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Application of Near-Infrared Reflectance Spectroscopy (NIRS) to the Evaluation of Carotenoids Content in Maize

ORESTE V. BRENNA*,† AND NICOLA BERARDO§

Department of Food Science and Microbiology, University of Milan, Milan, Italy, and Istituto Sperimentale per la Cerealicoltura, Bergamo, Italy

The importance of including antioxidant compounds in the diet is well recognized. These compounds remediate the detrimental activity on animal cells of the so-called reactive oxygen substances (ROS). Many papers have reported on the determination of both hydrophilic and hydrophobic antioxidant compounds present in a large number of vegetables, and all methods involve the extraction from the matrix of the compounds to be determined. Because some problems may arise, such as the completeness of the extraction and the stability of the extracted compound during the extraction steps, the possibility of analyzing these compounds in the native matrix would be useful. Here is reported the application of near-infrared spectroscopy (NIRS) to the determination of the content of carotenoids in maize, comparing the obtained data with those derived from high-performance liquid chromatography (HPLC) determination of the extract obtained from the same samples. Equations for predicting carotenoid content in maize were derived using scores from modified partial least-squares (MPLS) as independent variables. Cross-validation procedures indicated good correlations between HPLC values and NIRS estimates. The results show that NIRS, a well-established and widely applied technique, can be applied to determine the maize carotenoids and that samples are readily analyzed in minutes, the only required step being their grinding.

KEYWORDS: Carotenoids; maize; NIRS; nondestructive method

INTRODUCTION

Carotenoids are chemical compounds widely present in both vegetables and animals. All of these pigments are based on a tetraterpenic skeleton, which may be cyclized at one or both ends. When only C and H atoms are present, as in α-carotene, β-carotene, and lycopene, they are named carotenes. When oxygen is also present, they are named xanthophylls; in particular lutein, zeaxanthin, and cryptoxanthin have a hydroxyl group, cantaxanthin a ketonic group, and violaxanthin an epoxidic one. A further distinction can be made between acyclic or bicyclic carotenoids, such as lycopene and β-carotene, respectively. Some of them, such as lutein, are present in a large variety of vegetables, whereas others, such as lycopene and zeaxanthin, are mainly present in tomatoes and maize, respectively (*1*).

Animals cannot synthesize carotenoids (in vivo, α-carotene, β-carotene, and β-cryptoxanthin are transformed in vitamin A), so that they must eat vegetables. They are an essential intake, as many degenerative diseases, such as atherosclerosis, catharat, diabetes, cancer, cardiovascular problems, and other health problems related to aging, are thought to be originated by some reactive oxygen subtance (ROS) (*2*).

Due to their chemical structures, carotenoids are recognized to act as radical scavengers (*3*) and oxygen quenchers, so they are able to react with ROS and protect the organism from their attacks. Lutein and zeaxanthin, the main carotenoids present in maize, possess a specific protective function on the retinal membrane, in particular, versus the yellow spot (macula lutea), in which they are the predominant pigments, and to which is due its characteristic yellow-orange (latin: luteus) color. They can reduce the effects of diffused light and photo-oxidative damage, due to their ability to absorb blue light (*4*). Furthermore, the retina is particularly susceptible to the ROS, being in direct contact with light and air and containing many polyunsaturated fatty acids, which act as ROS substrates.

HPLC methods useful to determine carotenoids in different foods (*5*−*10*) were reported in recent years, and most of the techniques used included a diode array detector (DAD). All of these methods required the previous extraction of the analyte from the matrix, so many difficulties may arise regarding their stability over the whole procedure. In fact, carotenoids and xanthophylls are very sensitive to heat and acids, which may cause trans−cis isomerization and structural changes, these problems being strengthened by light and/or oxygen.

To analyze carotenoids present in foods, a method must include some essential parameters, such as a rapid extraction

* Address correspondence to this author at the Department of Food Science and Microbiology, University of Milan, Via Celoria 2, 20133 Milan, Italy (telephone +39-02-503-16633; fax +39-02-503-16632; e-mail Oreste.Brenna@unimi.it).
† University of Milan.
§ Istituto Sperimentale per la Cerealicoltura.

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procedure, the presence of an antioxidant such as butylhydroxytoluene (BHT), the use of amber glassware and of volatile solvents, and a final storage at −20 °C under nitrogen of the extracts.

