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ARTICLES

Development of a New Method for the Complete Extraction of Carotenoids from Cereals with Special Reference to Durum Wheat (*Triticum durum* Desf.)

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Due to the growing interest in the role of carotenoids in human health, their qualitative and quantitative analysis in foods is becoming more and more important. High-performance liquid chromatography has become the method of choice for the determination of these phytochemicals. A crucial step prior to the chromatographic separation is the quantitative extraction from the food matrix which was proven to be impeded in durum wheat. To optimize the extraction procedure, several factors with influence on extractability of carotenoids were investigated. Finally, it was shown that soaking of samples in water for 5 min prior to extraction with organic solvents had the strongest impact on extraction yield and led to the most rapid and gentle method. Contents of carotenoids in the extracts of several durum wheat and corn samples were doubled by soaking in water before extracting with methanol/tetrahydrofuran (1/1, v/v). In light of these findings, literature data on contents of carotenoids in cereal grains have to be viewed critically regarding the extraction procedures employed.

KEYWORDS: Cereals; durum wheat; extraction; carotenoids; lutein; yellow pigment

INTRODUCTION

Carotenoids are one of the most important classes of plant pigments. Today, about 700 carotenoids are known, which are divided into carotenes (e.g., α-carotene, *β*-carotene, lycopene) and the oxygenated xanthophylls (e.g., lutein, zeaxanthin, *β*-cryptoxanthin). Among them, α-carotene and *β*-carotene as well as *β*-cryptoxanthin are the major provitamin A active carotenoids. In plants, carotenoids play a crucial role in light harvesting for photosynthesis and in protection of chlorophyll against oxidative damage. These functions can also be the reason for their properties in humans. Epidemiological studies have shown associations between intake of fruits and vegetables rich in carotenoids and reduced risks of different types of cancer, cardiovascular diseases, and age-related macular degeneration (AMD) (*1*). In particular, the carotenoids in cereals, lutein and zeaxanthin, play an important role in the prevention of frequently occurring eye diseases like AMD, cataracts, and retinitis pigmentosa (*2*). Even though cereal grains contain far fewer carotenoids than most vegetables and fruits, they are consumed frequently in considerable amounts.

Besides their nutritional value, carotenoids are suggested to be responsible for the bright yellow color of durum wheat semolina and pasta products. The color of the end products, which is one of the most important quality criteria for customers, is based on pigmentation of durum wheat varieties used for manufacturing, as well as on processing conditions and enzyme activities (*3*). Thus, breeders and manufacturers endeavor to produce raw materials with high pigmentation and to retain the color during pasta production, respectively. Today, three major methods to evaluate the color of durum semolina and pasta are in use: visual comparison with standard samples, light reflectance measurement (colorimetric method), and spectrophotometrical determination after chemical pigment extraction (*4*). Usually, yellow pigments of durum wheat semolina, flour, or pasta are extracted according to ICC standard method 152 (*5*) or AACC standard method 14–50 (*6*). Both are based on the extraction of pigments with water-saturated 1-butanol and subsequent spectrophotometrical measurement with *β*-carotene as the reference substance. If samples do not contain any *β*-carotene, obtained values might differ by about 5% from real contents (*5*). In fact, since the 1930s, several investigations have shown that the predominant pigments in durum wheat are xanthophylls (*7*) with a preponderance of lutein (*8*). Regarding the occurrence of carotenes, the results have been contradictory. Besides measuring total absorption at a specific wavelength

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related to a reference substance, these data were obtained by open column chromatography and spectrophotometrical quantification of the separated fractions (*9*).

