AACCI Approved Methods Technical Committee Report: A New AACCI Approved Method for the Determination of the Total Carotenoid Content of Cereal Whole Grain and Refined Flours

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Carotenoids are of great interest due to their importance in food coloration and durum quality. They also play significant roles in human health and nutrition because they have vitamin A activity and are linked with reduced risk of a number of chronic diseases and promotion of human health, in particular the eyes and skin. Lutein is the main carotenoid present in wheat, durum, and corn (3,4,11,13) and is associated with reduced incidence of cataracts (18), age-related macular degeneration (6), cancer (15), and cardiovascular disease (19). Lutein and zeaxanthin (lutein relative) constitute the macular pigments in the yellow spot of the human retina. These pigments provide beneficial functions such as protection of the macula from damage by blue light (22), improved visual acuity (17), and scavenging of harmful reactive oxygen species (9). Other carotenoids found in wheat and corn, including β-cryptoxanthin, α-carotene, and β-carotene, possess vitamin A activity and play significant roles in vision.

Differences in carotenoid content and composition have been found between ancient and modern wheats (3,4) and between spring and winter wheats (14), suggesting there is the potential to develop high-carotenoid wheats. Einkorn (diploid ancient wheat) contains the highest lutein levels among wheat species, ranging from 7.4 to 13.4 mg/kg compared with 1.9 mg/kg in common wheat (3,4,11). Durum or pasta wheat and Kamut, a specialty ancient khorasan wheat, contain intermediate levels of lutein at 5.5–6.5 mg/kg. High-lutein or -carotenoid wheat is essential for making high-quality pasta products, as well as for supporting a healthy diet. Corn contains an exceptionally high level of carotenoids, mainly lutein and zeaxanthin, which makes it a potentially useful blending flour in the development of high-lutein wheat-based functional foods (3). The availability of a standard, simple, and reliable method for quantifying total carotenoid content in grains is needed, in particular for breeding programs focused on developing high-carotenoid wheat or corn for their important health attributes.

Why a New Method for Quantifying Total Carotenoid Content?

Due to their health benefits, carotenoids are essential dietary substances that also positively influence the quality of semolina and pasta products. The new method provides a simple and reliable procedure for the determination of total carotenoid content (TCC) in whole grain and refined flours. The method is designed to address two concerns associated with the total yellow pigment content measured by AACC International (AACCI) Approved Method 14-50.01 (1). In the new method, absorbance is read at 450 nm, the average maximum absorption (λ_{max}) of carotenoid wheat extract, instead of 438.5 nm, as in AACCI Approved Method 14-50.01 (1). Lutein, zeaxanthin, and β-cryptoxanthin are the predominant carotenoids present in wheat and corn, and their λ_{max} is 445, 452, and 452 nm, respectively. The new method measures TCC as a lutein equivalent based on the use of an authentic lutein standard instead of β-carotene, as is used in AACCI Approved Method 14-50.01 (1) and ICC Standard Method 152 (12). The new method also uses smaller amounts of sample and solvent that produce high throughput. The method is considered a good predictor of lutein or TCC in wheat when significant positive associations between TCC and lutein or TCC and the sum of individual carotenoids determined by HPLC are found (r = 0.9442 and 0.9886, respectively) (3).

The new method can be used to identify high-carotenoid wheat or corn or the milling fractions from various wheat species such as durum, einkorn, emmer, Kamut, and khorasan. Recently, a micromethod for the determination of yellow pigment content in durum has been developed, but the method only addresses sample size and solvent volume (5). The new method described here addresses several factors, including sample size, solvent volume, wavelength, and a carotenoid standard to improve accuracy and reliability. In addition, the new method complements the existing AACCI (1) and ICC (12) methods as a micromethod that provides more accurate quantification of total carotenoids.

