Front-Surface Absorbance Spectra of Wheat Flour: Determination of Carotenoids

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Front-surface absorbance spectra of wheat flours in the 250−650 nm region can be obtained by measuring reflectance spectra with a conventional spectrofluorometer suitably set to detect light scattered from powder samples. The spectra recorded on flour samples, obtained from seeds of four bread and five durum wheats, show high-intensity absorption bands due to aromatic amino acids of wheat proteins and low-intensity bands due to chromophores bound to low-molecular-weight compounds. The intensity of these last bands is proportional to the concentration of the corresponding chromophores present in the flour; thus, it can be used to measure the content of the compounds containing the chromophore(s). In particular, a quantitative determination of the carotenoids actually present in the flours is made, obtaining information on the original content of the seeds. This determination is important, as, for example, xanthophylls are well-known antioxidants and free-radical scavengers involved in aging processes of seeds. Reflectance measurements on powder samples are far more economic in terms of time and materials consumption than methods such as extraction and HPLC analysis of extracts and, in addition, give an evaluation of the overall content of carotenoids with absorption bands in the spectral range 450−500 nm. Application of the technique to other food powders with low-intensity absorption bands in the near-UV and vis region is possible.

Keywords: *Reflectance; absorbance; front-surface spectroscopy; wheat flour; carotenoids*

INTRODUCTION

Reflectance measurements are acquiring increased importance in agricultural and food chemistry, as testified by papers recently appearing in the scientific literature. For instance, vis and near-IR reflectance measurements were carried out on fresh and dry leaves and on plant canopies, by either near or remote sensing techniques, and an estimation of the photosynthetic pigment content per unit area was achieved through spectral indices containing the ratios of reflectance intensities at selected wavelengths (Lichtenthaler et al., 1996; Blackburn, 1998a,b; Datt, 1998). Some authors report on the discriminant analysis of food ingredients (Reeves, 1998) by means of principal component analysis of mid-IR diffuse reflectance data measured on powdered food. In other studies, vis and near-IR reflectance on single wheat kernels was used for classification purposes and, in particular, to check the mixing of cultivars (cvs.) of different classes (Delwiche and Massie, 1996; Wang et al., 1999a−c). All these works indicate that reflectance data are full of information, even if, at present, problems connected to both reflectance measurement and information extraction are far from a general solution.

To the best of our knowledge no diffuse reflectance measurement on food powders in the vis−UV spectral region has been reported in the literature until now. Recently, a method for measuring fluorescence and light absorption of such powders through a reflectance technique was presented by one of the authors (Zandomeneghi, 1999). In that work, particular emphasis was on acquisition and interpretation of the *fluorescence* spectra of cereal flours, while in the present work we focus on the application of the technique to obtain *absorption* spectra of durum and bread wheat flours. A phenomenological approach is employed to extract analytical information from these spectra. In fact, a general theory to extract information from reflectance measurements is still lacking, since the absorption of light in a strongly diffusive environment, such as a powder, is a much more complex phenomenon than the absorption in nondiffusive media, such as the clear solutions in the usual transmission spectroscopy. The absorbance of low-intensity bands has been found to be proportional to the concentration of the corresponding chromophores, as in the usual transmission absorbance spectroscopy, hence rendering front-surface absorbance spectroscopy of food powders a useful technique to quantify their content of some important molecules.

In flours, absorption bands of carotenoids in the 430−530 nm range are well-resolved and suitable for evalu-

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ation by this technique. Carotenoids, which in the analyzed flours are almost exclusively xanthophylls, turn out to be particularly useful because of their importance as both antioxidants and natural dyes. In fact, these substances act as singlet oxygen quenchers and as quenchers of molecules in triplet excited states (Krinsky, 1994; Palozza and Krinsky, 1994). Among other mechanisms of action, carotenoids are able to transfer one electron to radicals giving stable carotenoidic radical cations and regenerating the original molecules (Mortensen and Skibsted, 1997). Moreover, a correlation between xanthophylls content and aging has been recently found in wheat seeds (Pinzino et al., 1999).

Thus, we calculated the relative xanthophyll contents from the front-surface absorbance spectra of the flours obtained from four bread and five durum wheats, all characterized by the fact that lutein in the free form (i.e., nonesterified form) is the dominant carotenoid. Lutein and other xanthophylls present in detectable amounts were also determined by means of HPLC analysis of exhaustive methanol extracts of flours (Pinzino et al., 1999) in order to test the reliability of the spectroscopic evaluations.