Thus, a nondestructive method could greatly simplify the analysis of such compounds. Near-infrared spectroscopy (NIRS) (*11*) may be this technique.

Here we report on the possibility of applying NIRS to the determination of carotenoids in maize, by comparing the data obtained using an HPLC method (*10*) with those obtained with NIRS. This technique is also widely used to control milk and dairy products (*12*) and for determining proteins, lipid, and humidity in meat products (*13*) and has been applied to evaluate carotenoids in durum wheat (*14*) and other minor components in some vegetables (*15*). Recently, a new method to detect carotenoids in wheat flour, based on reflectance spectra collected by a conventional spectrofluorometer, has been reported (*16*),

MATERIALS AND METHODS

Chemicals and Reagents. Crystalline carotenoids used as standards were as follows: α-carotene, β-carotene, lycopene, lutein, and astaxanthin (Sigma-Aldrich srl, Milan, Italy); β-cryptoxanthin and zeaxanthin (Extrasynthese, Genay Cedex, France); and canthaxanthn, a gift from F. Hoffmann-La Roche (Basel, Switzerland). The concentrations of stock standard solutions were determined spectrophotometrically using a DU 650 Beckman spectrophotometer (Beckman Instruments Inc., Fullerton, CA). All standard solutions were stored under nitrogen in the dark at −20 °C and dissolved in the mobile phase to give individual working standards in the range of 0.1−10 μg mL^{-1} immediately prior to analysis. All solvents were of HPLC grade (Merck, Darmstadt, Germany).

Samples. One hundred and twenty samples from 61 different maize varieties grown at the experimental fields of the Istituto Sperimentale per la Cerealicoltura (Bergamo, Italy), were analyzed. Two sets of samples, composed by different hybrids and varieties, were considered. The larger set (80 samples) was used to calibrate and to cross-validate the equation derived. The second set (40 samples) was used to test the goodness of fit of the developed equations. All samples were analyzed in duplicate. For each variety the harvested grains varied in color from almost white to dark yellow. Seed samples for each entry were dried in an oven until constant weight. The samples were milled using a Cyclotec mill with a 1 mm sieve and stored in polypropylene bottles in the dark at room temperature. Meals obtained from each sample was utilized both for NIRS analysis and chemical wet analysis.

Extraction Procedure. Ground maize samples were extracted in duplicate as in Buratti et al. (*10*), with some important modifications due to the rather high lipid content of the whole maize. Briefly, 5 g of ground maize, brought to a constant humidity at 40 °C in a desiccator, were suspended in 3 mL of methanol; internal standard (canthaxanthin) was added, and the mixture was extracted in the dark with ∼50 mL of unstabilized tetrahydrofuran (THF). The sample was homogenized by using an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at a moderate speed for few minutes, keeping the sample refrigerated in an ice bath to avoid overheating and potential carotenoid damage. Following a 10 min centrifugation at 10000*g*, the supernatant was collected and the residue extracted again, until a white solid resulted. The pooled THF extracts were partitioned in petroleum ether, and the ether extracts were kept refrigerated in an ice/solid NaCl mixture with stirring for 15 min to solidify the contained lipids. The ether was transferred in another amber flask, and the solid lipid was re-extracted with petroleum ether by brief sonication of the melted lipid mass. The freezing−sonication step was repeated at least a third time, or further, until an $A_{450nm} < 0.01$ resulted for the ether supernatant. All solvents used included 0.1% BHT. The ethereal extracts were concentrated under a nitrogen steam, transferred into 5 mL glass tubes, and finally dried under nitrogen flow and stored at −20 °C in the dark.

Chemical Analysis. Standard solution of carotenoids were prepared by dissolving the required amount in petroleum ether (α- and β-carotene), ethanol (lutein and zeaxanthin), or benzene [the internal standard (canthaxanthin)]. To obtain the calibration curves, the mother solutions were diluted with a mixture of methanol/*tert*-butyl methyl ether (TBME)/water (70:24:6), containing 0.1% BHT. A linear relationship was obtained for all of the tested compounds, with an $R^2 > 0.998$ in the range of 1−10 mg/L and detection limits ranging from 0.05 to 0.2 mg/L.

