Fluorescence of Cereal Flours

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Front-surface fluorescence spectra of cereal flours are easily measured with a commercial spectrofluorometer and a simple cell. The emission spectra are intense in the range 290−600 nm in which three emission bands are active. The most intense is due to the aromatic amino acid residues present in the proteins of flours. At least two other fluorophores are active in the above spectral range and compete for light with the chromophores present in the flours. Four absorption bands are revealed in the same spectral region by reflectance spectra, the most intense being due to the amino acidic fluorophores. Thus, the measured emission is the result of absorption, scattering, emission, and reabsorption processes. Information on the microenvironment of some fluorophores can be obtained. The possibility to recognize different species of cereals, even of different cultivars, is shown.

Keywords: *Front-surface spectroscopy; fluorescence; reflectance; cereal flours; species recognition*

INTRODUCTION

Fluorescence from solutions has had a long and rich history in biological sciences. On the contrary, surface emissions from solids, in particular from noncrystalline solids, have been scarcely studied (Wrighton et al., 1974; Ramasamy et al., 1986). Reflectance and thermal emittance spectroscopies of surfaces of particulate materials have had many applications in the field of remote sensing and imaging (Hapke, 1993a), meanwhile epifluorescence has had an explosive development in the field of fluorescence microscopy applied to biological and medical sciences. To the best of the knowledge of the author, no use of fluorescence spectroscopy of food powders such as cereal flours or powders obtained from gluten, leguminous seeds, pollens, eggs, milk, etc. exists in scientific literature. This is quite surprising as natural fluorophores, mainly those associated with aromatic amino acids of proteins, are present in these systems and their emission could be significant both in scientific research and in technical and commercial applications. Some emission spectra from cereal flours will be presented here and a qualitative interpretation of these spectra will be proposed.

MATERIALS AND METHODS

Emission, excitation, and synchronous spectra of powdered samples can be easily achieved by using, for example, a cell as shown in Figure 1 with a standard commercial spectrofluorometer in the "L" configuration and with the slit image of the monochromator parallel to the "L" plane, as the Jasco FP 770 machine actually used. The 30° incidence angle of the excitation beam on the cell window eliminates the light reflected by the quartz surfaces and reduces the reflections of the sample contained between the windows. With the above apparatus, intense fluorescence spectra of the samples (vide infra) have been obtained by using narrow spectral bandwidths (1.5 and 3 nm for the excitation and emission monochromators, respectively) and with a low voltage applied to the photomultiplier. This means that the excitation light, scattered by our powdered samples, is effectively reduced by the emission monochromator so that its intensity at the phototube is negligible with respect to true fluorescent light.

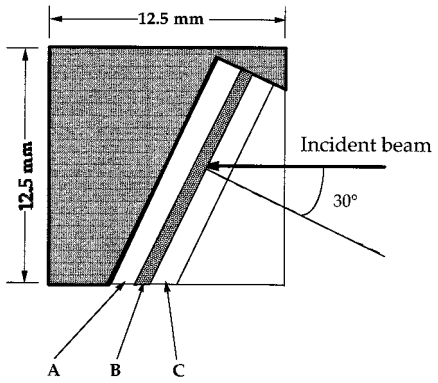

Figure 1. Front-face fluorescence cell: A and C optically polished quartz windows, B sample. The windows are held against the support, in gray, by a laminar spring, not shown here, avoiding any interception of light.

Reflectance spectra, relative to a reference light scatterer, have been obtained by using the above spectrofluorometer in the synchronous mode of scanning and with nominally equal wavelengths for both the monochromators. A strong reduction of the excitation light was required to avoid signal saturation (Zandomeneghi and Lenzi, 1999). The spectral bandwidths of the excitation and of the emission monochromators were 1.5 and 5 nm, respectively. The reference light diffuser (Wrighton et al., 1974; Hapke, 1993) used was powdered crystalline Na_2SO_4; however, identical results were obtained by using MgO powder, a classical scatterer.

The determination of the xanthophylls content of some cereals was made by means of HPLC analysis of exhaustive methanol extracts of the flours (Pinzino et al., 1998). An HPLC Jasco 880 Pu pump and a spectrophotometric Shimadzu SPD 10A detector were used. The column was a S5 ODS2 Spherisorb column 4.6 × 250 mm and the mobile phase was 100% MeOH. Retention times for lutein and zeaxanthine were 15.5 and 16.7 min, respectively, with a flux of 0.5 mL/min.

The glutens and the cereal flours, whose cultivar is reported in the work, have been obtained from the Department of Botanical Sciences, Pisa.