For several decades, these findings have not been checked using modern analytical methods. However, to maintain the yellow color during pasta processing, the knowledge of the accurate chemical composition of the yellow pigments is a precondition. High-performance liquid chromatography (HPLC) has become the method of choice for the determination of carotenoids. Recent investigations aimed at verification of former findings with modern chromatographical methods. Panfili et al. (*9*) and Fratianni et al. (*10*) used normal-phase HPLC to confirm the predominant occurrence of lutein in durum wheat. Besides this, the authors detected low amounts of zeaxanthin as well as α- and β-carotene, which could not been separated. Minor compounds were tentatively identified as (*Z*)-isomers of lutein (*9*). Other authors also ascertained (all-*E*)-lutein as the main carotenoid in wheat, followed by (all-*E*)-zeaxanthin (*11–13*). Further compounds were identified as different (*Z*)-isomers of lutein (*11*, *12*) and zeaxanthin (*12*). Abdel-Aal et al. additionally found (all-*E*)-β-carotene in traces (*11*). Nevertheless, until today the complete identification of the yellow pigments in durum wheat and products thereof is lacking. Hentschel et al. (*14*) compared the contents of yellow pigments and carotenoids of durum wheat. The authors found lutein and very low amounts of zeaxanthin in different durum wheat cultivars. Surprisingly, the fraction of carotenoids amounted to only 30–50% of the yellow pigment content, indicating the presence of additional color-producing compounds in durum wheat which have not yet been identified. This assumption is supported by recent studies of Leenhardt et al. (*13*) and Abdel-Aal et al. (*11*) who also found discrepancies between yellow pigment content measured photometrically and content of carotenoids analyzed with HPLC. Our investigations on whole durum wheat grain, however, have shown an incomplete extraction of xanthophylls from the food matrix using common extraction with organic solvents. These findings challenge the contribution of yellow compounds other than carotenoids to the yellow pigment content.

For hydrophilic phytochemicals, e.g., phenolic compounds, some investigations on extraction efficiency exist. In general, these phytochemicals are difficult to extract because many of them are bound to cell wall materials (*15*). Thus, phytochemical contents and hydrophilic antioxidant capacities of grains are commonly underestimated in the literature because bound phytochemicals are not included (*16*). In contrast, literature data on the extractability of lipophilic phytochemicals from cereals are scarce, and possible associations among carotenoids and other ingredients in cereals are not currently known.

Thus, the present investigation aimed at the evaluation of several factors affecting the extractability of carotenoids from whole durum wheat grain. On this basis, the extraction procedure for carotenoids of durum wheat was optimized. As a reference for complete extraction, the content of yellow pigments, extracted with water-saturated 1-butanol, was used. The improved extraction method was also tested on durum wheat semolina and different corn (*Zea mays* L.) samples.

MATERIALS AND METHODS

Chemicals. All chemicals for extraction were of analytical grade; solvents for chromatography were of HPLC quality. The carotenoid standards (all-*E*)-lutein, (all-*E*)-zeaxanthin, (all-*E*)-β-cryptoxanthin, (all-*E*)-β-carotin, and (9*Z*)-β-carotin were purchased from CaroteNature (Lupsingen, Switzerland). They were dissolved in cyclohexane/toluene (4/1, v/v) and stored in the dark at −30 °C. Concentrations of stock solutions were calculated periodically using their absorption maxima and appropriate extinction coefficients. For preparing working solutions, stock solutions were diluted daily 1:50 with methanol.

Sample Preparation. Different durum wheat cultivars (Prowidur, Orjaune) harvested in 2001 and 2002, respectively, were obtained from the Federal Research Center for Nutrition and Food (BfEL, Detmold, Germany). Three different samples of durum wheat semolina as well as one corn semolina and one corn flour were purchased at local markets. One corn sample (whole grain) was received from the Institute of Animal Physiology and Animal Nutrition, Georg August University Göttingen (Göttingen, Germany). For all steps of the method improvement, Prowidur from 2001 was used. Whole grains were ground before analysis using a laboratory mill model Grindomix GM 200 (Retsch, Haan, Germany). To obtain various particle sizes, whole grain was gradually ground and separated by sieving. Three fractions with the following particle sizes were collected: 1–2 mm, 0.2–0.5 mm, and 0.1–0.2 mm.

Extraction of Carotenoids. The procedures mentioned below apply to durum wheat samples. Modifications that were made for corn samples are given in parentheses at the appropriate position. All operations were performed under dim light conditions. For common solvent extraction, MgO and β-apo-8′-carotenal (internal standard) were added to approximately 5 g of durum wheat (2 g of corn sample). Samples were extracted with methanol/tetrahydrofuran (1/1, v/v) containing 0.1% BHT by homogenization on ice for 5 min using an ultra turrax (type T25, IKA-Werke, Staufen, Germany). The extract was filtered under vacuum through filter paper no. 390 (Filtrak, Niederschlag, Germany) on a Büchner funnel. This extraction was repeated twice. Combined extracts were rotary-evaporated under reduced pressure at 30 °C until dryness. The residue was redissolved in 5 mL methanol (10 mL of extraction-solvent) using an ultrasonic bath. Fifty microliters of this solution was injected into the HPLC system.