HPLC Analysis. Carotenoids present in all samples were evaluated according to the method described by Buratti et al. (*10*), with minor modification. The HPLC equipment consisted of a 996 photodiode array detector (Waters, Milford, MA) and a 600E multisolvent delivery system (Waters) equipped with a 20 μL loop. The chromatographic conditions were as follows: the column was a YMC-Pack column (4.6 × 250 mm, YMC Inc., Wilmington, NC) protected by a Waters Nova-Pak C18 guard column and maintained at 20 °C using a Waters column heater. Mobile phase A was a solution of methanol/water (92:8) and mobile phase B a solution of TBME/methanol (96:4). The flow rate was set at 1 mL min^{-1}; the gradient was run by increasing linearly the percentage of B from 15.5 to 55% in 45 min and then to 77.5% in the following 10 min. This value was kept for 10 min, then dropped to 0% in the following 10 min, and maintained there for 10 min; finally, B was brought again in 5 min to the initial value. Peak responses for carotenoids were measured at 450 nm. A photodiode array detector supported by the Millenium[32] chromatography manager computing system (Waters) was used to assess or confirm the spectral identity of carotenoids, registering the spectra in the range between 250 and 600 nm. Recoveries, checked by using canthaxanthin as the internal standard, ranged between 91 and 96%.

NIRS Analysis. Approximately 4 g of each corn meal sample was packed into a black aluminum sample cup containing a rectangular quartz window and a paper/polystyrene pressure pad backing. Samples were irradiated with near-infrared monochromatic light and the diffuse reflectance collected with lead sulfide detectors in a FOSS NIRSystems 6500 scanning monochromator (NIRSystems Inc., Silver Spring, MD). All spectral data were recorded in duplicate as log R^{-1} (R = reflectance) in the wavelength range 400−2500 nm at every 2 nm to give a total of 1050 data points per sample, and these were stored on an IBM-compatible PC. The software for scanning, mathematical processing, and statistical analysis was supplied with the spectrophotometer by Infrasoft International (ISI, Port Matilda, PA).

Data Processing. Equations for NIRS prediction were developed using the algorithm "CALIBRATE" (version 4.2, Infrasoft International, NIR Systems Inc.) with the modified partial least-squares regression (MPLS) option and two passes to eliminate outliers (*17*). The mathematical treatments (1, 4, 4), (2, 5, 5), and (2, 8, 6) (first or second derivative, gap over which derivative was calculated, number of data points used in first smoothing, and no second smoothing) were used for prediction regression equation models. The equation selected as the best for each chemical fraction was obtained using the lowest standard error of cross-validation; this was obtained by dividing the data into sets of four and predicting each fourth value from calibrations developed from the other three values. Samples with large residuals were omitted, and cross-validation was performed again.

RESULTS AND DISCUSSION

Ranges, mean values, and standard deviations (SD) for α-carotene, β-carotene, α-cryptoxanthin, β-cryptoxanthin, isolutein, lutein, violaxanthin, zeaxanthin, and total carotenoids in the samples used in the calibration set are shown in **Table 1**. In the sample set, there was a wide variation in chemical composition, and the samples covered most of the variability reported in the literature for maize grain (*9*, *18*, *19*).

In **Figure 1** are shown some typical NIR spectra obtained for the different maize samples analyzed. Clearly it can be seen that a considerable contribution is due to the visible wavelength range (400−700 nm), and this may be of relevance for highly colored maize samples.

This range has been indeed used to measure durum wheat pigments (*20*). However, most of the analyzed samples contain,

Table 1. Summary of Different Carotenoid Fractions (from HPLC Analysis), Expressed as Milligrams per Kilogram, of the Calibration and Cross-Validation Set for Maize Flour, Showing Number of Samples (*N*), Range and Mean of Values, Standard Deviation (SD), Standard Error of Analysis (SE), and Outliers

trait	*N*	min	max	mean	SD	SE	outliers
α-carotene	82	0	4.5	2.00	1.38	0.02	4
β-carotene	82	0	2.8	0.94	0.70	0.01	12
α-cryptoxanthin	82	0	8.9	1.41	1.64	0.03	32
β-cryptoxanthin	82	0	6.1	2.86	1.87	0.03	6
isolutein	82	0	8.0	2.33	1.26	0.02	5
lutein	82	0	29.7	13.07	5.71	0.10	2
violaxanthin	82	0	3.8	1.52	1.09	0.02	22
zeaxanthin	82	0.3	38.2	20.03	9.54	0.16	0
total carotenoids	82	0.5	68.8	42.35	13.99	0.24	0