Aromatic amino acids, microselect quality, and bovine and

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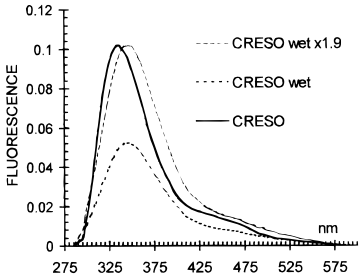

Figure 2. Fluorescence of wheat Creso flours on 275-nm excitation. The wet sample is shown, also, 1.9 times magnified.

human serum albumins, both ≥97% and fatty acids ≤0.02%, were from Fluka.

RESULTS AND DISCUSSION

Light in the Powdered Samples. When monochromatic light impinges on a more or less compact aggregate of small-sized noncrystalline and nonhomogeneous grains (for example cereal flours) reflection and refraction at the grain surfaces as well as absorption, emission, and scattering inside each grain occur. It is clear that the intensity of the light emitted from the aggregate depends on many factors: first, on the absorption features of the fluorophores at the excitation wavelength, λ_{exc}, in comparison with those of the other chromophores competing with them for excitation light. In addition, the emissive properties of each fluorophore are allied to its actual physicochemical microenvironment. Also the presence of chromophores able to absorb the light emitted by primary fluorophores and eventually emitting themselves at lower frequencies must be taken into account. Moreover, energy transfer (Lakowicz, 1983) to the above chromophores could be active within substructures of each particle. Clearly, the concentration and distribution of fluorophores within the grains may be important.

When the grains are different from each other not only in size (25−150 μm), or in form, but also in chemical composition, as in the case of wheat flours, fluorescence can be emitted also from grains illuminated or "back-illuminated" by light transmitted and diffused in the bulk of the material through more transparent grains. Grains containing much starch and a small quantity of proteins are very good light diffusers and, in addition, are much more transparent to UV light than grains with higher protein content. Through them excitation light can diffuse in the interior of the material. With wheat flours a few layers of grains are sufficient to give emission spectra whose intensity does not grow on further increasing the thickness of the material. In fact, on passing from some 300- to 600-μm thickness of the sample, the emission intensity, with λ_{exc} = 275 nm, was observed to increase only by some 10%. In general, a limiting fluorescence spectrum should be expected on augmenting the thickness of powdered materials. At this effective penetration depth, defined by the fluorescence, excitation light is completely extinguished by fluorophores and by chromophores absorbing it. The higher the absorbance of nontransparent grains and/or their quantity, the lower the penetration depth.

Fluorescence of Flours under UV Excitation. In Figure 2 the emission, λ_{exc} = 275 nm, from *dry* flour, durum wheat cultivar Creso, is reported in comparison with the emission of a *wet* sample of the same flour obtained after addition of water (60:100, v:w). In both spectra a dominant band in the near-UV is clearly evident which, very likely, is due to emission of the aromatic fluorophores of the proteins present in the flour. The red shift, 12 nm, and the band broadening, 10 nm, of spectral bandwidth at half-maximum intensity, are clearly due to the change, on wetting, of the environment of these fluorophores. Almost identical spectroscopic changes are observed when addition of water to dry powdered gluten (60:100 v:w) transforms it into an elastic and viscous material. Finally, the fluorescence spectra of the glutenins from wheat flour, in hydro alcoholic solution and on 275-nm excitation, show an intense band centered at 345 nm, i.e., exactly where the emission bands of wet flour and wet gluten are centered. Thus, the changes observed in the emission show that proteins in dough are in a very similar state to their state in aqueous solution. It can to be remembered that many functional properties of dough are connected (Lasztity, 1994; Cornell and Hoveling, 1997) to those of hydrated gluten. In particular, its elasticity has been related to the spiral structure of high-molecular-weight protein glutenins (HMWG) contained in it (Tatham et al., 1984; Kasarda, 1987; Shewry et al., 1992). Hence, studying the conformation *in solution* of the HMWG is correct for understanding the molecular origin of some interesting macroscopic properties of dough such as the viscoelastic ones. The increased Stokes shift in wet materials should be due to the relaxation of the polar aqueous environment of excited tryptophans and tyrosines *before* light emission: an environment very different and much more orientationally free with respect to the environment present in their dry state. Since the overall intensity is reduced about 2-fold on hydration, some quenching of the emission intensity seems to be present too. This reduction, however, is partially due to lower scattering, and lower absorption and back-absorption of light, by wet samples with respect to dry ones: in other words the penetration depth strongly increases on wetting and actually is higher than the present thickness of the material. On hydration of dry flour, or of dry gluten, air is expelled by water from interfaces and lower refraction index gradients are encountered by the incoming radiation: hence internal and external surface reflections as well as scattering are greatly reduced (Hapke, 1993b).