With regard to achieving a complete extraction, several factors influencing extraction yield were examined. These were (i) extraction time, (ii) extraction temperature, (iii) permanent shaking of samples with solvent, (iv) soaking in water prior to solvent extraction, and (v) particle size of ground durum wheat. For shaking samples with solvent, a water bath model GFL 1086 (GFL, Burgwedel, Germany) was used. Shaking was performed at 25 ± 1 °C at a frequency of 13 min^{-1}. After shaking, samples were extracted using an ultra turrax as described above. Fractions with different particle sizes were extracted using an ultra turrax or a shaker-incubator model ES-20 (Peqlab, Erlangen, Germany). The shaker was used instead of the ultra turrax, shaking samples 3 times each for 5 min with a frequency of 250 rpm at room temperature (22 ± 2 °C). A summary of different extraction procedures is given in **Figure 1**.

Analysis of Carotenoids. Chromatography was performed by means of a Merck (Darmstadt, Germany) HPLC analytical system composed of a solvent delivery system model L-6200A, an autosampler model AS-2000A, and a photodiode array detector (DAD) model L-4500. Chromatographic separation of durum wheat extracts was achieved within 40 min using a C_{30} reversed-phase column (250 mm × 4.6 mm, 5 μm) (Trentec, Gerlingen, Germany), preceded by a ProntoSil 120-5-C18 H guard column (10 mm × 4.0 mm, 5 μm) (Bischoff, Leonberg, Germany). Columns were tempered at 25 ± 1 °C by means of a column oven model CTO-10AC (Shimadzu, Duisburg, Germany). The mobile phase was methanol/water (99/1, v/v) at a flow rate of 1.3 mL min^{-1}. For corn samples, a separation of carotenoids was achieved within 70 min at 23 ± 1 °C at the same flow rate using methyl *tert*-butyl ether (solvent A) and methanol (solvent B) as the mobile phase. The gradient procedure was as follows: 35 min linear gradient from 10% to 45% solvent A, 10 min linear gradient from 45% to 60% solvent A, 60% solvent A for 13 min, and 12 min linear gradient to initial conditions of 10% solvent A. Identification of several carotenoids was accomplished by comparing the retention times and DAD absorbance spectra to those of external reference materials. (*Z*)-Isomers of lutein and zeaxanthin were tentatively identified by comparison to retention times and DAD absorbance spectra of isomerized standard solutions and quantified using (all-*E*)-lutein and (all-*E*)-zeaxanthin, respectively. (*Z*)-Isomers of lutein and zeaxanthin standards were obtained by iodine-