EXPERIMENTAL PROCEDURES

Materials. Seeds of durum wheat cvs. Grazia, Cirillo, Creso, Solex, and Colosseo and of bread wheat cvs. Pandas, Centauro, Idra, and Sagittario were provided by the Dipartimento di Agronomia of the Universita' di Pisa. The seeds of bread wheat cv. Pandas had been stored at room temperature since their harvest, in 1995, while all the other seeds were harvested in 1998 in Tuscan fields. The original very low content of esterified lutein was unaffected by storage and at the limit of detection with the high-performance liquid chromatography (HPLC) appropriate mobile phase (Kaneko et al., 1995).

Pure lutein and zeaxanthin solutions in MeOH were obtained by semipreparative HPLC from extracts of *Zea mais* flour. These solutions, used as HPLC standards or for artificial pigmentation of flour, were kept in the dark, under N_2, at −18 °C. Their purity was checked by HPLC before using, since these xanthophylls are thermally and photochemically labile compounds. Solvents used were of Carlo Erba HPLC quality.

Apparatus. A Labormill 4RB, Italy, break roller mill was used to obtain flours from wheat seeds. For vis−UV transmission spectrophotometry a Cary 219 instrument was used.

HPLC analyses and separations were performed on a Jasco 880 Pu pump equipped with a spectrophotometric Shimadzu SPD 10A detector. For analyses, a RP S5 ODS2 Spherisorb column (4.6 × 250 mm), with an RP-C18 precolumn (10 × 4.6 mm) and a 5 μm filter, was employed. A Spherisorb S5 ODS2 column (250 × 10 mm) was used in semipreparative separations.

A Jasco FP770 spectrofluorometer was used in the front-surface fluorescence and reflectance experiments. The samples, about 0.6 mm thick, were enclosed in a homemade cell, suitably designed to avoid specular reflections in measurements on powders (Zandomeneghi, 1999). The cell windows were 30° tilted with respect to the incident beam. Powdered Na_2SO_4 was used as a reference light scatterer (Wrighton et al., 1974; Hapke, 1993).

Front-Surface Spectroscopy. Reflectance measurements require scanning synchronous spectra with identical wavelength for both excitation and emission monochromators. Synchronous spectra were recorded setting the spectral bandwidths to 1.5 and 5 nm for the excitation and emission monochromators, respectively, and setting the photomultiplier voltage to the lowest available value. Such spectral bandwidths were chosen in order to have an acceptable resolution, together with a low dependence of the signal on residual asynchronism between the monochromators.

Since spectrofluorometers are capable of detecting and quantifying very faint fluorescence, the intensity of light coming from the highly diffusive powders used in this study was, as expected, much higher than the maximum intensity measurable by the Jasco FP770 machine. To reduce this intensity, a neutral density filter with absorbance $A = 2$ was placed in the optical train of the incoming excitation beam. This filter was used in the spectral region between 250 and 650 nm. Its absorbance exceeds the nominal value on approaching 250 nm, just where the emission intensity of the Xe lamp sharply decreases (vide infra).

To obtain emission or excitation spectra of the same powder, it is sufficient to take off the filter from the sample compartment and set the instrumental parameters for these scanning modes.

Carotenoid Extraction From/Addition to Flour. For the extraction of carotenoids from flour, 4 mL of MeOH was added to ca. 300 mg of fresh flour. After stirring for 2 h, the mixture was centrifuged in a bench centrifuge to separate the supernatant. The extraction was repeated three times and the overall liquid phase was reduced to 3 mL by evaporation under a N_2 flow. We used the same extraction procedure that proved to be accurate for HPLC analysis of xanthophylls in flours by Pinzino and co-workers (1999).

In experiments in which lutein was added to flour, 200 μL of MeOH solution, containing an amount of pigment measured by UV spectrophotometry or HPLC analysis, was added to ca. 200 mg of flour and left to stand for ca. 12 h at room temperature, in the dark under N_2 atmosphere. Then methanol was allowed to evaporate slowly during manual mixing.