It is interesting to note that crystalline tryptophan powdered with Na_2SO_4 in the weight ratio 1:100 gives a very strong emission (λ_{exc} = 275 nm) centered at 334 nm, as the above dry flours/glutens, while when dissolved in water the fluorescence band shifts to 350 nm and the bandwidth broadening is about 13 nm.

In Figure 2 the fluorescence excited by 275-nm light extends up to 600 nm, a spectral region where no aromatic amino acids emit. In fact, common proteins such as bovine, or human, serum albumin, in aqueous solution or in dry mixtures with Na_2SO_4, are not fluorescent at λ higher than 450 nm. Hence, more fluorophores are present in wheat. In Figure 3 are reported the emission spectra obtained on changing the excitation wavelength in the 275−350 nm spectral range and using flour from rice. The spectra of rice are shown because the presence of fluorophores in addition to the natural fluorophores of proteins is more evident here than in wheat. Thus, an emission band centered at ca. 430 nm emerges, and an isoemissive point can be

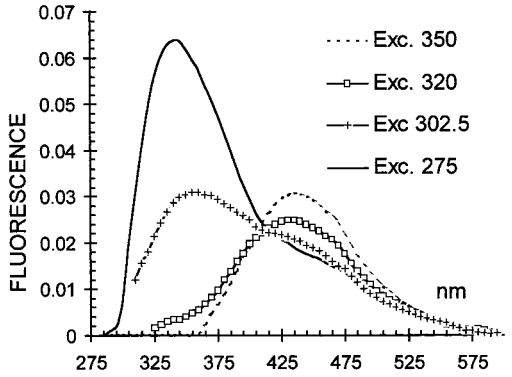

Figure 3. Fluorescence spectra of rice flour at four different wavelengths of the excitation light.

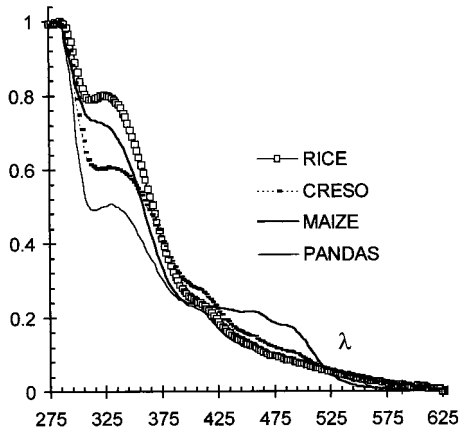

Figure 4. Logarithm of the reflectance of cereal flours relative to the Na_2SO_4 scatterer. The curves have been normalized to 1 at 280 nm.

localized at about 410 nm. Information on the origin of this 430-nm-centered emission is offered by the excitation spectrum with $\lambda_{em} = 430$ nm. An excitation band, centered at about 335 nm, of the fluorophore(s) responsible for the above emission is measured. Thus, the presence of relatively intense emissions around 430 nm in Figure 2, Creso line, and in Figure 3, 275 nm line, can be explained with a trivial mechanism of partial reabsorption, by these fluorophores, of the light emitted around 335 nm by tryptophans and tyrosines excited by 275-nm light. Nonradiative energy transfer mechanisms (Lakowicz, 1983) are also possible. As a matter of fact, substances such as 4-aminobenzoic acid, pyridoxine, tocopherols, etc. are present in cereals and could be responsible for the above emission. Similar emission bands emerge on changing the excitation wavelength from 275 to 350 nm with other cereals, including wheat. As with rice, intense excitation bands in the 300−400-nm range are measured. In conclusion, two different fluorescence bands with maxima located at 335 and 430 nm can be excited with UV light: the corresponding excitation bands being centered at about 280 and 335 nm, respectively.

