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Improved Normal-Phase High-Performance Liquid Chromatography Procedure for the Determination of Carotenoids in Cereals

GIANFRANCO PANFILI,* ALESSANDRA FRATIANNI, AND MARIO IRANO

DISTAAM, Università degli Studi del Molise, via De Sanctis, 86100 Campobasso, Italy

Besides the health benefits associated with whole-grain consumption, cereals are recognized sources of health-enhancing bioactive components such as carotenoids, which are a group of yellow pigments involved in the prevention of many degenerative diseases and which have been used for a long time as indicators of the color quality of durum wheat and pasta products. This work reports a fast, sensitive, and selective procedure for the extraction and determination of carotenoids from cereals and cereal byproducts. The method involves sample saponification and extraction followed by normal-phase high-performance liquid chromatography, allowing the separation of the main carotenoids pigments of cereals, especially lutein and zeaxanthin. An application of the established method to various species of cereals and cereal byproducts is also shown. The highest carotenoid levels were found in maize (∼11.14 mg/kg of dry weight), which contains high amounts of β-cryptoxanthin (2.40 mg/kg of dry weight), and, among the cereals considered, has the highest content of zeaxanthin (6.43 mg/kg of dry weight) and α+β-carotene (1.44 mg/kg of dry weight). With the exception of maize, lutein is the main compound found (from 0.23 to 2.65 mg/kg of dry weight in oat and durum wheat, respectively). Moreover, whereas α+β-carotene and zeaxanthin are principally localized in the germ, lutein is equally distributed along the kernel.

KEYWORDS: Carotenoids; lutein; pigments; HPLC; cereals; food

INTRODUCTION

Carotenoids are a diverse group of yellow-orange pigments found in many biological systems, which can be divided in two general classes: carotenes and xanthophylls.

Carotenes, such as α- and β-carotene and lycopene, are hydrocarbons, whereas xanthophylls are oxygenated derivatives of carotenes and include the compounds β-cryptoxanthin, lutein, and zeaxanthin.

The nutritional importance of carotenoids comes mainly from the provitamin A activity of β-carotene, β-cryptoxanthin, and others with at least one intact nonoxygenated β-ionone ring. In addition to their role as precursors of vitamin A, carotenoids are antioxidants (*1*), and besides β-carotene, other carotenoids without provitamin A activity have recently been involved in the prevention or protection against serious human disorders such as cancer and cardiovascular disease (*2*). Lutein and zeaxanthin are found in the eye and have been associated with reduced risk of cataract development and age-related macular degeneration (AMD) (*2*).

Fruits and vegetables are the main sources of carotenoids, and there have been several reports on the qualitative and quantitative distributions of these compounds in foods (*3*). Moreover, in light of this abundance and of the new roles of carotenoids in health, the determination of accurate qualitative and quantitative data on these compounds has become increasingly important.

Historically most of the carotene data in tables of food composition have been obtained by measuring total absorption at a specific wavelength and quantified against β-carotene (*4*) or, more usually, by open column chromatography (AOAC method), widely used to separate carotenoid pigments, which are then quantified spectrophotometrically.

In recent years, high-performance liquid chromatography (HPLC) has become the method of choice for the analysis of carotenoids because of its ability to distinguish between similar geometrical structures of carotenoids.

In general, both normal-phase and reverse-phase HPLC have shown the capability to separate closely related pigments.

The majority of the carotenoid separation involves the use of reverse-phase HPLC. Quite a large number of procedures have been reported for the HPLC analysis of carotenoids on C18 columns (*5*, *6*) and the use of a C30 stationary column has been reviewed (*7*). Reverse-phase HPLC is able to distinguish between α- and β-carotene (*8*); however, it is less efficient than the normal-phase in separating lutein, zeaxanthin, and their geometric isomers. Apart from a recent work (*3*) that uses a normal phase column, several works available in the literature (*9*−*11*) report the complete HPLC profiles of carotenoids in most common fruits and vegetables determined by means of a reverse-phase column, thus giving only the combined concentration of lutein, zeaxanthin, and their geometrical isomers.

* To whom correspondence should be addressed. Tel: +39 0874 404620. Fax: +39 0874 404652. Email: panfili@unimol.it.