Analysis of Carotenoids in Extracts. The determination of the content of lutein, and other carotenoids with similar retention times in the methanol extracts, was made by isocratic RP-HPLC at room temperature using a flow rate of 0.5 mL/min and MeOH as mobile phase. Lutein peaks at 445 nm were reproducible within 2% when 20 μL of the methanol extracts was injected (five injections). The lutein content was calculated by comparing these elongations with those of a standard solution of pure lutein in methanol, the concentration of which was determined by measuring the absorbance at 445 nm ($\epsilon_{445} = 133\,000\ M^{-1}\ cm^{-1}$).

RESULTS AND DISCUSSION

Physical Background. To obtain a front-surface reflectance spectrum, the synchronous spectra of both the flour sample and a reference diffuser (a nonabsorbing light scatterer) have to be recorded. In fact, the reflectance, $R(\lambda)$, of the flour relative to a chosen reference light scatterer is defined (Hapke, 1993) as the ratio between the intensities of the two spectra at the same wavelength (λ):

$$R(\lambda) = I_{\text{sample}}(\lambda)/I_{\text{scatter}}(\lambda)$$

Clearly, in spectral regions where none of the flour chromophores absorb light, the light diffused by the flour sample (the "reflected light") will have an intensity equal to that of the scatterer; otherwise the intensity of light diffused by the flour will be lower to an extent depending on both the concentration of chromophores and their absorption features. The first case occurs for all the flours examined between 550 and 600 nm, where reflectance is equal to 1. At $\lambda < 550$ nm reflectance becomes considerably lower than 1.

To quantify the chromophore absorption, it turned out to be useful to define the quantity:

$$A(\lambda) = -\log(I_{\text{sample}}(\lambda)/I_{scatter}(\lambda)) = -\log(R(\lambda)) \quad (1)$$

We call this quantity "reflection absorbance" (the "pseudo-absorbance" of Blackburn, 1998b) because its definition

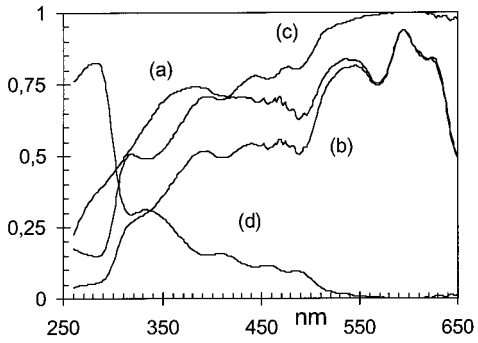

Figure 1. (a) Front-surface synchronous spectrum of a Na_2SO_4 powder sample; (b) front-surface synchronous spectrum of a flour sample (durum wheat cv. Solex); (c) front-surface reflectance spectrum of the flour sample relative to the Na_2SO_4 powder sample; (d) reflection absorbance spectrum of the flour (eq 1).

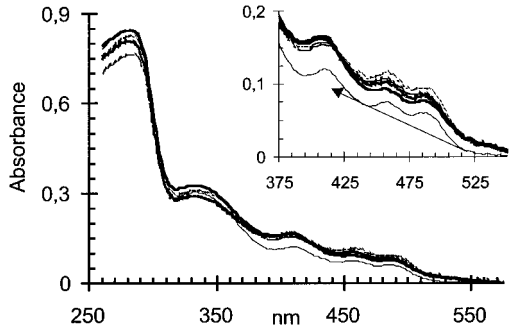

Figure 2. Reflection absorbance spectra of flours obtained from seeds of durum wheat cvs. Grazia (dashed line), Cirillo (solid line), Creso (solid bold line), Solex (dotted line), and Colosseo (dotted−dashed bold line). In the insert the procedure to evaluate the carotenoid absorbance is shown for the Cirillo flour.