Reflectance Spectra of Flours. A direct view of the absorption bands present in the spectral range 275−650 nm is given by the spectra reported in Figure 4. In Figure 4 the spectra of flours obtained from rice, maize, a durum wheat cultivar, Creso, and a soft wheat cultivar, Pandas, are reported. The quantity in ordinate is $-\log(I^{\text{cereal}}/I^{Na_2SO_4})$, a definition resembling that of the absorbance in the usual transmission spectroscopy. The log function is here used to better define the bands present in the relative reflectance spectra, i.e., $I^{\text{cereal}}/I^{Na_2SO_4}$. To be clear, for example, I^{rice} at the wavelength λ is the measured intensity of the light, at λ, scattered by the rice powder when irradiated in the front-surface cell with light of wavelength λ, while $I^{Na_2SO_4}$ is the intensity of the light diffused, at λ, by the reference scatterer under identical conditions. In Figure 4 the curves reported have been normalized to 1 at 280 nm to emphasize the relative weight of the bands of each cereal. The measured values of the intensity at 280 nm were in the ratios 100 (maize):95 (rice):94 (Creso):79 (Pandas). Four absorption bands are distinguished in each spectrum: band I, centered at 280 nm, is the most intense and is due to the aromatic amino acids present. The second, band II, is an intense band located in the 315−370 nm spectral zone and is due to the fluorophores causing the 430 nm emission band, Figure 3, 320 nm line. The third band, band III, is observed as a shoulder at about 400 nm, and, finally, band IV centered at ca. 450 nm is substantial only in the maize spectrum. This absorption band is the reason for the usual yellow color of maize flour and it is mostly due to the very high xanthophyll content of the maize here used, 4.3 parts per million (ppm) of lutein and 4.9 ppm of zeaxanthine. The reflectance spectra of maize flour *after* extraction of materials soluble in MeOH, including xanthophylls, showed an almost complete disappearance of the absorption band IV. We note that in rice and maize the intensity of band II is higher than with wheat so that the above-hypothesized trivial mechanism of reabsorption of the fluorescence light of aromatic amino acidic residues should be more effective with these cereals than with wheat. This might be the explanation for the relatively low intensity of the aromatic amino acid fluorescence in rice and maize in comparison to that of the flours under 275-nm excitation. The fluorescences of the four cereals are reported in Figure 5.

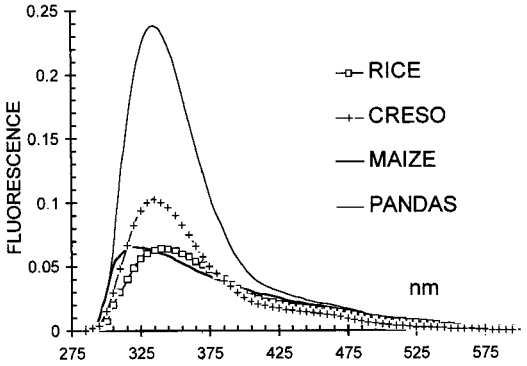

Figure 5. Fluorescence of maize, rice, durum wheat Creso, and soft wheat Pandas on 275-nm irradiation.

Species Recognition. The strong differences between the four species observed both in the emission, Figure 5, and in the reflectance spectra, Figure 4, appear sufficient for a clear recognition of the species there considered. In Table 1 the values of the intensity maxima of the fluorescence spectra of 11 cereal powders, under identical experimental conditions and excitation wavelengths of 275 and 325 nm, are reported. A quantitative index based on these intensity data appears to be a possible parameter to discriminate between cereal species or cultivars. The last column reports the *ratios* of the maximum fluorescence excited by 275-nm light and the maximum excited by 325-nm light, for each cereal. Notice the strong variations of the fluorescence maxima under 275-nm excitation, see also Figure 5, compared with the relatively minor variations

Table 1. Maximum Intensities of the Fluorescences under 275 and 325 nm Wavelength Excitation, *I*(275) and *I*(325), Respectively, and Their Ratio

cereal	cultivar	$I_{max}(275)$	$I_{max}(325)$	$I(275)/I(325)$
maize		0.0654	0.0611	1.07
rice		0.064	0.0302	2.12
durum wheat	Creso	0.105	0.0283	3.71
wheat	Alisei 1	0.148	0.0317	4.67
durum wheat	Lira	0.245	0.0501	4.91
wheat	Wru6983	0.157	0.0299	5.22
wheat	Nibt	0.149	0.0272	5.48
bread wheat	Mercia	0.152	0.0271	5.63
wheat	Alisei 2	0.222	0.0375	5.92
bread wheat	Pandas	0.238	0.0391	6.09
wheat	Wru6979	0.195	0.0264	7.39

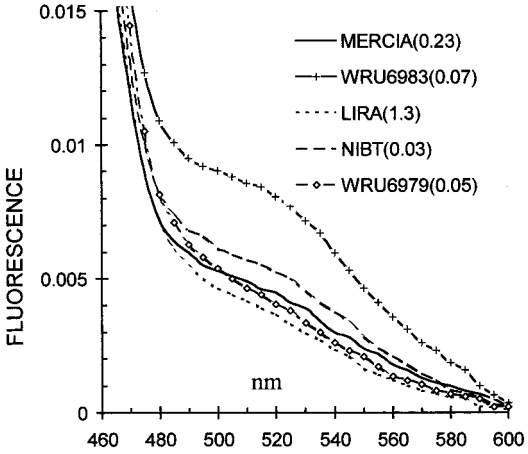

Figure 6. Fluorescence of five different wheat cultivars on 445-nm excitation. In the parenthesis are reported the value, in ppm, of the lutein present in each sample.

of the maxima under 325-nm irradiation. Because of these features we observe the wide range of values of the index reported in the last column.