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Carotenoids are very minor constituents in cereal grains. Besides their nutritional and health benefits, they are responsible for the attractive bright yellow color of durum wheat. Color due to carotenoids is an important factor in the use of cereal grains in food manufacture, especially for durum wheat and the quality of pasta, and in fact much of the research published on cereal grain carotenoids has been focused on this aspect.

Cumulative data on the yellow pigment composition of several durum wheat varieties showed that >90% of the pigments of durum wheat consist of xanthophylls. β-Carotene amounts to ~1%, and it is also commonly found as a minor component in cereals, although some authors (*12*) report higher levels in barley grains. Among xanthophylls, free lutein accounts for ~84.8% of the yellow pigments, lutein monoesters account for 9.8%, and lutein diesters account for 5.3% (*13*). These data have never been questioned and, above all, have never been checked by using modern analytical methods such as HPLC. As a result, an accurate account of the levels and the relative distribution of carotenoids in cereals is essential.

Extraction of analytes from sample matrix is an another important step prior to the HPLC analysis. Carotenoids are highly sensitive to light, heat, air, and active surfaces, and their isolation and analysis may be accompanied by degradation, formation of stereoisomers, structural rearrangements, and other physicochemical reactions. A literature review has shown that procedures for the extraction of analytes from food involve various types of solvent, solvent combinations, and procedures; however, the relative efficacy of the existing methods has been poorly evaluated (*14*).

As far as cereals are concerned, apart from scarce literature related to HPLC analysis of carotenoids, a systematic comparison of different extraction procedures suitable for a quantitative recovery from cereal samples is completely lacking.

This study aims at developing a procedure involving saponification and normal-phase HPLC detection for a fast and reliable simultaneous determination and quantification of the most common carotenoids of cereals.

Evaluation of the proposed method was made by (i) comparison of extraction yields of the analytes obtained by using different reported methodologies, (ii) validation of the established method to indicate its accuracy and repeatability, and (iii) investigation of the possible application of the proposed method to cereal matrices.

MATERIALS AND METHODS

Chemicals. α-Carotene, β-carotene, and lutein were from Sigma (St. Louis, MO). β-Cryptoxanthin and zeaxanthin were from Extrasynthese (Z. I. Lyon-Nord, Genay, France). All other reagents were of analytical or HPLC grade and were purchased from Carlo Erba (Milano, Italy).

Samples. Different cereal products were purchased in local stores (maize, oat) or supplied by research institutes (wheat, spelt, barley). Cereal samples were collected according to standard sampling procedures and were stored at 4 °C. Immediately before analysis, the samples were ground in a Cyclotec 1093 laboratory mill with a 0.5 mm sieve (FOSS Italia, Padova, Italy) and carefully mixed.

The certified reference material was a BCR (Institute for Reference Materials and Measurements, Geel, Belgium) lyophilized mixed vegetable sample (CRM 485).

Equipment. Chromatography was performed using an HPLC analytical system comprising a Waters model 510 solvent delivery system (Milford, MA) equipped with an injector with a 50 μL loop (Rheodyne, Cotati, CA) and a 2996 diode array detector. Results were evaluated by a Waters Millennium Chromatography system.

Standard Solutions. Stock solutions of carotenoids were prepared at a concentration of nearly 25 μg/mL of isopropyl alcohol (10%) in *n*-hexane and stored under nitrogen at −20 °C in the dark. These solutions were diluted in *n*-hexane, apart from β-cryptoxanthin diluted in petroleum ether, and were confirmed for potency against the known extinction coefficient of each compound (*15*). The working solution was prepared by pooling suitable volumes of each stock solution and diluting with isopropyl alcohol (10%) in *n*-hexane to obtain concentrations ranging from 0.48 to 8.36 μg/mL for each compound.

Sample Preparation. *Saponification*. Carotenoids were extracted according to the method reported by Panfili et al. (*16*): 2 g of cereal sample was saponified under nitrogen in a screw-capped tube by adding 5 mL of ethanolic pyrogallol (60 g/L) as antioxidant, 2 mL of ethanol (95%), 2 mL of sodium chloride (10 g/L), and 2 mL of potassium hydroxide (600 g/L). The tubes were placed in a 70 °C water bath and mixed every 5−10 min during saponification. After alkaline digestion at 70 °C for 45 min, the tubes were cooled in an ice bath and 15 mL of sodium chloride (10 g/L) was added. The suspension was then extracted twice with 15 mL portions of *n*-hexane/ethyl acetate (9:1 v/v). The organic layer was collected and evaporated to dryness, the dry residue was dissolved in 2 mL of isopropyl alcohol (10%) in *n*-hexane.