is similar to that of the absorbance in the more common transmittance spectrophotometry. However, it must be emphasized that in the front-surface reflectance measurements the light path length is not clearly defined as in transmittance spectroscopy where this length is generally coincident with the distance between the windows of the sample cell. In fact, in the case of powders, each photon passes through an extremely heterogeneous sample and its path length depends on the scattering processes it encounters; thus, only an average path length can be defined, which, unfortunately, cannot be measured or calculated in a simple way. For highly absorbing powders, the photons entering into a grain are absorbed and only those reflected or diffracted at the grain surface can contribute to $I_{sample}(\lambda)$ in eq 1. Clearly, the average path length of detected photons is different from that due to the scattering of nonabsorbing powders. In addition, absorption of light is not as complete as in transmission spectrophotometry, so that all strongly absorbing bands tend to have similar values (see Figures 1−5). In this extreme situation, quantitative information hardly can be extracted from front-surface absorbance spectra. On the contrary, these spectra should give useful analytical results for samples showing low absorption. In this case, the path of photons is essentially determined by scattering processes, the absorption processes being a minor phenomenon, so that in eq 1 a comparison is made between two light beams with the same average path length and a correlation of absorbance with concentration is expected, similar to the Lambert & Beer law. Thus, at low values of *A*, some

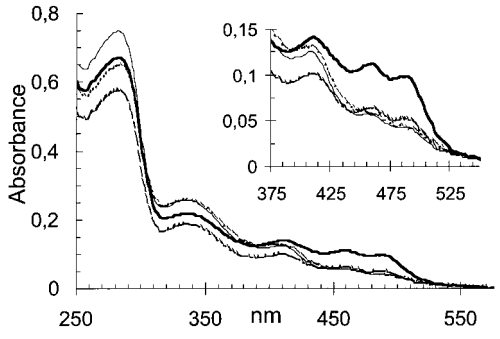

Figure 3. Reflection absorbance spectra of flours obtained from seeds of bread wheat cvs. Pandas (dotted line), Centauro (dashed line), Idra (solid bold line), and Sagittario (solid line).

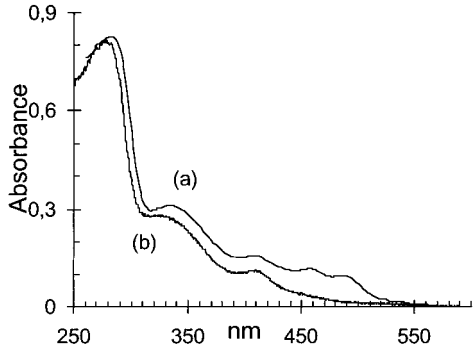

Figure 4. Reflection absorbance spectra of flour from seeds of durum wheat cv. Solex: (a) before and (b) after exhaustive extraction with methanol.

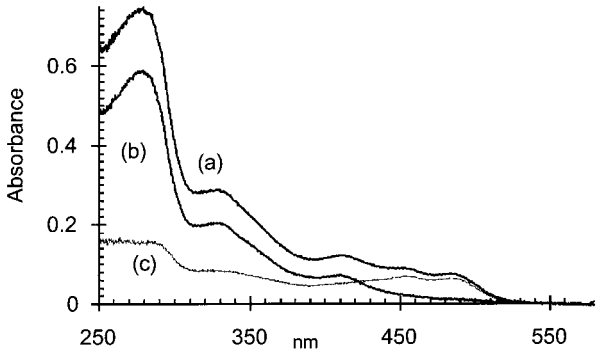

Figure 5. Reflection absorbance spectra of flour from seeds of bread wheat cv. Pandas depigmented by extraction with methanol (b) and repigmented by addition of 7.8 ppm of lutein (a). Curve c represents the difference between the two spectra and coincides with curve d of Figure 6.

of the useful properties of classical absorbance should be retained in the reflection absorbance. In particular, it should be an additive property with respect to chromophore contributions and ultimately proportional to the concentration of the molecules bearing the chromophores.

Flour Spectra. The front-surface absorbance spectrum of a flour sample obtained from seeds of durum wheat cv. Solex is shown in Figure 1 curve d, together with the synchronous spectrum of a Na_2SO_4 reference sample (curve a), the synchronous spectrum of the flour sample itself (curve b), and the relative reflectance spectrum (curve c). As described in the previous section, the spectra a−c represent intermediate steps in the procedure employed to obtain the reflection absorbance spectrum d.