Fluorescence of Flours under Vis Excitation. In Figure 6 the emissions in the visible spectral region of flours of five different wheats are shown with $\lambda_{exc} = 445$ nm. One emission band, centered at about 520 nm, is present in some of the samples examined, particularly in WRU6983. The correspondig excitation band, already seen as the absorption band IV, Figure 4, is located in the 430-nm zone i.e., exactly in the *emission* region of the 330-nm-absorbing fluorophore, and it should be due to a new, different, fluorophore. Thus, a mechanism of absorption of the fluorescence emitted in the violet and subsequently re-emission in the green is possible by this third, unknown, fluorophore. Three subsequent absorption−emission processes in which the photon energy diminishes by the Stokes losses are possible; this mechanism could *contribute* to the extension to the visible of the fluorescence excited by irradiation with 275-nm light of protein fluorophores, Figures 2, Creso line, 3, and 5. We have to take into consideration that the fluorescence "inner filter effects", typically present in concentrated solutions, could be strongly effective in our cereal powders.

The differences in the emissions, under vis excitation, appear useful to recognize the species. One of the causes of the differences observed in the vis fluorescences, on violet light excitation of flours, is indicated by the emission of two flours of *the same cultivar*, the durum wheat Cappelli, obtained from seeds of *very different age* (37 and 2 years, respectively). The spectra look quite different, in a manner very similar to that shown in Figure 6 between the different WRU cvs. Here the difference of the fluorescences seems connected to the difference in the concentrations of the carotenoids contained, mainly lutein, a yellow xanthophyll strongly absorbing violet light, $\epsilon_{max} = 135\,000\ M^{-1}\ cm^{-1}$ at 445 nm. In the flour from younger Cappelli seeds we found more than 3 ppm of lutein, while in the older one only 0.2 ppm of the above antioxidant is present (Pinzino et al., 1998). When the xanthophyll content in the younger seed flour is reduced by extraction with MeOH, the fluorescence intensity in the visible (due to a yet unknown fluorophore) increases appreciably. Clearly, in the younger seed a strong light absorber, xanthophyll, which is also a very weak emitter (Jorgensen et al., 1992), is present in concentration sufficient to successfully compete for the 445-nm excitation light with the unknown fluorophore. However, the WRU samples, both with negligible content of xanthophylls (about 0.06 ppm), show very different intensities of the 525 nm emission band, Figure 6. This, probably, implies a different content of the fluorophore that generates the band centered at 525 nm. The above dependence of the intensity of the fluorescence on the content of both the unknown fluorophore and the xanthophylls in flours indicates some caution is necessary in using visible fluorescence intensity. In fact, we proved that the xanthophyll content depends strongly on the aging conditions of seeds.

CONCLUSIONS

Complex phenomena of scattering, absorption, emission, and reabsorption of primary-emitted light occur during frontal-surface illumination of cereal flours. A quantitative analysis of these events is very difficult and probably requires the use of some approximate realistic models. At least three *emission* bands are present in the 290−600-nm spectral range. Four *absorption* bands centered at about 280 nm, band I, at 330 nm, band II, at 400 nm, band III, and at 450 nm, band IV, are revealed by reflectance spectra. The two most intense bands, i.e., those at 280 and 330 nm, and that at 450 nm are revealed also by excitation fluorescence spectra: therefore, these absorption bands correspond to definite fluorophores. The origin of band I (light absorption maximum 280 nm; emission maximum 335 nm) is essentially due to the aromatic fluorophores present in cereal proteins. Band II (maximum absorption at 330 nm, emission maximum at ca. 430 nm) is probably due to low-molecular-weight molecules such as tocopherols, pyridoxine, 4-aminobenzoic acid, etc. present in flours. Actual experiments do not allow us to correlate any emission to the low-intensity band III. Band IV (absorption in the 450-nm region, emission in the green spectral region) should be connected to the presence of both strong light absorbers as xanthophylls and some yet unknown fluorophores. Fluorescence spectra of cereal flours are strongly dependent on the species or, even, on the cultivar: a numerical index obtained from the maximum intensities of the fluorescence spectra caused by excitation of band I and band II could be used to this scope. Vis fluorescence, generated on excitation of band IV, could be useful in this respect. Information on the microenvironment of protein fluorophores can be obtained from excitation of band I.

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