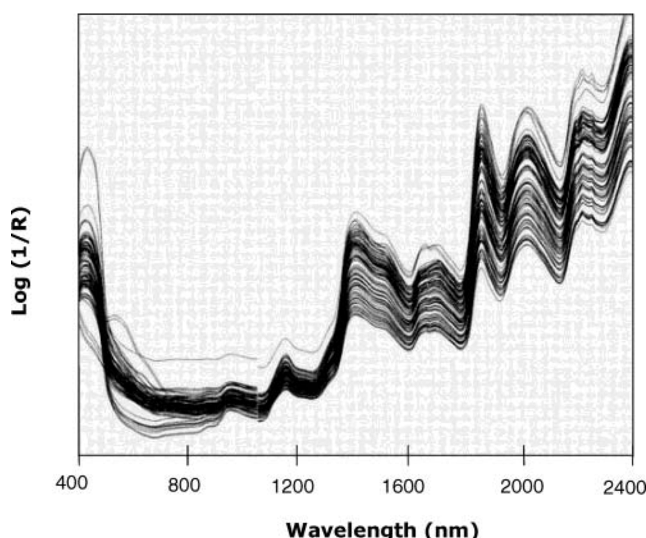


Figure 1. Typical NIR spectra obtained for the different maize samples analyzed.

besides zeaxanthin, other carotenoids that can be evaluated only at higher wavelengths, typical of the near-infrared range.

In **Table 2** are reported the statistics of the calibrations and of the cross-validations for the different carotenoids, including standard errors of calibration (SEC) and R^2 values for the equations of best fit obtained for each of the traits. The r^2 values for the cross-validation and standard error of cross-validation (SECV) are shown also in the same table. SEC values ranged from 0.16 for isolutein to 5.24 for total carotenoids, and R^2 values ranged from 0.82 for β-carotene to 0.98 for total carotenoids; for the cross-validation, the r^2 values ranged from 0.70 for β-carotene to 0.96 for total carotenoids, lutein, and violaxanthin, and SEC(V) values ranged from 0.27 for isolutein to 6.91 for total carotenoids.

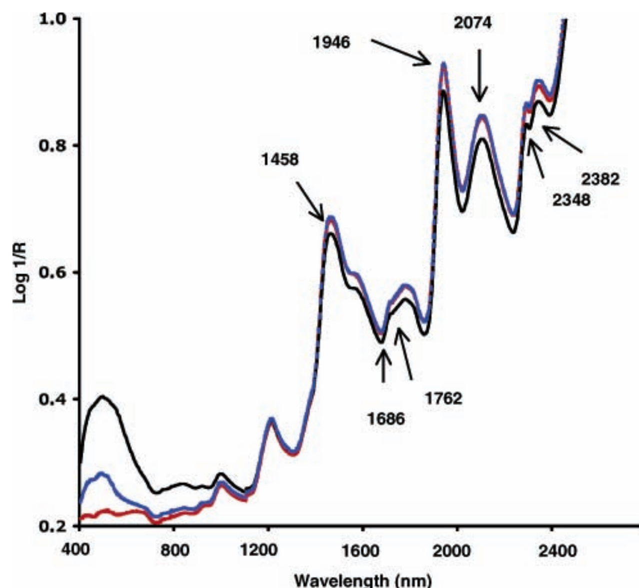


Figure 2. Vis–NIR spectra for zeaxanthin (black), lutein (blue), and lutein mixed at 25 mg kg^{-1} with dry G 25 Sephadex superfine (red).

The possibility of evaluating the single carotenoid content utilizing only spectra in the visible region (400−1100 nm) is shown in **Table 3**. Using this spectral region lower R^2 (from 0.64 to 0.96) and higher SEC (from 0.20 to 6.93) values are obtained, better than the values obtained using the whole Vis−NIR range (400−2500 nm), that is, from 0.82 to 0.98 for R^2 and from 0.16 to 5.24 for SEC, respectively (**Table 2**).