Some observations can be made on these spectra. First of all, the intensity of light reflected by the flour

is almost identical to that of the reference scatterer in the 550−600 nm spectral region (see curves a and b) where, evidently, no chromophore is able to absorb light; correspondingly, reflectance R approaches 1 (curve c). It must be emphasized that the *absolute* intensities of light scattered by two samples of the same material might be slightly different even if they remain strictly proportional. Thus, the overlap of the synchronous spectra of the flour and the scatterer at $\lambda > 550$ nm can always be achieved by a simple normalization of experimental intensities. Curves a and b reflect, at each wavelength, the optical properties of the sample, the emission intensity of the Xe lamp, the efficiency of the optics of the monochromators, the characteristics of the photomultipliers, and the effect of the added neutral filter. Notice the spikes typical of the Xe lamp discharge in the 450−480 nm wavelength range, which survive notwithstanding the voltage feedback at the emission photomultiplier. All the synchronous spectra should present these spikes at identical wavelengths so that they can be used as a reference for the wavelength axis. In particular, the error in the λ repeatability of the monochromators, ca. 0.5 nm, present in curves a and b, can be minimized by shifting one spectrum with respect to the other till the optimum overlap of the spikes is obtained. Thus, optimum reflectance and absorbance spectra can be achieved.

When λ approaches 250 nm, the intensity values decrease (i.e., they are about 1 order of magnitude lower than those recorded at 400 nm), particularly for the flour sample. The lowering of both the lamp brilliance and the transmission of the filter contributes to this result, as described in the Experimental Procedures. This means that a decrease in precision is to be expected in the middle-UV region. We evaluate it as the loss of a significant figure in the reflectance and absorbance values.

The differences in intensity between curves a and b for $250 < \lambda < 550$ nm reflect the absorption of light by the chromophores acting in this spectral range and result in the four bands (Zandomeneghi, 1999) present in the reflection absorbance spectrum (curve d of Figure 1). These bands are observed in the absorbance spectra of all nine flour samples obtained from seeds of both durum (Figure 2) and bread (Figure 3) wheats investigated in this work. The different intensities are dependent on the species and cultivar of wheat.

Two of these bands (280 and 485 nm) can be assigned to well-defined chromophores. The band centered at 280 nm shows high intensity in all spectra: the absorbance maximum ranging from 0.60 to 0.85; in other words, at least 15% of the radiation diffused by a pure scatterer survives to the strong light absorption of the aromatic amino acids of flour proteins to which light absorption is here essentially due (Figures 2 and 3). This physically relevant finding was confirmed by the reflection absorbance of a pure crystalline tryptophan sample and of a mixture of tryptophan, 1% by wt, and Na_2SO_4. In the first case the maximum absorbance value in the 280−290 nm spectral range resulted close to 1, while in the second the maximum absorbance value was about 0.5 showing that no proportionality of absorbance with chromophore concentration holds for this band. Hardly reliable quantitative information can be achieved from such an absorption band.

On the contrary, useful analytical results can be obtained analyzing the low-intensity band occurring in the spectral region 430−530 nm: this band can be attributed to the absorption of flour carotenoids on the basis of two complementary experiments. In the first experiment, a flour sample, obtained from seeds of durum wheat cv. Solex, was submitted to exhaustive extraction with methanol, a method to extract xanthophylls from flours (Pinzino et al., 1999). In Figure 4 we report the absorbance spectra of the sample before extraction (curve a) and after extraction (curve b). The complete disappearance of the absorption band at 430−530 nm is evident in curve b, together with the reduction of the intensity of the other bands. In the second experiment, a flour sample, bread wheat cv. Pandas, previously depigmented by means of methanol extraction, was repigmented by two subsequent additions of pure lutein: the first of 3.4 ppm and the second of 4.4 ppm. The absorption spectra before (curve b) and after (curve a) lutein addition (7.8 ppm) are reported in Figure 5, together with their difference (curve c). Notably, the absorbance spectrum of the flour sample added with lutein resembles the spectra of untreated flours shown in Figures 2 and 3.

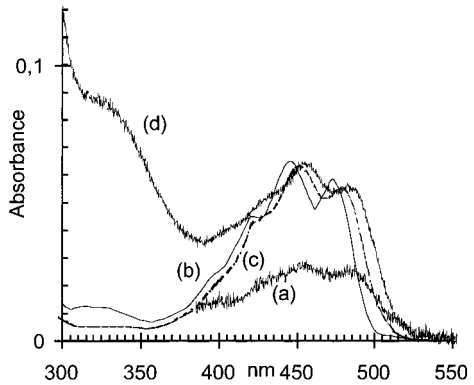

Figure 6. Reflection absorbance spectra (300−550 nm) of lutein added to depigmented Pandas flour (eq 2): (a) 3.4 ppm and (d) 7.8 ppm. Curves b and c represent the spectra of methanol solutions of lutein and zeaxanthin, respectively.

