BaSO_4$ with a 1.8 μm particle diameter (1.03 times the emission of $BaSO_4$). Thus, we assume that emission spectra from organic or inorganic scatterers in the spectral region of interest have a shape that is independent from the particular source of scattering.

In the case of a powder containing fluorophores, at any emission wavelength, we have two contributions to the measured emission intensity: the scattered excitation light (including stray light of the excitation monochromator) and the fluorescence light. However, the findings previously described allow the light scattered by a powder to be determined and, hence, subtracted from the measured emission to obtain the true fluorescence. To better explain this point, an experiment was set up.

We prepared some samples of $BaSO_4$, 0.9 μm diameter, added with different amounts (1.5, 3.5, 7.5, and 14.5 μg/g) of RBF, and we recorded their emission spectra on excitation with light at 450 nm; the results are reported in **Figure 3** together with the fluorescence spectrum (multiplied by 30) of a 5.0×10^{-6} M ethanol solution of RBF.

This last spectrum, completely due to RBF fluorescence, shows a maximum at 520 nm and vanishes at $\lambda > 750$ nm. A component showing a similar maximum is clearly evident in the emission spectra of the RBF-added powders. Thus, it is reasonable to assign this component to the fluorescence of RBF in the powders and to expect it to vanish at $\lambda > 750$ nm, so that the emission intensity measured in this last spectral region must be due only to scattering. The contribution of scattering to the emission spectrum of the sample at each wavelength, $I^{\text{scatt·sample}}$, can be calculated as the emission spectrum of a pure scatterer, $I_{\text{scatterer}}$, multiplied by the ratio of the intensity of the sample, averaged over the 750−800 nm range, and that of the scatterer averaged over the same spectral range:

$$I^{\text{scatt·sample}} = I_{\text{scatterer}} \times (\text{average } I_{\text{sample}}/\text{average } I_{\text{scatterer}}) \quad (1)$$

Clearly, this fraction of the scattering of the pure scatterer is able to reproduce the "emission" intensity of the sample in the spectral range where only scattering is present. If we assume that the scattering of the sample in the spectral region ($\lambda <$ 750 nm) where fluorescence occurs is unaffected by absorption/emission processes, except for the intensity, we can calculate the fluorescence of the sample by subtracting the calculated scattering intensity from the total emission at each wavelength. The fluorescence spectra so obtained are reported in **Figure 4** together with the fluorescence of the 5.0×10^{-6} M solution of RBF in EtOH (multiplied by 90), for comparison. It is worth noticing that the calculated intensity of the scattering of RBF-added $BaSO_4$ is 94−98% the intensity of the pure salt.

Notwithstanding the extremely different environments of RBF in solid $BaSO_4$ and in EtOH solution, fluorescence is notably similar. To better relate the fluorescence features of these samples, excitation spectra of $BaSO_4$ pure and added with 3.5, 7.5, and 14.5 μg/g of RBF were recorded at the emission wavelength of 520 nm. The intense scattering of the pure $BaSO_4$ salt, curve **d** in **Figure 5**, was simply subtracted from the measured excitation spectra of RBF-added samples to obtain semiquantitative excitation spectra (curves **a**−**c** in **Figure 5**). These spectra show features, such as the position and the width of the bands, similar to those observed in the fluorescence excitation spectra of RBF in solution, curve **e** in **Figure 5**. The fluorescence spectra of **Figure 4** were also quantitatively analyzed in order to establish a relationship between the intensity of the calculated fluorescence and the RBF concentration in the scatterer.

In **Figure 6**, we report the integral, between 520 and 775 nm, of the fluorescence curves, obtained under the excitation wavelengths 420, 450, and 480 nm, as a function of added RBF. In all cases, a good linear relationship of the experimental data is obtained. We observe that the slope of the curves depends both on the absorption of RBF chromofluorophores at the excitation wavelength and on their quantum yield of fluorescence. We can conclude that the above method for evaluating scattering in this spectral region is correct and that the calculated fluorescence is, in this case, linearly dependent on the RBF concentration.