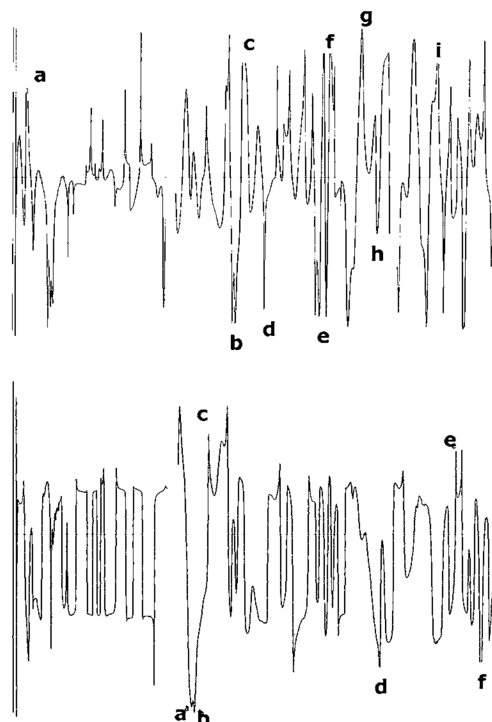
A further confirmation to utilize the whole NIR spectral region for developing a model for measuring the different carotenoids in maize flour is shown in **Table 4**, where three different zeaxanthin models are compared. The statistical variables SEC, R^2, SECV, and r^2 range from 2.55, 0.95, 3.22, and 0.92 to 3.44, 0.88, 3.84, and 0.85, for zeaxanthin models utilizing the whole NIR spectra range (400−2500 nm) and the visible spectra range (400−1100 nm), respectively.

In **Figure 2** are shown the NIR spectra of the main carotenoids (zeaxanthin and lutein) present in corn flour, both mixed at 25 mg kg^{-1} with an eccipient (dry Sephadex G25

Table 2. Vis–NIR Modified Partial Least-Squares (MPLS) Regression Statistics of Calibration and Cross-Validation for the Different Carotenoids in Maize Flour and the Wavelength Range Used To Develop the Calibration of Each Carotenoid

	statistical variables										
trait	*N*[a]	mean[b]	SD	SEC	R^2	SECV	r^2	*n*[c]	λ_{range} (nm)	*t*[d]	math[e]
α-carotene	67	2.10	1.31	0.41	0.90	0.51	0.85	259	400–2500	8	1, 4, 4
β-carotene	64	0.94	0.66	0.28	0.82	0.37	0.70	170	1100–2500	5	2, 8, 6
α-cryptoxanthin	35	1.35	0.88	0.30	0.88	0.40	0.81	259	400–2500	5	1, 4, 4
β-cryptoxanthin	73	3.55	2.46	0.52	0.96	0.74	0.91	256	400–2500	8	2, 5, 5
isolutein	43	2.16	0.67	0.16	0.94	0.27	0.84	259	400–2500	6	1, 4, 4
lutein	81	15.92	19.22	3.41	0.97	4.06	0.96	252	400–2500	8	2, 8, 6
violaxanthin	59	2.38	3.88	0.63	0.97	0.78	0.96	252	400–2500	7	2, 8, 6
zeaxanthin	74	21.60	11.79	2.55	0.95	3.22	0.92	252	400–2500	8	2, 8, 6
total carotenoids	71	47.49	35.09	5.24	0.98	6.91	0.96	173	1100–2500	8	1, 4, 4

[a] Samples used to develop the model; SD, standard deviation; SEC, standard error of calibration; R^2, determination coefficient of calibration; SECV, standard error of cross-validation; r^2, determination coefficient of cross-validation. [b] Expressed in mg kg^{-1}. [c] Number of variables for each constituent. [d] Number of PLS loading factors in the regression model MPLS. [e] Mathematical transformation of spectral data: the first number is the order of the derivative function, the second is the length in data points over which the derivative was taken, and the third is the segment length over which the function was smoothed.