A different kind of spectrum, amenable to the absorbance of the lutein added to the depigmented flour, can be calculated as

$$A(\lambda) = -\log(I_{\text{added}}(\lambda)/I_{\text{extracted}}(\lambda)) \qquad (2)$$

by using the reflectance of the repigmented flour relative to the depigmented one. The resulting spectra, corresponding to 3.4 and 7.8 ppm of added lutein, are reported in Figure 6, curves a and d, respectively. We observe that they resemble the absorbance spectrum of a methanol solution of lutein (Figure 6, curve b), the differences reasonably being due to the drastically different environments of the chromophore in solution and in the solid state.

It is important to observe that the intensity of the band between 430 and 530 nm is proportional to the lutein content of the flour within the experimental error, here less than 5%. From these results, we calculated that 137 ppm of lutein corresponds to 1 absorbance unit.

It is known that, *in solution*, the absorption spectra of many important carotenoids contained in cereal flours show bands with similar position, shape, and intensity in the visible spectral region; as an example, the spectra of lutein (curve b) and zeaxanthin (curve c) are reported in Figure 6. On this basis we expect most carotenoids to show similar absorption bands also when dispersed

Table 1. Xanthophyll Content (ppm) and Reflection Absorbance of Flours at 485 nm Evaluated by Means of the Procedure Represented in Figure 2[a]

wheat	lutein from HPLC	carotenoids from HPLC	reflctn absorb	calcd carotenoids from absorb
durum wheat cv. Solex	2.75	3.35	0.0358	3.35[a]
durum wheat cv. Grazia	2.24	2.71	0.0309	2.82
durum wheat cv. Colosseo	2.12	2.58	0.0274	2.45
durum wheat cv. Cirillo	2.03	2.43	0.0237	2.36
durum wheat cv. Creso	1.69	2.12	0.0218	2.09
bread wheat cv. Idra	1.90	2.23	0.0378	2.23[b]
bread wheat cv. Centauro	0.87	1.05	0.0176	1.04
bread wheat cv. Sagittario	0.61	0.77	0.0108	0.64
bread wheat cv. Pandas	0.09	0.13	0.0040	0.23

[a] For the linear relationship between HPLC lutein concentration and reflection absorbance, correlation coefficients (R) equal to 0.94 and 0.99 are found for durum and bread cultivars, respectively. These coefficients are 0.95 and 0.99 when carotenoids are considered instead of the sole lutein. The a and b values have been taken equal to the corresponding overall xanthophyll contents in the third column.

in solid particles as in flour samples, so that the band in the 430−530 nm spectral region is representative of the whole class of compounds. Thus, the possibility arises to measure the content of carotenoids in flours using the described front-surface spectroscopic technique.

To this aim, a method is needed to extract quantitative information from reflection absorbance spectra. The first problem is to evaluate the background absorption due to the tails of bands of chromophores different from carotenoids: this background absorbance has to be subtracted from the measured absorbance in the spectral region where xanthophylls absorb. Comparing the absorbance spectra of depigmented and pigmented flours (see Figures 4 and 5), we can see that all curves tend to overlap at $\lambda \geq 530$ nm. On the basis of these observations, the absorbance values measured at $\lambda \geq 530$ nm can be used to extrapolate the baseline of the carotenoid absorption band. Clearly this baseline will be more correct as the wavelength is closer and closer to 530 nm, so that it is convenient to calculate the absorbance due to carotenoids in correspondence of the maximum nearest to 530 nm, i.e., the maximum at ca. 485 nm. Different extrapolation procedures can be adopted; however, all of them are affected by not easily estimable errors. We have chosen the simple graphic approach shown in the insert of Figure 2 for a flour sample of durum wheat cv. Cirillo. The absorbance values, measured at 485 nm applying this procedure, are reported in Table 1.