Other Extraction Procedures. *(i) Extraction with Water-Saturated n-Butyl Alcohol (WSB).* The extraction was made according to AACC method 14-50 (*4*) with slight modifications. Five grams of cereal sample was extracted with 25 mL of water-saturated *n*-butyl alcohol on a horizontal shaker for 60 min at 150 oscillations per minute and overnight at 90 oscillations per minute. The extract was then filtered by means of a Whatman no. 1 paper, and the residue was washed two times with 25 mL of water-saturated *n*-butyl alcohol until a colorless extract was obtained. Finally, the solvent was first evaporated under vacuum at 40 °C and then dried under a stream of nitrogen.

(ii) Soxhlet Extraction. Ten grams of sample was introduced into the extraction thimble of a Soxhlet apparatus and extracted for 8 h by means of petroleum ether (40−60 °C) (*17*).

(iii) Folch Method. Fifteen grams of sample was extracted with 200 mL of chloroform/methanol (1:1) according to the method of Folch et al. (*18*).

The dry residues of all extraction procedures were redissolved in 2 mL (saponification, WSB) or 5 mL (Soxhlet, Folch) of isopropyl alcohol (10%) in *n*-hexane.

A sample volume of 50 μL was injected for chromatographic analysis. For the recovery procedure 2 g of a semolina sample was spiked with 250 μL of a solution of β-carotene (2.18 μg/mL), lutein (8.36 μg/mL), and zeaxanthin (3.80 μg/mL). The sample was submitted in triplicate to the entire procedure of saponification, extraction, and chromatographic determination.

HPLC Analysis. The chromatographic separation of the compounds was achieved by means of a 250 mm × 4.6 mm i.d., 5 μm particle size, Kromasil Phenomenex Si column (Torrance, CA). The mobile phase was *n*-hexane/isopropyl alcohol (5%) at a flow rate of 1.5 mL/min. The mobile phase was previously degassed by sonication for 10 min. Spectrophotometric detection was achieved by means of a diode array detector set in the range of 350−500 nm. Peaks were detected at 450 nm. Carotenoids were identified through their characteristic spectra and comparison of their retention times with known standard solutions. After every 10 injections the column was reactivated with a solution of 10% isopropyl alcohol in *n*-hexane (v/v).

Statistical Analysis. Results were statistically evaluated by means of the Student's *t* test.

RESULTS AND DISCUSSION

The qualitative and quantitative distribution of lutein and zeaxanthin in cereals and cereal byproducts was determined by normal-phase HPLC by using a silica column. Contrarily to reverse-phase conditions, this column fails in separating α- and β-carotene. However, lutein and zeaxanthin have been found as major components of cereals and cereal byproducts. Because the main aim of our study was to measure the individual concentrations of lutein and zeaxanthin in cereals, less attention was given to the other minor carotenoids. Moreover, contrarily to other fruits and vegetables, in wheat products no epoxy