Fluorescence of Wheat Flours. The above method allows the determination of the fluorescence spectra of the RBF added to a scatterer powder. Here, we explore the possibility of applying the same method for obtaining the fluorescence of fluorophores contained in wheat flours. The scattering present in the emission of flour, from the bread wheat cv. Idra, was calculated, as previously described, as the fraction of the scattering of $BaSO_4$ reproducing the average scattering intensity of the flour at $\lambda > 750$ nm. Then, it was subtracted from the emission spectra to obtain the pure fluorescence spectra, some of which, with different excitation wavelengths, are reported in **Figure 7**.

A simplification of the spectra and a decrease of the average fluorescence intensity are observed on increasing the excitation wavelength from 430 to 500 nm. These features are due both to the presence of fluorophores, other than RBF, absorbing in the near-UV and in the violet spectral region, and to the light absorption of RBF, which decreases at wavelengths higher than 450 nm (see also the excitation spectra of RBF in **Figure 5**). The progressive disappearance of the fluorescence secondary maximum at 475 nm (see **Figure 7**) is evidence that the absorption of non-RBF fluorophores decreases much more

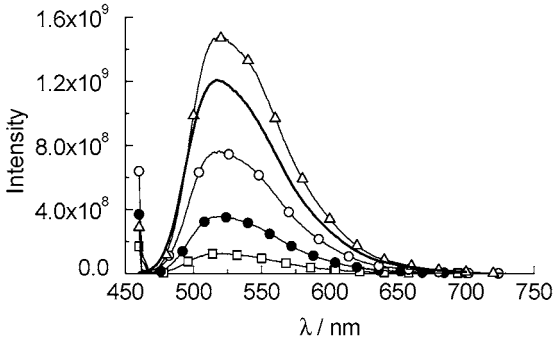

Figure 4. Fluorescence spectra on 450 nm excitation of RBF (—) in EtOH solution (×90) and of barium sulfate added to variable amounts of RBF after subtracting the contribution of scattering (see text). From bottom to top: 1.5 (□), 3.5 (●), 7.5 (○), and 14.5 (△) μg/g.

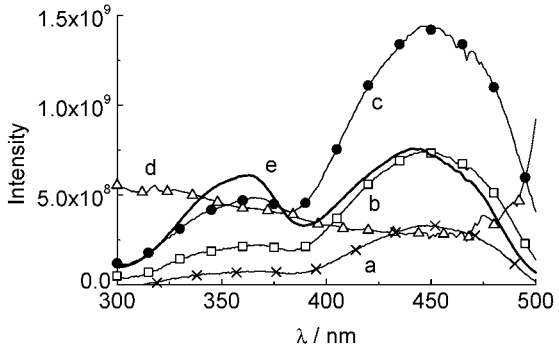

Figure 5. Excitation spectra recorded at 520 nm emission of $BaSO_4$ added with (**a**) 3.5 (×), (**b**) 7.5 (□), (**c**) 14.5 (●) of RBF, (**d**) 0.0 (△) μg/g, and (**e**) —, spectrum of a RBF solution. Curve **d** was subtracted from each of the measured excitation spectra (not reported) to obtain curves **a**–**c**.

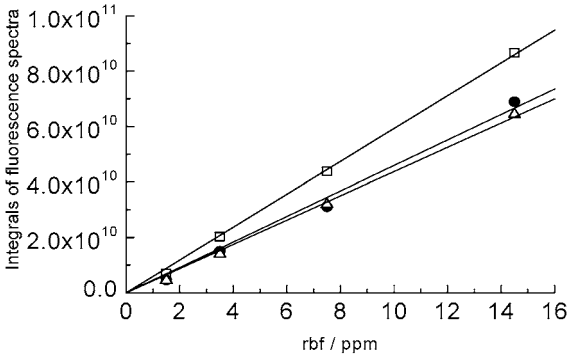

Figure 6. Integral of the fluorescence spectra, calculated from 520 and 750 nm, against RBF added to barium sulfate. Spectra were recorded on excitation at 450 (□), 480 (●), and 420 (△) nm.