Grain Materials

Grain samples included in the collaborative study were amber durum wheat, yellow einkorn wheat, white wheat, red wheat, and yellow corn. The grains were obtained from the University of Saskatchewan, Saskatoon, SK, Canada, and the retail market in Guelph, ON, Canada, in 2009 and 2010. The red, white, and einkorn wheat grain samples were ground using a sample mill (Cyclone, Udy Co.) equipped with a 0.5 mm sieve to obtain whole grain flours. Durum semolina and corn flour samples were obtained from the retail market, and a subsample of semolina was milled into flour using a sample mill (Cyclone). The ground whole grain or refined materials were mixed thoroughly, packaged in plastic bags, coded, wrapped in dark paper bags, and stored in a freezer at –20°C (up to a few days) to avoid exposure to light or heat prior to shipping. Three replicates of each sample were prepared.

Extraction of Carotenoids

Water-saturated 1-butanol (WSB) was used to extract carotenoids from wheat and corn flours. In a previous study, WSB and 80% ethanol were more efficient in extracting carotenoids from wheat among the five solvents (WSB, 80% ethanol, 80% methanol, methyl tert-butyl ether, and tetrahydrofuran) studied, providing the highest absorbance and lutein concentration measured by HPLC (3). Because WSB is used in AACCI Approved

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Method 14-50.01 (1) and ICC Standard Method 152 (12) for the determination of total yellow pigment content in durum, it was used in the current method. Extraction of carotenoids was performed in a fume hood and under dim light to avoid isomerization and oxidation damage due to heat and light. These conditions should be considered particularly in the preparation of lutein standard solution. The carotenoid extracts were then measured spectrophotometrically as in the AACCI and ICC methods, with some modifications in wavelength and carotenoid reference as mentioned above.

Collaborative Study

In 2010 we ran a minicollaborative study with a small number of labs to evaluate the performance of the new method and to modify the method accordingly before running a regular interlaboratory study. Four labs participated in the minicollaborative study: Guelph Food Research Centre, AAFC, Canada; School of Food Science and Engineering, Nanjing University of Finance and Economics, China; Department of Food Science, University of Manitoba, Canada; and VDF FutureCeutical Inc., USA. Five different grain materials were used to evaluate the method. The statistical data (Table I) from the minicollaborative study demonstrated good method performance and showed its potential to become a standard method. The data were discussed at the 2010 AACC International Annual Meeting in Savannah, GA, and the AACCI Bioactive Compounds Technical Committee (BCTC) agreed to proceed with a regular interlaboratory study, which was performed in 2011.

Fifteen labs participated in the regular collaborative study (Table II), and each laboratory received six different grain materials and the preweighed lutein standard. A minimum of five different materials is required for a collaborative study (16). The two durum samples obtained from the same material were considered blind duplicate samples. The samples were identical durum wheat with coarse and fine particle sizes (i.e., durum semolina and durum flour, respectively) and were coded differently. The durum samples had very close TCCs of 6.3 and 6.2 mg/kg for flour and semolina, respectively, based on the results of the minicollaborative study (Table I). Thus, the samples were considered as blind duplicates in the regular collaborative study.

Because the purity of lutein may vary between suppliers and between batches from the same supplier, it is crucial to use high-quality lutein that is obtained from a single supplier to avoid additional variables that will impact the method results. The purity of the lutein used was confirmed in our lab by both liquid chromatographic and spectrophotometric methods. After the lutein with the highest purity was chosen from among the three suppliers available in the market, a package of lutein standard (25 mg) was purchased, and each lab was supplied with 1 mg of authentic lutein standard. The lutein was weighed on a certified semianalytical balance that was automatically calibrated each time with an accuracy of 0.1 mg. The semianalytical balance was double-checked with certified weights of 1 and 2 mg (Troemner). Over the past several years we have found that purchasing preweighed lutein may produce high absorption coefficient values and, thus, verification of weight and purity is important.