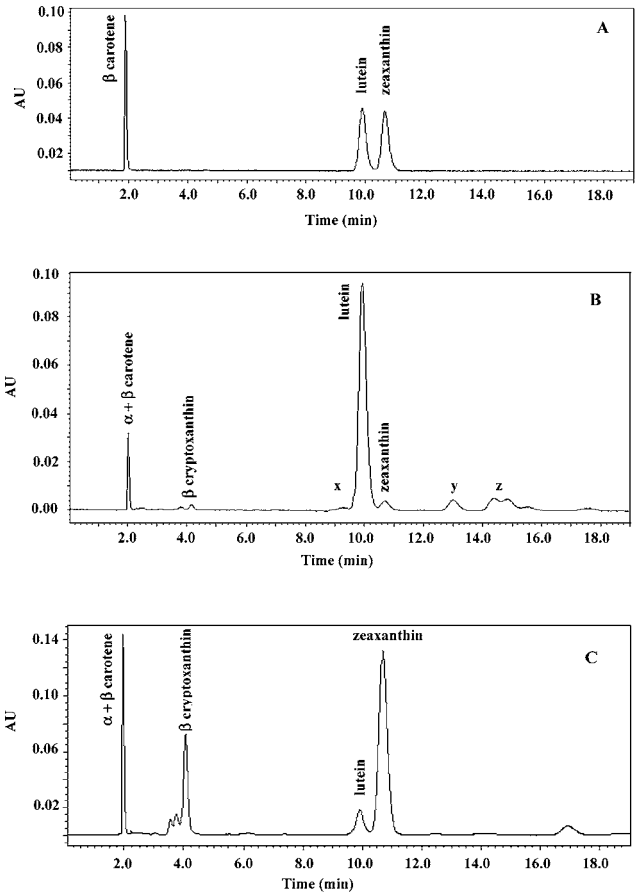

Figure 1. Typical chromatograms of carotenoids in a mixture of commercial standards (**A**), in wheat (**B**), and in maize samples (**C**). x–z represent compounds tentatively identified as other cis isomers of lutein (*3*).

carotenoids were present; therefore, the extracts of these food samples could be analyzed by HPLC under isocratic conditions, which allow separation of compounds of interest in a time suitable for routine analysis.

The chromatographic separation of carotenoids in a standard solution is reported in **Figure 1A**, whereas the separation of carotenoids in durum wheat and in maize samples is shown in panels **B** and **C**, respectively, of **Figure 1**. Apart from maize, lutein is the main carotenoid found in cereals, with a ratio to zeaxanthin ranging from 0.1 to 12 in the samples under investigation. Others minor peaks were detected during the chromatographic run. Some peaks were eluted near β-cryptoxanthin, in either the maize sample or the standard solution, and are probably isomers of β-cryptoxanthin (*3*). Other compounds (x−z) with retention times from 9 to 16 min have been tentatively identified as cis isomers (9, 9′, 13, 13′) of lutein as reported by some authors (*3*). All of these peaks are still under investigation.

Repeated calibrations with standard solutions during the analysis of a batch of samples were used to verify the assay precision. Results provided a successful determination of carotenoids within an acceptable precision range, the CV% being <4% for every carotenoid (data not shown). The linearity test for quantification was carried out over the range of 0.48−8.36 μg/mL for each carotenoid standard. Calibration curves were obtained by plotting the peak area of β-carotene, β-cryptoxanthin, lutein, and zeaxanthin versus the concentration of each compound. An excellent relationship was found with the r (correlation coefficient) values for each carotenoid near unity (>0.99). The detection limit values, defined as field blank + 3σ, where σ is the standard deviation of the field blank value signal (*19*) and expressed as the amount injected, were 0.03 ng for $\alpha+\beta$-carotene and zeaxanthin and 0.02 ng for lutein.

The isolation of carotenoids from natural products involves extraction and chromatography with organic solvents. There is no universally accepted or standard method for the extraction of carotenoids because of the wide variety of food products containing these compounds and the great range of carotenoids that can be found in these samples. Moreover, the instability of carotenoids can produce problems during their manipulation, which therefore requires particular attention.

To achieve a preparative procedure convenient to speed the extraction of cereal carotenoids with a reduced loss of carotenoid compounds and suitable for this food matrix, four different extraction techniques were evaluated: conventional extraction techniques such as Folch and Soxhlet, the traditional standard method for the extraction of durum wheat yellow pigments AACC 14-50, and hot saponification followed by solvent extraction.

Saponification, involving heating of the sample in a strong alkaline environment, reduces the amount of organic extractables. This is accomplished by hydrolysis of lipid esters to more hydrophilic fatty acids and alcohols, which, in a solvent system extraction, will remain in the aqueous phase. This decreases the load of material that will be extracted with carotenoids into the organic phase and injected into the analytical column. However, depending on the nature of the carotenoid and the food type (*20*), saponification may result in destruction or structural transformation; therefore, it is important to state the mildest conditions of extraction to do the job in the best way. These conditions usually involve protection from oxidation by addition of antioxidants, nitrogen flushing, limited exposure to light and oxygen, and rapid cooling of the reaction mixture after the saponification.

Table 1 reports carotenoid extraction yields from semolina samples and the percentage of recovery by using hot saponification followed by solvent extraction and the other tested methods. The saponification procedure used was chosen on the basis of that previously reported for the extraction of tocols from several products, including cereals (*16*, *21*, *22*), which proved to give good results with a reduced loss of analytes from the food matrix.