rapidly with respect to that of RBF on going to the red side of the 400−500 nm excitation spectral region. Thus, only a fluorescence band results on excitation with $\lambda > 450$ nm light, as it is shown by fluorescence curves corresponding to 460 and 470 nm excitations in **Figure 7**. The similarity of these spectra with the fluorescence spectrum of RBF in solution (solid line in **Figure 7**) or with those of RBF-added $BaSO_4$ samples (**Figure 4**) suggests that fluorescence at 460−490 nm excitation wavelengths is essentially due to RBF. Some errors due to noiselike causes can be seen on close inspection of the same **Figure 7**. Even if this noise could be strongly reduced by choosing suitable experimental conditions, we preferred to use rather narrow spectral bandwidth on the excitation side (0.25 nm) and large wavelength increments (5 nm) in scanning spectra, to minimize photolysis of samples during the many

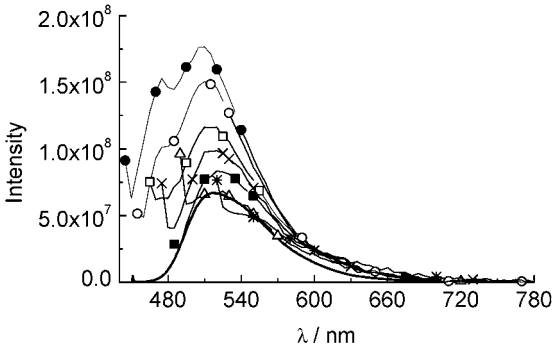

Figure 7. Fluorescence spectra of Hydra wheat flour on 430 (●), 440 (○), 450 (□), 460 (×), 470 (■), 480 (△), 500 (∗) nm excitation; and spectrum of RBF in EtOH solution on 450 nm excitation (—).

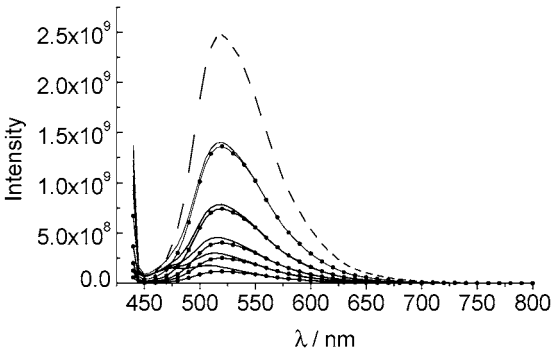

Figure 8. Fluorescence spectra on 430 nm excitation of Idra wheat flour added with variable amounts of RBF; from top to bottom: dashed line, 25 μg/g; solid lines, 12.48, 6.25, 2.94, 1.47, and 0 μg/g. Spectra calculated as a fraction of the most enriched sample (–●– lines) are reported too.

spectrofluorometric determinations on each flour sample. The consequences of the above noise were reduced by using averaged values of fluorescence, i.e., integrals of the fluorescence spectra.

Fluorescence and RBF Concentration in Flour. Now, we have to face the problem of quantifying the concentration of the RBF fluorophore contained in flour. The determination of the content of RBF fluorophore in flours cannot be found out on the basis of quantitative fluorescence data of RBF solutions because of the large differences between the physics behind front-face emission of solids and traditional emission in liquid state. Therefore, we resort to controlled addition of RBF dissolved in volatile solvents to wheat flour, in a similar way to the previous addition of RBF to an inorganic pure scatterer. Measurement of the resulting fluorescence could be used to find out the dependence of the fluorescence intensity on the concentration of the fluorophore giving rise to it in flours. Thus, we prepared Idra flour samples enriched in RBF (1.47, 2.94, 6.25, 12.48, and 25.0 μg/g) in a controlled way. The fluorescence spectra of these samples as well as the native ones, obtained with the excitation wavelength 430, 450, 470, and 490 nm, are reported in **Figures 8**−**11**, respectively. Removal of scattering from the emission spectra was made by the method described above.