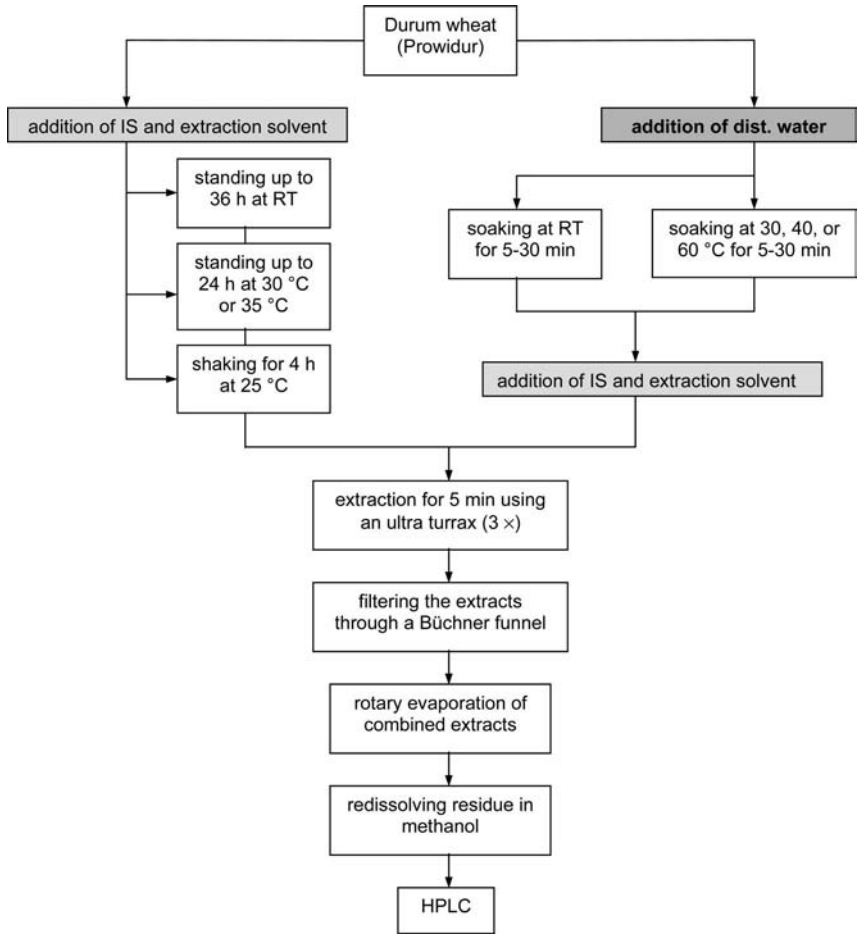

Figure 1. Overview of procedures tested for quantitative extraction of carotenoids from durum wheat. IS: internal standard. RT: room temperature.

catalyzed photoisomerization of the (all-*E*)-carotenoid standards according to Zechmeister (*17*). Quantification of individual carotenoids was conducted via the peak areas considering the recovery of the internal standard.

Determination of Yellow Pigment Content. Yellow pigments were extracted overnight (16–18 h) with water-saturated 1-butanol according to the ICC standard method 152 (*5*). In light of the preponderance of lutein in durum wheat, the method was modified regarding the reference substance. Hence, absorbances of sample extracts as well as of (all-*E*)-lutein standard solutions (0.25–1.25 μg/mL) were determined at the absorption maximum of lutein in 1-butanol (446 nm) by using an UV–vis spectrophotometer model V-530 (Jasco, Gross-Umstadt, Germany).

Statistical Analysis. Determinations were conducted in triplicate. Results are presented as means ± standard deviation (SD). To ascertain differences between means, the *t* test or one-way analysis of variance (ANOVA) followed by the Student–Newman–Keuls (SNK) procedure was performed using *SPSS 13.0* for Windows (SPSS Inc., Chicago, IL). Differences were considered to be significant at $p < 0.05$. The influence of extraction time on extraction yield was determined as a single measurement. Due to the analysis of carotenoids at defined time intervals, multiple determinations could not be realized in practice. Thus, a statistical evaluation was not possible.

RESULTS AND DISCUSSION

Yellow Pigments. Yellow pigments (expressed as lutein) in durum wheat grains and commercial semolina ranged between 0.39 and 0.58 mg/100 g dry matter (dm), respectively (**Table 1**). The ICC standard method 152 is suitable for durum semolina and flour as well as for pasta with and without eggs (*5*). In the case of corn, the photometric value related to a single reference substance was not comparable to carotenoid contents determined using HPLC, because corn samples contained several carotenoids other than lutein in appreciable amounts (**Table 2**). Hence, for corn samples yellow pigment content was not measured.

Table 1. Contents of Carotenoids [mg/100 g dm] of Six Different Durum Samples Depending on Extraction Procedure in Comparison To Yellow Pigment Content [mg/ 100 g dm][a,b,c]

sample	carotenoids[A] (HPLC) SE[B]	carotenoids[A] (HPLC) water + SE	yellow pigments (photometrically)
Prowidur 2001	0.168 ± 0.009[a]	0.371 ± 0.006[b]	0.393 ± 0.006[c]
Prowidur 2002	0.208 ± 0.012[a]	0.509 ± 0.018[b]	0.568 ± 0.018[c]
Orjaune 2002	0.199 ± 0.014[a]	0.446 ± 0.005[b]	0.476 ± 0.004[c]
semolina 1	0.214 ± 0.009[a]	0.593 ± 0.035[b]	0.577 ± 0.004[b]
semolina 2	0.264 ± 0.004[a]	0.524 ± 0.009[b]	0.504 ± 0.003[c]
semolina 3	0.244 ± 0.012[a]	0.542 ± 0.010[b]	0.540 ± 0.017[b]

[A] Lutein + zeaxanthin. [B] Solvent extraction. [a,b,c] Values with different superscript letters in the same row are significantly different (ANOVA, $p < 0.05$).