The paired t test proved that the difference between saponification and WSB was not statistically significant, although, when water *n*-butyl alcohol was used, the CV% values for some of the analyzed compounds rose to 25% in contrast with those of saponification, which were below 4%. Results obtained with the WSB method may be attributed to different factors such as the sample size, the several steps that make the analytical recovery difficult, and the probable presence of interfering compounds that may affect the HPLC determination. Moreover, due to the lack of difference found between the two tested methods, the data above-reported, in agreement with previous works (*23*), show that, in semolina samples, carotenoids are present as free compounds instead of their esters or other bounded forms.

If compared with the Soxhlet and Folch procedures, hot saponification followed by solvent extraction gave significantly ($p < 0.01$) higher recoveries. As to saponification, Soxhlet provided carotenoid recoveries varying from 38% for lutein to 78% for $\alpha+\beta$-carotene. As for the Folch method, which uses solvents with a higher polarity and therefore a higher extractive capacity, these recoveries ranged from 78% for $\alpha+\beta$-carotene to 108% for zeaxanthin.

Table 1. Carotenoid Content[a] (Milligrams per Kilogram of Dry Weight) in a Semolina Sample and Percentage of Recovery Using Different Extraction Procedures

carotenoid	hot saponification followed by solvent extraction (*A*)	WSB (*B*)	% recovery (*B*/*A* × 100)	Soxhlet (*C*)	% recovery (*C*/*A* × 100)	Folch (*D*)	% of recovery (*D*/*A* × 100)
α+β-carotene	0.09 ± 0.00	0.11 ± 0.02	122	0.07 ± 0.00	78	0.07 ± 0.00	78
lutein	2.69 ± 0.05	2.80 ± 0.33	104	1.01 ± 0.01	38	2.25 ± 0.14	84
zeaxanthin	0.13 ± 0.00	0.12 ± 0.03	92	0.06 ± 0.00	46	0.14 ± 0.00	108

[a] Mean of three determinations.

Table 2. Analytical Recoveries[a] of Carotenoids Added to a Semolina Sample

carotenoid	theoretical (natural + added) (μg)	found (μg)	recovery (%)
β-carotene	0.70	0.72 ± 0.02	102.86 ± 2.81
lutein	6.59	6.29 ± 0.16	95.44 ± 2.22
zeaxanthin	1.02	0.95 ± 0.02	93.14 ± 1.50

[a] Mean ± SD of three determinations.

Table 3. Carotenoid Content[a] (Milligrams per Kilogram of Dry Weight) in a Certified Reference Sample Using Hot Saponification Followed by Solvent Extraction

carotenoid	declared[b]	found
α+β-carotene	35.4 ± 1.9	32.2 ± 0.8
lutein	12.5 ± 0.8	11.4 ± 0.4
lutein + zeaxanthin	22.3 ± 1.3	21.1 ± 0.5

[a] Mean ± SD of three determinations. [b] Determined by reverse-phase HPLC.

In comparison with the tested methodologies, the higher effectiveness of the saponification procedure was also confirmed by clearer extracts and shorter analytical times, which did not require further purification steps to remove interfering substances.

In light of these results saponification was chosen as an elective method for the extraction of carotenoids from cereals.

To determine recovery of added analytes under the established extraction procedure, a standard solution of β-carotene, lutein, and zeaxanthin was added to a semolina sample at concentration levels close to those expected in this sample, which was submitted to the entire procedure of saponification.

Table 2 shows the relevant results. A quantitative recovery of the analytes was found, being ∼100% for β carotene, 95% for lutein, and 93% for zeaxanthin; the good repeatability was confirmed by the standard deviations (SD) ranging from 1.50 to 2.81.

The proposed method was also tested on a sample certified for carotenoid content. There is no certified material for cereal carotenoids available; therefore, a standard BCR reference sample for mixed vegetables was used. Three replicates of the reference material were assayed according to the proposed method. Mean data are reported in **Table 3**. The differences between found values and certified ones are not statistically different at 95% confidence interval. Results reported above demonstrate that the used assay procedure for the determination of carotenoids in cereals provided reliability, a good precision, accuracy, and repeatability, without significant losses.

Several samples of different cereal species and their byproducts were analyzed using the proposed method. Average results are reported in **Table 4**, where carotenoids are expressed as single compounds and as total carotenoids.

Maize was the sample with the highest level of carotenoids [11.14 mg/kg of dry weight (dw)]; this is mainly due to the higher contributions of α+β-carotene, β-cryptoxanthin (2.40 mg/kg of dw), not detectable in other cereals, and above all zeaxanthin (6.43 mg/kg of dw).