For each excitation wavelength, the resulting fluorescence curves are similar to each other and resemble the fluorescence spectrum of RBF or FAD in solution. However, they are not strictly proportional to each other, as it would be expected, especially in the spectral zone close to the excitation wavelength, where scattering largely dominates the recorded emission intensity. Unfortunately, it was not possible to perfectly remove

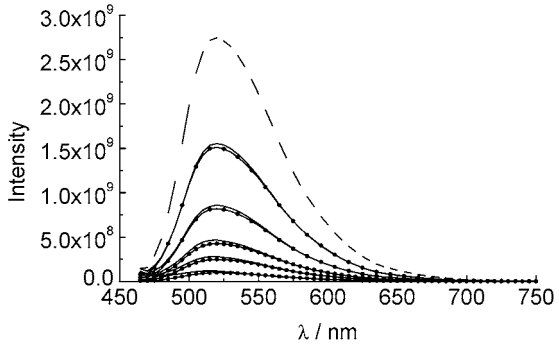

Figure 9. Fluorescence spectra on 450 nmexcitation of Idra wheat flour added with variable amounts of RBF; from top to bottom: dashed line, 25 μg/g; solid lines, 12.48, 6.25, 2.94, 1.47, and 0 μg/g. Spectra calculated as a fraction of the most enriched sample (–●– lines) are reported too.

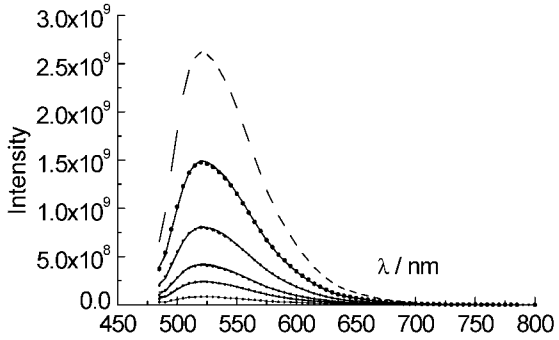

Figure 10. Fluorescence spectra on 470 nm excitation of Idra wheat flour added with variable amounts of RBF; from top to bottom: dashed line, 25 μg/g; solid lines, 12.48, 6.25, 2.94, 1.47, and 0 μg/g. Spectra calculated as a fraction of the most enriched sample (–●– lines) are reported too.

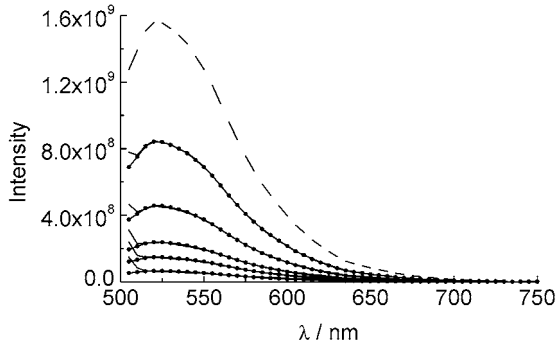

Figure 11. Fluorescence spectra on 490 nm excitation of Idra wheat flour added with variable amounts of RBF; from top to bottom: dashed line, 25 μg/g; solid lines, 12.48, 6.25, 2.94, 1.47, and 0 μg/g. Spectra calculated as a fraction of the most enriched sample (–●– lines) are reported too.

this scattering, especially when the intensity exceeded the linearity limits of the measurement devices. In this spectral region, fluorescence curves deviate from the expected behavior; this feature is particularly evident in **Figure 11**, for the spectra with $\lambda_{exc} = 490$ nm. In addition, contributions from fluorophores emitting in the blue side of the RBF fluorescence spectrum are present in the spectra of flours, particularly at shorter excitation wavelengths (see **Figure 8**, $\lambda_{exc} = 430$ nm), as was already observed for the fluorescence at 430 nm excitation in **Figure 7**. On the contrary, there is a good proportionality among the curves on the red side of the fluorescence spectra.