To obtain quantitative information on the carotenoid content of the samples from spectral data, we proceeded to determine the concentration of some xanthophylls contained in the flours by HPLC analysis (Pinzino et al., 1999). Besides lutein, which generally is the most relevant xanthophyll in wheat, four other xanthophylls were found, with retention times close to that of lutein. Among them, we could identify zeaxanthin (with retention time 1 min longer than that of lutein) and isolutein; the assignment of the remaining two pigments could not be made on the basis of the sole retention times and absorption spectra (Roussef et al., 1996). The contents (in ppm) of lutein and lutein plus these carotenoids determined for the nine samples HPLC-analyzed are reported in Table 1. It can be seen that absorbance values, at 485 nm, are proportional to the lutein concentrations within each set of durum and bread flours. This linear relationship is even better when absorbance values are related with the overall xanthophylls contents (Table 1).

On the basis of these results, we conclude that the *relative* content of carotenoids of flours obtained from wheats of the same class can be evaluated by means of reflection absorbance measurements. In the last column of Table 1 we report the carotenoid contents of the examined flours calculated from their absorbance values, assuming that the highest absorbance measured within each class corresponds to the carotenoid content measured by HPLC. Thus, once a conversion factor between absorbance and carotenoid content is established, absolute quantitative determinations of carotenoids can be done for the other flours from wheats of the same class.

In our experiments we found that 1 absorbance unit corresponds to 94.0 ± 2.3 and 59.6 ± 2.2 ppm of carotenoids in durum and bread wheats, respectively. It must be observed that both these values are lower than the 137 ppm value determined from the absorbance measurements on depigmented flour added with lutein (vide supra). This result underlines that the *absolute* quantity of carotenoids is not immediately determinable from front-surface spectra.

To shed light on the physical reasons of this finding, the following experiments were performed: 176 mg of flour, from seeds of bread wheat cv. Idra, was exhaustively extracted with methanol. The xanthophylls content of the extract was measured by HPLC. Then the solution, reduced in volume to 150 μL by evaporation under a N_2 flow, was added to the previously extracted flour. The front-surface absorbance at the 485 nm maximum, measured in identical experimental conditions, was 0.090 for the untreated Idra flour and 0.051 for the repigmented flour, respectively. Then, the repigmented flour was submitted to a new exhaustive extraction with MeOH. By means of HPLC analysis approximately the same xanthophyll content was found in the repigmented flour and in the original flour (for instance, 7.4% of lutein was lost in the overall workup operations). Thus we have to admit that different reflection absorbance values in the spectra of repigmented and original flour correspond to the same quantity of pigments, the absorbance of repigmented flour being 43% reduced with respect to the original one.

This difference can be qualitatively explained by the decrease in size of flour grains observed after extraction with MeOH, since grains with different sizes give different interactions with light. In fact, in large grains, photons have a long internal path during which they are absorbed according to the Lambert−Beer law, while diffusion events originate only at the surface and at the internal imperfection sites. For smaller grains more surface reflections correspond to an equal internal path length; hence a higher proportion of diffused light and a lower absorbance are expected. This means that grains with equal surface-to-volume ratios are needed in order to have the same average path length of light for different flours. When this condition holds, a correct comparison between the front-face absorbance of different samples can be made and reliable information on the carotenoid content is obtained. Evidently, in our experiment the average path length of photons in the repigmented flour is shorter than in the original flour and this causes the lowering of absorbance.

CONCLUSIONS

We have shown that front-surface reflectance in the UV−vis spectral region of wheat flour is easily measured on a commercial spectrofluorometer by recording synchronous spectra with zero-lag between the excitation and emission wavelengths. Technical details were given to overcome difficulties in obtaining precise reflectance data. A phenomenological approach was adopted to deduce reflection absorbance spectra of flours from reflectance spectra relative to a reference scatterer. In fact, *reflection* absorbance is a much more complex function than the usual *transmission* absorbance, because it depends also on variables typical of light diffusion, in particular on grain size. Thus, as we have shown, a similar distribution of grain size is required for a correct comparison among absorption spectra of different cultivars or species.

In reflection absorbance spectra low-intensity bands are proportional to chromophore concentration thus allowing quantitative determination of the compounds bearing chromophores. The applicability of this novel technique to the measurement of xanthophylls in wheat flours was shown. In particular, it was demonstrated that the relative concentrations of carotenoids within each class of durum and bread flours could be quickly obtained from absorbance at 485 nm, while for an absolute concentration determination a normalization procedure is required.