Correlations for Zeaxanthin

	Wavelength	r (2nd derivative)	chemical bond
a)	480	0.73	electronic vibration
b)	1366	-0.73	C-H combination band -CH_3
c)	1392	0.82	C-H combination band -CH_2
d)	1486	-0.73	O-H stretching 1st overtone
e)	1720	-0.91	C-O stretching 3rd overtone C-H stretching 1st overtone
f)	1766	0.87	C-H stretching 1st overtone
g)	1898	0.94	OH- deformation 3rd overtone
h)	1960	-0.65	$(-CH=CH-)_n$ 3rd overtone
i)	2200	0.72	C-H stretching

Correlations for Lutein

	Wavelength	r (2nd derivative)	chemical bond
a)	1158	-0.92	-CH stretching -CH= 3rd overtone
b)	1168	-0.95	C-H 2nd overtone / $(-HC=CH-)_n$
c)	1230	0.74	C-H 2nd overtone -CH
d)	1950	-0.79	$(-CH=CH-)_n$ 3rd overtone
e)	2270	0.61	OH stretching/C-O combination band
f)	2378	-0.76	C-H stretching

Figure 3. Spectral/chemical relationship of the main antioxidant zeaxanthin and lutein in standard samples (*X*-axes = wavelength; *Y*-axes = *r*). Correlations are based on ref *21*.

Table 3. Vis (400–1100 nm) Modified Partial Least-Squares (MPLS) Regression Statistics of Calibration and Cross-Validation for Different Carotenoids in Maize Flour

trait	N^a	mean^b	SEC	R^2	SECV	r^2	n^c	t^d	math^e
α-carotene	67	2.50	0.39	0.94	0.62	0.86	82	5	2, 8 ,6
β-carotene	64	0.88	0.20	0.76	0.34	0.28	82	8	2, 8 ,6
α-cryptoxanthin	35	2.26	0.41	0.92	0.66	0.79	82	7	2, 8 ,6
β-cryptoxanthin	73	3.56	0.66	0.93	0.87	0.88	82	7	2, 8 ,6
isolutein	43	2.18	0.30	0.64	0.47	0.22	82	2	2, 8, 6
lutein	81	15.75	3.95	0.96	4.60	0.94	82	7	2, 8, 6
violaxanthin	59	2.47	0.73	0.96	0.88	0.95	82	6	2, 8, 6
zeaxanthin	74	21.60	3.44	0.88	3.84	0.85	82	5	2, 8, 6
total carotenoids	71	41.45	6.93	0.77	7.23	0.74	82	5	2, 8, 6

[a] Samples number used to develop the model; SD, standard deviation; SEC, standard error of calibration; R^2, determination coefficient of calibration; SECV, standard error of cross-validation; r^2, determination coefficient of cross-validation. [b] Expressed in mg kg^{-1}. [c] Number of variables for each constituent. [d] Number of terms in the regression model. [e] Mathematical transformation of spectral data: the first number is the order of the derivative function, the second is the length in data points over which the derivative was taken, and the third is the segment length over which the function was smoothed.

Superfine). The differences among the three NIR spectra are quite clear and evident mainly in the visible region (400−700 nm). However, many differences can be revealed also in the NIR range, as shown in **Figure 3**, which reports the spectral/chemical relationship of the main antioxidant zeaxanthin and lutein in standard samples (*21*). One of the higher correlations (−0.91) for zeaxanthin is to 1720 nm, corresponding to C−O stretching (third overtone) and to the first overtone of C−H, which could be ascribed to the OH group of zeaxanthin. In any case, the higher correlations between spectra and chemical composition for zeaxanthin fall into the NIR range 1366−2200 nm, with *r* values (defined as the relationship among the substance and wavelengths, absorptivity maxima as derived by second derivative) from −0.91 to 0.94. Therefore, we can assume that the major contribution for developing the calibration model for zeaxanthin is the whole NIR spectral range. This hypothesis is confirmed by a calibration developed using only the visible region (**Tables 2−4**). As well as for zeaxanthin, higher lutein correlation falls into the NIR range from 1158 to 2378 nm, with *r* values from −0.95 to 0.74, whereas in the visible region the correlation coefficients are definitely lower.