Now, it can be observed that the flour sample added massively with RBF, e.g., here with 25 μg/g, RBF exhibits a fluorescence curve with relatively small contributions to its intensity from

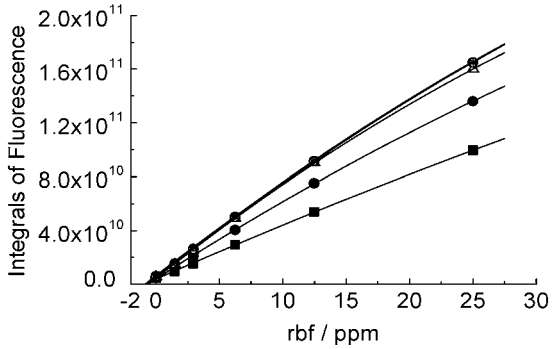

Figure 12. Integral of the fluorescence spectra, calculated from 520 and 775 nm, against RBF added to Idra wheat flour. Spectra were recorded on excitation at 490 (■), 480 (●), 470 (△), 460 (○) nm. The equation for 470 nm excitation parabola is $Y = -4.914 \times 10^7 x^2 + 7.548 \times 10^9 x + 4.582 \times 10^9$, $R^2 = 0.99$.

both the residual scattering and the tail of the fluorescence of fluorophores different from RBF. Thus, we could assume that the true fluorescence of samples added with smaller amounts of RBF is better represented by fluorescence curves proportional to the fluorescence of the 25 μg/g RBF sample and with the same intensity, on the red end of the fluorescence spectrum previously calculated. In practice, for each flour sample enriched with less than 25 μg/g of RBF, we considered also a curve (dashed−dotted lines in **Figures 8**−**11**) with the shape of the 25 μg/g RBF curve and tangent to the previously calculated fluorescence curve (solid lines **Figures 8**−**11**) at 575 nm. These spectra overlap the previous fluorescence spectra to a different extent depending on the excitation wavelength: the highest overlap was found for $\lambda_{exc} = 470$ nm. Non-RBF fluorescence and residual scattering are, now, more easily recognizable, especially at $\lambda_{exc} = 430$ and 490 nm.

As anticipated, we preferred to find the relationship between the averaged value of the fluorescence and the RBF added to each sample. Thus, we calculated the integrals of the fluorescence spectra over the 520−775 nm range, that is, starting from the maximum of the curves in order to avoid the errors on the fluorescence intensity near the excitation wavelength, as discussed before. Integrals were preferred to maximum intensity of fluorescence in order to reduce errors due to the noise in fluorescence spectra. The dependence of the integrals on the RBF amount added to flour samples is shown in **Figure 12**, where the best-fitting parabolic curves for $\lambda_{exc} = 460$, 470, 480, and 490 nm are reported. Experimental data and calculated curves corresponding to 450 and 440 nm excitation wavelength overlap the 460 nm one when plotted. For the sake of clarity, the 440 and 450 curves are not shown in **Figure 12**.

In all cases, a good fit (correlation parameter R^2 very close to 1, always higher than 0.98) of the experimental data was obtained with a quadratic function. There are sound physical reasons, in particular the partial reabsorption of fluorescence photons, to justify a parabolic trend of fluorescence intensity as a function of added RBF concentration.

It is worth noticing that the zero RBF addition point is almost perfectly lined up with the other points on each curve and that the same best fit curves are obtained irrespective of the fact that data relative to native flour were considered or not in the fitting. These findings indicate that the contribution of native RBF to the fluorescence of RBF-enriched flours remains present and constant on RBF addition.

RBF Contents in Native Flours. The quantity of RBF fluorophore present in the native Idra flour can be calculated

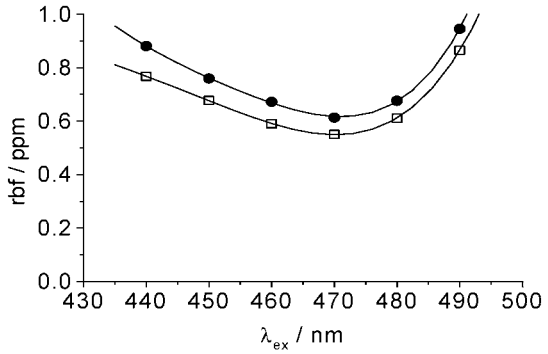

Figure 13. Plots of the contents of RBF (μg/g) of native Idra flour against the excitation wavelength, as calculated on the basis of the data and the correspondent quadratic fits reported in **Figure 8**; from fluorescence spectra, ●; from fluorescence spectra calculated as a fraction of the most enriched sample (see text), □.