In any case, diffuse reflectance spectroscopy allows the measurement of the overall content of important pigments in flours, such as carotenoids, while HPLC analysis of flour extracts allows the measurement of single pigments. It is important to remark that the spectroscopic approach is far simpler and less time-consuming and requires less materials than the chromatographic one. In addition, problems connected to the extraction and manipulation of labile compounds such as carotenoids are avoided.

Finally, since xanthophyll content in flours was found to be correlated to the aging of wheat seeds (Pinzino et al., 1999), we believe that this technique could represent a way to easily and quickly evaluate seed preservation. Moreover, carotenoid determination can be similarly performed in substrates derived from wheat such as pasta: this determination could be important in measuring the color of these materials, a factor contributing to their commercial value.

LITERATURE CITED

Blackburn, G. A. Spectral indices for estimating photosynthetic pigment concentrations; a test using senescent tree leaves. *Inte. J. Remote Sens.* **1998a**, *19*, 657−675.

Blackburn, G. A. Quantifying chlorophylls and carotenoids at leaf and canopy scales: an evaluation of some hyperspectral approaches. *Remote Sens. Environ.* **1998b**, *66*, 273−285.

Datt, B. Remote sensing of chlorophyll a, chlorophyll b, chlorophyll a+b, and total carotenoid content in eucalyptus leaves. *Remote Sens. Environ.* **1998**, *66*, 111−121.

Delwiche, S. R.; Massie, D. R. Classification of wheat by visible and near-infrared reflectance from single kernels. *Cereal Chem.* **1996**, *73*, 399−405.

Hapke, B. In *Theory of Reflectance and Emittance Spectroscopy*; Arvidson, R. E., Rycroft, M. J., Eds.; Cambridge University Press: Cambridge, U.K., 1993; Chapters 10−11.

Kaneko, S.; Nagamine, T.; Yamada, T. Esterification of endosperm lutein with fatty acids during storage of wheat seeds. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 1−4.

Krinsky, N. I. The biological properties of carotenoids. *Pure Appl. Chem.* **1994**, *66*, 1003−1010.

Lichtenthaler, H. K.; Gitelson, A.; Lang, M. Nondestructive determination of chlorophyll content of leaves of a green and an aurea mutant of tobacco by reflectance measurements. *J. Plant Phys.* **1996**, *148*, 483−493.

Mortensen, A.; Skibsted, L. H. Importance of carotenoid structure in radical-scavenging. *J. Agric. Food Chem.* **1997**, *45*, 2970−2977.

Palozza, P.; Krinsky, N. I. Antioxidant properties of carotenoids. In *Retinoids: From Basic Science to Chemical Applications*; Livrea, M. A., Vidali, G., Eds.; Birkhäuser Verlag: Basel, Switzerland, 1994.

Pinzino, C.; Capocchi, A.; Galleschi, L.; Saviozzi, F.; Nanni, B.; Zandomeneghi, M. Aging, Free Radicals, and Antioxidants in Wheat Seeds. *J. Agric. Food Chem.* **1999**, *47*, 1333−1339.

Reeves, J. B.; Zapf, C. M. Mid-infrared diffuse reflectance spectroscopy for discriminant analysis of food ingredients. *J. Agric. Food Chem.* **1998**, *46*, 3614−3622.

Rouseff, R.; Raley, L.; Hofsommer, H.-J. Application of Diode Array detection with a C-30 Reversed Phase Column for the Separation and Identification of Saponified Orange Juice Carotenoids. *J. Agric. Food Chem.* **1996**, *44*, 2176−2181.

Wang, D.; Dowell, F. E.; Lacey, R. E. Single wheat kernel classification by using near-infrared reflectance spectra. *Cereal Chem.* **1999a**, *76*, 30−33.

Wang, D.; Dowell, F. E.; Lacey, R. E. Single wheat kernel size effects on near-infrared reflectance spectra and color classification. *Cereal Chem.* **1999b**, *76*, 34−37.

Wang, D.; Dowell, F. E.; Lacey, R. E. Single wheat kernel color classification using neuronal networks. *Trans. ASAE* **1999c**, *42*, 233−240.

Wrighton, M. S.; Ginley, D. S.; Morse, D. L. A technique for the determination of absolute emission quantum yields of powdered samples. *J. Phys. Chem.* **1974**, *78*, 2229−2233.

Zandomeneghi, M. Fluorescence of Cereal Flours. *J. Agric. Food Chem.* **1999**, *47*, 878−882.

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