In **Table 5** the statistics of the validation set for the different carotenoids in maize flour not used in calibration, including the outliers, are shown. It can be seen that very good correlation factors (r^2 ranging from 0.64 to 0.95 for β-carotene and

Table 4. NIR Modified Partial Least-Squares (MPLS) Regression Statistics of Calibration and Cross-Validation for Zeaxanthin on Different Spectroscopic Ranges

	statistical variables										
trait	N^a	mean	SD	SEC	R^2	SECV	r^2	n^c	λ_{range} (nm)	t^d	math^e
zeaxanthin	74	21.60	11.79	2.55	0.950	3.22	0.920	252	400–2500	8	2, 8 6
zeaxanthin	74	21.60	11.79	3.26	0.918	3.95	0.879	170	1100–2500	8	2, 8, 6
zeaxanthin	74	21.60	11.79	3.44	0.882	3.84	0.852	82	400–1100	5	2, 8, 6

[a] Samples number used to develop the model; SD, standard deviation; SEC, standard error of calibration; R^2, determination coefficient of calibration; SECV, standard error of cross-validation; r^2, determination coefficient of cross-validation. [b] Expressed in mg kg^{-1}. [c] Number of variables for each constituent. [d] Number of PLS loding factors in the regression model MPLS. [e] Mathematical transformation of spectral data: the first number is the order of the derivative function, the second is the length in data points over which the derivative was taken, and the third is the segment length over which the function was smoothed.

Table 5. Statistics of the Validation Set for Different Carotenoids in 40 Maize Flour Samples Used for Testing the Model of Calibration (Spectral Region as in **Table 2**)

trait	statistical variables[a]						
	$mean_{lab}$	$mean_{NIR}$	bias	SEP	r^2	SE_{lab}	SE_{NIR}
α-carotene	2.58	2.48	0.01	0.39	0.86	0.19	0.18
β-carotene	0.78	0.85	–0.07	0.18	0.64	0.06	0.05
β-cryptoxanthin	3.71	3.71	0.00	0.46	0.91	0.27	0.24
isolutein	2.19	2.18	0.01	0.21	0.92	0.13	0.13
lutein	11.47	11.53	–0.06	2.06	0.86	0.86	0.81
zeaxanthin	17.54	17.53	0.01	2.40	0.95	1.71	1.66
total carotenoids	37.06	37.52	–0.46	3.56	0.94	2.42	2.41

[a] Lab, laboratory values obtained by HPLC; NIR, predicted values obtained by NIR; SEP, standard error of prediction; r^2, correlation coefficient of prediction; SE, standard error of accuracy.

zeaxanthin, respectively) were obtained, confirmed also by the bias values that range from −0.46 to 0.10 for total carotenoids and α-carotene and by the standard errors of prediction (SEP) that range from 0.18 to 3.56 for β-carotene and total carotenoids, respectively.

The carotenoids presented in **Table 1** are highly correlated between them, as can be expected, as the biosynthetic pathway for the different xanthophylls is the same (*22*). Moreover, the algorithm used for developing calibration equations (MPLS) is useful to avoid overfitting.

For what is taken as the detection limit for NIRS, usually recognized to be ∼0.1% of the considered analyte, in our hands this can be assumed to be even lower, as shown in **Figure 2**, which reports the spectra obtained with lutein and zeaxanthin at 25 mg kg^{-1}.

Using NIRS for the determination of humidity, proteins, and lipids in soft and durum wheat, barley, oats, maize, soy, soft and durum wheat flours, and oleaginous seeds is widely accepted in the trade of these products (*11*, *23*). Furthermore, the Canadian Grain Commission (*24*) promoted this kind of instrumental analysis in wheat grading and classification, as the data they produce possess a precision comparable with that of the respective official methods of analysis and can be obtained at much lower unit cost. Our results show that NIRS can be routinely applied to determine the carotenoid content in maize. This nondestructive method could greatly simplify the analysis of such compounds, because no extraction step with organic solvents is required and samples are readily analyzed in minutes, the only required step being their grinding.

When compared to conventional laboratory analyses, NIRS appears to be an attractive alternative technique because of its rapidity, simplicity, safety, and low operational costs.

This is of particular importance in nutritional quality evaluation, quality plant-breeding programs, species resource identification, and health food processing of maize in which a large number of samples must be analyzed (*25*, *26*).

As the sample set used in this study was not very large, we are going on to extend and to increase the robustness of the model, acquiring other corn meal samples from different locations, hybrids, agronomic trials, and treatments.

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