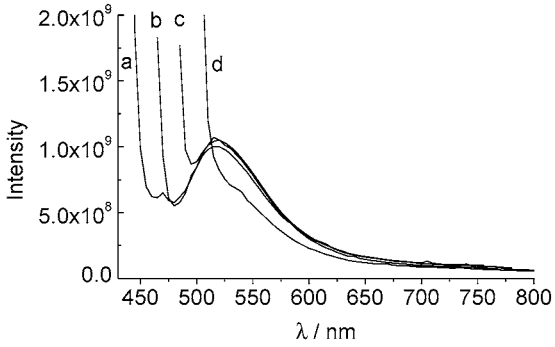

Figure 14. Emission spectra of the Idra flour sample added with 6.25 μg/g of RBF on excitation at (**a**) 430, (**b**) 450, (**c**) 470, and (**d**) 490 nm.

either from the integrated fluorescence spectra of native and RBF-enriched samples or from the corresponding integrated fluorescence curves proportional to the spectrum of the sample enriched with 25 μg/g of RBF. In both cases, the absolute value of the intercept of best fit curves with the RBF concentration axis (**Figure 12**) represents the amount of RBF naturally present in flour, which accounts for the measured fluorescence of native flour. Clearly, the underlying assumption is that the fluorescence of the RBF fluorophore is independent on the precise molecular species in which it is inserted. This assumption has strong experimental support as will be shown later.

The RBF contents in native Idra flour (expressed in μg/g) calculated using fluorescence data relative to excitation wavelengths $430 < \lambda_{exc} < 500$ are reported in **Figure 13** as a function of the excitation wavelength. Surprisingly, the calculated quantity of the RBF fluorophore present in native flour depends on the excitation wavelength with a flat minimum at about 470 nm excitation. Clearly, our previous efforts to isolate the RBF fluorescence from scattering and from the fluorescence of other chromophores present in the flour have not been entirely successful and the efficiency of the isolation procedure seems to depend on the excitation wavelength.

In particular, the steep rise of the curve at excitation wavelengths in the 480−500 nm range can be attributed to imperfect elimination of scattering. In fact, at these excitation wavelengths, scattering represents the main contribution to the overall emission intensity, being more intense than the fluorescence of the samples, at least when they are added with less than 25 μg/g of RBF. As an example, for 500 nm excitation light, the intensity of the scattering, at the maximum of the RBF fluorescence (520 nm), is about 10.3, 6.2, 4.4, 2.8, 1.5, and 0.84 times that of the fluorescence of the six samples containing 0, 1.47, 2.94, 6.25, 12.48, and 25.0 μg/g, respectively. At higher emission wavelengths, these ratios are even higher. Dominance of scattering over fluorescence on increasing excitation wavelength (from 480 to 500 nm) is in part due to the vanishing absorption of RBF. As an example, in **Figure 14**, the emission spectra of the sample added with 6.25 μg/g at excitation wavelengths between 430 and 490 are reported. Note the apparent progressive drop of the fluorescence, curves **b**−**d**, in particular curve **d**. It is clear that insufficient detraction of scattering in samples with low RBF contents automatically decreases the slope of the curves in **Figure 12** and shifts the intercept of the curve with the RBF μg/g axis at lower negative values. With shorter excitation wavelengths, the presence of scattering in the fluorescence spectral region becomes less and less important; see curves **a**−**d** in **Figure 14**.