Due to the extremely high cost of shipping with dry ice, lutein was shipped in a regular package without dry ice. Unfortunately, the lutein deteriorated during shipping due to the unexpected length of the shipping time, particularly for shipments to laboratories in Europe and Asia (shipping time was 5–8 days instead of the 1–2 days predicted). In this case, participants were asked to obtain fresh lutein or verify the purity of the lutein received. Five of the six laboratories that obtained fresh lutein were able to obtain consistent results for the calibration curve and absorption coefficient ($A^{1\%}$). Most suppliers recommend that lutein be stored at –20°C, and in the current study, the lutein standard was kept at –80°C; however, we could not afford to ship standards at low temperature to guarantee undamaged delivery. When lutein was stored in the lab at ambient temperature ≈27.1% loss in lutein concentration over 8 days was found when monitored spectrophotometrically. Significant losses in lutein also occurred at elevated temperatures during baking and storage (2,10).

The calibration curve constructed by the five labs (Fig. 1; N = 15) produced an $A^{1\%}$ of 2,471 in WSB, which was very close to the previously published $A^{1\%}$ of 2,550 in absolute or aqueous ethanol (7,8,20). The average $A^{1\%}$ was also close to that used by official organizations such as the National Institute of Standards and Technology (NIST) and the USDA Foreign Agricultural Service (21,23). Due to the accuracy and reliability of the calibration curve compared with the literature a decision was made to use the calibration curve to convert absorbance readings of unknown samples into concentrations of lutein equivalents.

The new method is provided with a calculator enhancement to guarantee a convenient and precise calculation process. The calculator enhancement includes Microsoft Excel Spreadsheet Part I for creating the calibration curve and regression equation to calculate the molar absorptivity (ε) or absorption coefficient

Table I. Summary of overall means and statistics for determination of total carotenoid content in wheat whole grain and refined flours (2010 minicollaborative study; N = 4)

Statistical Parameter[a]	Einkorn Wheat[b]	Durum Flour	Durum Semolina	White Wheat[b]	Red Wheat[b]
Total number of labs	4	4	4	4	4
Total number of replicates	10	10	9	10	10
Total number of outliers	–	–	1	–	–
Overall mean (mg/kg)	8.20	6.26	6.16	3.70	3.53
Largest within variance	0.058	0.012	0.021	0.006	0.004
Largest average lab result	8.66	6.58	6.76	3.81	3.82
Smallest average lab result	7.89	6.08	5.95	3.44	3.21
Repeatability SD	0.11	0.08	0.12	0.05	0.04
Reproducibility SD	0.37	0.22	0.39	0.16	0.31
Repeatability RSD	1.29	1.23	1.94	1.45	1.11
Reproducibility RSD	4.54	3.43	6.25	4.40	8.62
HorRat value	0.39	0.28	0.51	0.33	0.65

[a] SD = standard deviation; RSD = relative standard deviation.
[b] Whole grain flour.

($A^{1\%}$) value of lutein to verify purity, and Microsoft Excel Spreadsheet Part II to calculate TCC in milligrams of lutein equivalents per kilogram of unknown grain sample.

Method Performance

The purpose of the new method is to precisely measure TCC in wheat and corn milling products to identify and develop high-carotenoid grains or milling fractions with health-promoting effects. Because lutein is the main carotenoid found in wheat and corn (3,4,11,13), TCC is measured as lutein equivalents. To guarantee reliable results, the method includes a procedure for the verification of lutein purity. The purity and authentication of lutein is confirmed by measuring the absorption coefficient ($A^{1\%}$) or molar absorptivity (ε) and by HPLC analysis (3). In the current study, the average $A^{1\%}$ value was 2,471 in WSB. This value is based on the average of the five labs that received unspoiled lutein or obtained fresh lutein from suppliers and, thus, were able to produce consistent results. The $A^{1\%}$ value is comparable with that published in the literature for authenticated pure lutein (2,550) in absolute ethanol (7,8,20). The $A^{1\%}$ value can be converted to ε, where ε = ($A^{1\%}$ × molecular weight)/10. It is important that the storage and handling of the lutein standard or lutein solutions at ambient temperature and exposure to light be avoided because they can deteriorate. We found ≈27.1% loss in lutein concentration over 8 days when lutein was stored in brown vials inside envelopes similar to shipping packages at ambient temperature and under regular daylight.