With the exception of maize, which shows a typical carotenoid composition, the main compound detected in other cereals was lutein, followed by zeaxanthin and α+β-carotene. Lutein shows the highest levels in durum wheat (2.65 mg/kg of dw), followed by dicoccon (1.78 mg/kg of dw), spelt (1.46 mg/kg of dw), and soft wheat (1.31 mg/kg of dw). Oat was the species with the lowest amount of lutein (0.23 mg/kg of dw); its ratio to zeaxanthin was ∼2.

The highest levels of α+β-carotene were found in maize (1.44 mg/kg of dw), whereas in other species they range from a minimum of 0.01 mg/kg of dw in oat to a maximum of 0.14 mg/kg of dw in durum wheat.

A comparison with the literature related to HPLC analysis of carotenoids is very difficult due to the few data available obtained by different methods and the fact that these pigments vary depending on genotype and location.

Our data are quite in agreement with those of Chung (*24*), who reported xanthophyll amounts of 1.67 mg/kg in durum and

Table 4. Average Carotenoid Content (Mean ± SD) in Samples of Different Cereal Species and Cereal Byproducts

species	no. of samples	carotenoid (mg/kg of dw)				
		α+β-carotene	β-cryptoxanthin	lutein	zeaxanthin	totals
oat	1	0.01		0.23	0.12	0.36
spelt	3	0.04 ± 0.01		1.46 ± 0.44	0.12 ± 0.03	1.62 ± 0.45
dicoccon	3	0.05 ± 0.02		1.78 ± 0.37	0.19 ± 0.04	2.02 ± 0.41
durum wheat	14	0.14 ± 0.04	traces	2.65 ± 0.65	0.26 ± 0.04	3.05 ± 0.72
soft wheat	3	0.05 ± 0.01	traces	1.31 ± 0.10	0.14 ± 0.06	1.50 ± 0.16
maize	1	1.44	2.40	0.87	6.43	11.14
barley	3	0.05 ± 0.01		0.86 ± 0.12	0.30 ± 0.15	1.21 ± 0.27
flour	3	0.03 ± 0.01		1.43 ± 0.04	0.06 ± 0.03	1.52 ± 0.06
semolina	14	0.10 ± 0.06		2.48 ± 0.48	0.11 ± 0.02	2.69 ± 0.53
soft wheat germ	2	0.91 ± 0.08	traces	1.71 ± 0.05	2.96 ± 0.01	5.58 ± 0.44
durum wheat germ	2	0.68 ± 0.07	traces	2.60 ± 0.07	2.14 ± 0.01	5.42 ± 0.57

1.33 mg/kg in soft wheat, and with those of Hentschel et al. (*23*), in which lutein ranges from 1.5 to 4.0 mg/kg. Other authors (*3*, *25*) reported different levels depending on the carotenoid variability discussed above. For maize, lutein levels are reported by some authors (*26*) to range from 0.00 to 27.59 mg/kg of dw, whereas zeaxanthin and β-cryptoxanthin ranged from 0.01 to 7.73 mg/kg of dw and from 0.07 to 2.40 mg/kg of dw, respectively.

Few data (*23*, *24*), mostly obtained with methods that do not use HPLC and which are unable to distinguish the different carotenoids, are present in the literature on the localization of carotenoids along the kernel. Analysis of cereal fractions (**Table 4**) shows low differences between lutein levels in semolina and flour and those in durum and soft wheat germ; this accounts for its equal distribution along the kernel. On the contrary, in flour and in semolina samples lower amounts of $\alpha+\beta$-carotene and zeaxanthin were found if compared with their levels in wheat germ, where they seem to be principally located. Because wheat germ represents only 3−5% of the kernel, the above contents cannot influence levels in the whole grain or in whole meals.

Data reported show that the setup method could also be used to verify the qualitative and the quantitative distribution of carotenoid compounds in the kernel fractions and in cereal byproducts.

In conclusion, the proposed method is reliable and relatively fast for the determination of carotenoid pigments in cereals and cereal byproducts. It allows the determination of the different carotenoid compounds with particular regard to the concentration of lutein and zeaxanthin, which are the main carotenoids in cereal products and are of importance in view of their health implications. Moreover, taking into account the variability of the contents of these pigments, the method could also be used for genetic, biological, and technological purposes in cereal science.

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