Unfortunately, the fluorescence of other fluorophores present in the flour becomes more and more important; see also **Figure 7**. The presence of residual heterogeneous fluorescence explains the gentle rising of the curves in **Figure 13** for $\lambda_{exc} < 470$ nm. From these considerations, the best value for the concentration of RBF fluorophore in native flour seems to be that corresponding to the 470 nm excitation, i.e., to the minimum value of the curves of **Figure 13**.

The whole procedure for RBF determination in native flour described here can be resumed in the following points: (i) Prepare RBF-enriched flour samples. The maximum enrichment should be about two orders of magnitude higher than the contents of the native sample. (ii) Measure the emission of each sample and that of a reference scatterer at 470 nm excitation. The scatterer may be any one of those previously mentioned. Because its scattering spectrum does not change in the 0.3−1.8 μm size range, it is preferable that the grain average size of the reference scatterer is of this order of magnitude. (iii) For each cereal sample, calculate the scattering present in the emission spectra by using eq 1. (iv) Subtract the scattering calculated from the emission spectrum of each sample and integrate the fluorescence spectrum thus obtained from 520 nm up to its vanishing ($\lambda = 775$ nm). (v) Calculate the best quadratic curve fitting the fluorescence integrals against the quantity of RBF added to flour. (vi) Equate to zero the parabolic function and find the negative root: its absolute value is the RBF concentration of native flour.

Verifications. The above procedure rests on the hypothesis that the fluorescence quantum yield of added RBF is equal to that of the native forms in flour. Experiments suggest that this assumption is correct. In fact, the addition of flavin adenine dinucleotide (Na salt), FAD, to native flour gives, within experimental error, the same fluorescence as the addition of an equimolar quantity of RBF. No intramolecular quenching has been observed here, in the solid state, in strong contrast with that reported for the behavior of FAD with respect to flavin−adenine mononucleotide, or to RBF, in solution (*13*). Finally, the points representative of native flours, corresponding to 0 μg/g of RBF added in **Figure 12**, line up almost perfectly with points representing flours added with RBF.

To independently verify the accuracy of the RBF determinations carried out with our method, flour samples were subjected to analysis of B_2 vitamin contents with a different method by the ISV, Epalinges (CH) (*14*). Thus, two flour samples of bread wheat Idra and two samples of durum wheat Grazia have been analyzed by means of microbiological and HPLC analyses. For each wheat, one sample was the native flour and the other was native flour added with 3.1 μg/g of RBF. **Table 1** reports the results obtained by ISV and the present fluorometric method on samples from the same batches.

Table 1. Values of RBF Contents (in μg/g) of Two Native Wheat Flours, Idra and Grazia, and Their Corresponding Artificially RBF-Enriched Samples, Idra+ and Grazia+, As Determined by Fluorometric and ISV Methods

wheat	RBF (μg/g)[a]	RBF (μg/g)[b]	RBF (μg/g)[c]	RBF (μg/g)[d]
Idra	*X*	0.60	0.55	0.4
Idra+	*X* + 3.1	3.83	3.63	2.6
Grazia	*Y*	0.65	0.64	0.5
Grazia+	*Y* + 3.1	3.67	3.96	3.5

[a] *X* and *Y* = amounts of RBF in native Idra and Grazia flours, respectively. Amount of RBF added = 3.1 μg/g. [b] Determined by the integrals of the fluorescence spectra (470 nm excitation). [c] Determined by the integrals of fluorescence spectra recalculated by taking into account the form of the spectrum of the RBF richest sample; see text (470 nm excitation). [d] Determined by the ISV.

We should note the good agreement between the figures of the fluorometric results, columns b and c, the variations between equivalent samples being ca. 6%, on the average. Remarkable differences were found between RBF contents in native flours determined by fluorometry and by ISV methods.