Figure 1 illustrates the linearity of the relationship between lutein concentration and absorbance reading at 450 nm. The graph shows good linearity and an acceptable determination coefficient (R^2) of ≥0.99. The regression equation used to convert the absorbance of unknown samples into a lutein concentration is also shown in Figure 1. This equation is reliable and precise because it is based on the results of five labs, and the purity of the

Table II. Participants in the carotenoid interlaboratory study

Participant	Country
AAFC, Eastern Cereal and Oilseed Research Centre	Canada
AAFC, Guelph Food Research Centre	Canada
Canadian Grain Commission, Durum Wheat Research	Canada
Michigan State University, Department of Food Science and Human Nutrition	United States
Nanjing University of Finance and Economics, School of Food Science and Engineering	China
South Dakota State University, Department of Health and Nutritional Sciences	United States
Unidade Technologia Alimentar, INRB Labratório de Investigação Agrária	Portugal
Università degli Studi del Molise, Dipartimento di Scienze e Tecnologie Agro-alimentari Ambientali e Microbiologiche	Italy
Università degli Studi di Milano, DISTAM-Sez. Tecnologie Alimentari	Italy
Universität für Bodenkultur, Abteilung für Lebensmittelwissenschaften und Technologie	Austria
University of Guelph, Department of Food Science	Canada
University of Manitoba, Department of Food Science	Canada
University of Minnesota, Department of Food Science and Nutrition	United States
USDA, Western Wheat Quality Lab	United States
VDF FutureCeutical Inc.	United States

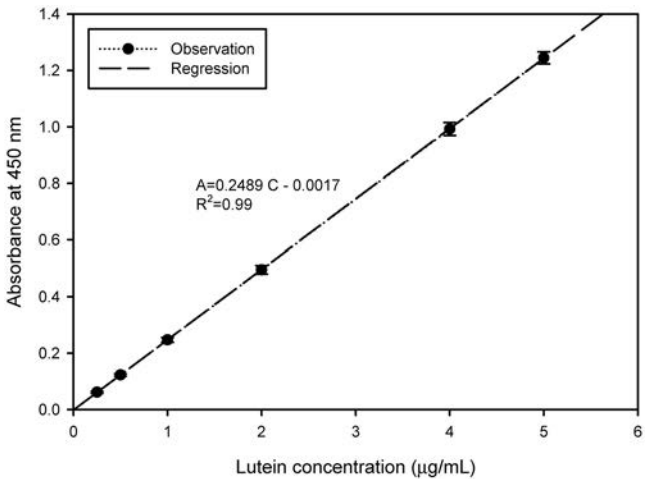

Fig. 1. Calibration curve for the lutein standard (5 labs; $N = 15$).

Table III. Overview of outlier test data

Grain Sample	Cochran (%)	Single Grubbs (%)	Double Grubbs (%)
Einkorn	33.2	14.2	26.1
Durum flour	20.6	19.6	27.8
Durum semolina	36.8	6.9	13.0
White wheat	27.0	10.8	22.5
Red wheat	31.9	8.5	16.8
Corn flour	21.8	4.7	11.6
Critical value (2.5% significance)	51.5	30.0	40.9

Table IV. Summary of overall means and statistics for total carotenoid content in wheat and corn whole grain and refined flours