To study the variance of our determinations, we measured the fluorescence of native as well as RBF-enriched samples of Grazia and of Idra flours on 470 nm excitation. From seven different samples of the same batch, we obtained fluorescence integrals with a relative standard deviation of 2.0 and 3.5% for native Idra and Grazia flours, respectively. The relative standard deviation for samples of the same cultivars added with ca. 7 μg/g of RBF was lower, 1.4 and 1.6%, respectively. These findings indicate the good homogeneity of samples and the good reproducibility in filling the measurement cell with flours. It is to be remembered that starch granules in wheat flour have a diameter of 1−30 μm and shapes varying from lenticular (15−30 μm) to spherical (1−10 μm) (*15*). Thus, most of the flour particles (see the size ranges reported in the Materials and Methods Section) have a nonhomogeneous structure from an optical point of view as they contain a large number of starch granules. This accounts for the intense scattering from starch and flours (which is comparable to that of ca. 1 μm scatterers). Given the above figures for the standard deviation of fluorescence integrals (from which the RBF concentration in flours is calculated), one could deduce that the error in concentration determinations should be of this order of magnitude.

For a more direct evaluation of the error in the RBF concentration, we again consider **Table 1** where fluorometric, as well as "standard methods", values of the RBF present in samples added with 3.1 μg/g are reported. By subtracting the measured native content of RBF from the measured content of these enriched samples, the fluorometric values for the added quantity 3.08, 3.23 and 3.32, 3.02 are calculated, for Idra and for Grazia flour, respectively. These values should be compared with 3.1 μg/g. The average of the four fluorometric values is 3.16 μg/g, and the maximum error was present for the value 3.32 μg/g, 5% distant from the above average and 7% from the true 3.1 μg/g. On the contrary, there is a larger relative error, in the measure of RBF-added flours analyzed by the ISV method. In fact, values of 2.2 and 3 are obtained, 15% distant from their average value 2.6 and maximum error 29% (2.2 vs the true 3.1). These errors are within the range expected for RBF determination in foods with low contents of vitamin B_2 (*7*, *8*) by standard methods.

Additionally, our findings indicate that fluorometrically determined RBF concentrations are representative of the overall content of the isoalloxazine fluorophore in flours. Thus, the chemical microenvironment of the fluorophore and the distribution of the fluorophore within the flour matrix seem to play a negligible role in determining the fluorescence intensity: the only important variable seems to be the number of absorbing/emitting centers per unit volume, i.e., the fluorophore concentration.

Whether the fluorometric method reported here is applicable also to other cereal flours or, in general, to other solid food must be verified by experiments. In this work, wheat flours showed conditions favorable to the isolation of RBF fluorescence, such as the absence of fluorophores emitting at wavelengths longer than 750 nm (allowing scattering to be evaluated in emission spectra) and the presence of a spectral window of excitation (around 470 nm) of the sole RBF fluorescence. However, similar conditions may be met also in other foods and for other fluorophores.

One may question if the knowledge of the relationship between fluorescence and RBF contents in a given flour sample (e.g., Idra) might be sufficient to determine the RBF contents in any other flour by a simple fluorescence measurement with excitation at 470 nm, thus avoiding the relatively cumbersome procedure of artificially enriching the flour under examination with exactly known RBF quantities. In this regard, we remember that in flours a very important cause of absorption of light in the spectral region around 450 nm is ascribed to carotenoids (*16*). Carotenoid content in flour is 2−5 times higher than that of RBF, and their molar extinction coefficient is 1 order of magnitude higher than that of RBF, at least in solution. Thus, carotenoids strongly compete with RBF for absorption of excitation light, and consequently, the fluorescence intensity of flours will depend also on their content of carotenoids. This implies that the results obtained here for Idra flour cannot be directly used to determine the RBF concentration of different flours.

Nevertheless, our method keeps the advantages of being less time- and material (both chemicals and substrate)-consuming and improving the precision with respect to other methods. We believe also that application of front-face fluorometry methods could be easily extended to other complex solid substrates containing fluorophores, including nonalimentary ones.

ABBREVIATIONS USED

Cv., cultivar; RBF, riboflavin, 7,8-dimethyl-10-D-ribitol-1-yl-10*H*-benzo[*g*]pteridin-2,4-dione; FAD, riboflavin 5′-adenosine diphosphate disodium salt; ISV, Istitut Suisse des Vitamines.

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