Statistical Parameter[a]	Einkorn Wheat[b]	Durum Flour	Durum Semolina	White Wheat[b]	Red Wheat[b]	Corn Flour
Total number of labs	15	15	15	15	15	15
Total number of replicates	44	43	42	40	39	42
Total number of outliers	1	2	3	5	6	3
Overall mean (mg/kg)	8.77	5.91	5.58	3.50	2.65	23.01
Largest within variance	0.32	0.09	0.32	0.03	0.04	0.43
Largest average lab result	9.09	6.40	5.88	3.70	2.78	23.71
Smallest average lab result	8.26	5.60	5.31	3.31	2.51	22.24
Repeatability SD	0.25	0.17	0.22	0.10	0.10	0.38
Reproducibility SD	0.32	0.26	0.26	0.14	0.12	0.64
Repeatability RSD	2.85	2.90	3.98	2.78	3.69	1.66
Reproducibility RSD	3.70	4.40	4.61	3.92	4.39	2.77
HorRat value	0.32	0.36	0.37	0.30	0.32	0.28

[a] SD = standard deviation; RSD = relative standard deviation.
[b] Whole grain flour.

lutein standard was verified through comparison with the value for the absorption coefficient ($A^{1\%}$) published in the literature.

Table III shows the data for the outlier tests expressed as percent reduction in standard deviation compared with the critical values. For 15 labs the critical values were 51.1, 30.0, and 40.9% for Cochran, single Grubbs, and double Grubbs tests, respectively. The percentage of outliers ranged from 2 to 13% of the total number of replicates, which is below the acceptable percentage of removal (22%) (16).

Table IV summarizes the statistical data for performance and validation of the new method. The overall means for wheat species and corn varied broadly, ranging from 2.65 mg/kg in red wheat to 23.01 mg/kg in corn. This indicates that the method worked in a diverse population of grains. The results also showed genetic diversity among wheat species in terms of TCC, with einkorn having the highest TCC level (8.77 mg/kg) and common or bread wheat exhibiting the lowest (2.65 mg/kg). Durum wheat had an intermediate TCC level (5.58–5.91 mg/kg), while corn was exceptionally high in TCC (23.01 mg/kg). According to Abdel-Aal et al. (3) the colorimetric method, compared with the HPLC method, overestimates lutein content by ≈23% and total carotenoids content by ≈20%. The largest and smallest average lab results for each grain also support the efficiency of the new method (Table IV).

The repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R) values were <5%, ranging from 1.66 to 3.98% for RSD_r and from 2.77 to 4.71% for RSD_R (Table IV). The method also had very low HorRat values, ranging between 0.28 and 0.37. These values appear to be very reasonable and demonstrate the reliability of the new method. In general the method performed better with high-carotenoid wheat and corn samples or yellow flours compared with low-carotenoid wheat samples or white and red wheats. The number of outliers in the red and white wheat samples was 6 and 5, respectively, compared with 1–3 outliers in the einkorn, durum, and corn samples (Table IV). White and red wheats or low-carotenoid wheats may contain compounds that can potentially interfere with TCC measurement, in particular red wheat, which contains red flavones pigments that may interfere with yellow pigment measurement.

In general the new method possesses several improvements over previous methods, including use of small sample weight, low solvent volume, more accurate and reliable results based on adjustments in the wavelength and carotenoid standard, and high throughputs. Such improvements provide a simple and reliable procedure that would be useful in identifying high-carotenoid wheat or corn for the development of grain-based functional food ingredients. In addition, the new method is equipped with a Microsoft Excel spreadsheet calculator to run data calculations precisely and conveniently.

Summary

A new AACCI Approved Method for the determination of TCC in cereal whole grain or refined flours has been developed and validated. The method is based on extraction of carotenoids with water-saturated butanol and subsequent spectrophotometric measurement using lutein as a standard. Because total carotenoids are expressed as lutein equivalents it is crucial to verify the purity of the lutein standard to be able to produce consistent results among different laboratories. Fifteen labs participated in the collaborative study, and six grain materials, including einkorn, durum, red wheat, white wheat, and yellow corn, were used to evaluate method performance. The RSD_r and RSD_R values were <5%, and the HorRat values were very low, ranging from 0.28 to 0.37, indicating high precision and reliability. In general the new method provides a simple and reliable procedure that would be useful in the development of high-carotenoid wheat or corn as functional food ingredients with health-promoting effects.

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