Carotenoids. In agreement with former investigations (*11–14*), the main carotenoid found in all durum wheat samples was (all-*E*)-lutein. (all-*E*)-Zeaxanthin was detected in very low amounts (<0.05 mg/100 g dm). Samples did not contain xanthophyll esters or any carotenes. Some small peaks were tentatively identified as (*Z*)-isomers of lutein and zeaxanthin (**Figure 2**). For better comparability to the yellow pigment content, carotenoids in durum wheat and semolina are defined in our investigations as the sum of lutein and zeaxanthin including their (*Z*)-isomers.

Extraction Solvents. For the determination of yellow pigments in durum wheat and products thereof, standard methods

Table 2. Comparison of Contents of Carotenoids [mg/100 g dm] in the Extracts of Different Corn Samples with and without Soaking in Water Prior To Solvent Extraction[A,C]

sample	lutein	zeaxanthin	β-cryptoxanthin	β-carotene[B]
whole grain	0.441 ± 0.067	0.182 ± 0.023	0.013 ± 0.002	0.047 ± 0.003
	1.113 ± 0.024*	**0.425 ± 0.008***	**0.034 ± 0.008***	**0.106 ± 0.011***
semolina	0.408 ± 0.048	0.388 ± 0.039	0.077 ± 0.011	0.043 ± 0.008
	0.972 ± 0.023*	**0.931 ± 0.072***	**0.170 ± 0.016***	**0.098 ± 0.004***
flour	0.556 ± 0.003	0.457 ± 0.002	0.058 ± 0.003	0.042 ± 0.002
	1.025 ± 0.065*	**0.847 ± 0.042***	**0.097 ± 0.010***	**0.063 ± 0.004***

[A] Bold values were obtained by soaking in water prior to solvent extraction. [B] (*E*)-β-carotene + (9*Z*)-β-carotene. [C] * denotes values significantly higher than those obtained without soaking in water (*t* test, $p < 0.05$).

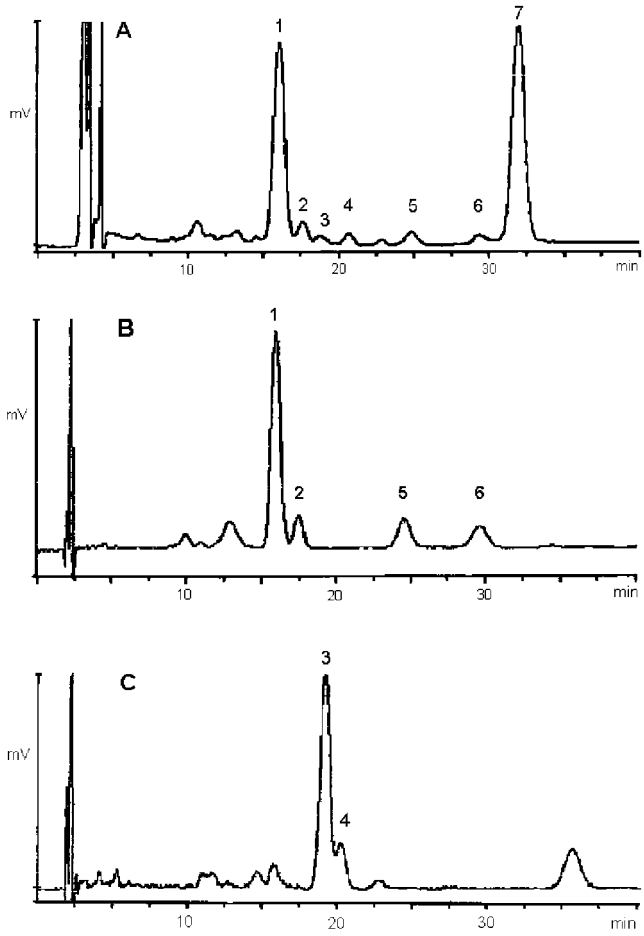

Figure 2. HPLC chromatograms of durum wheat cultivar Prowidur (A) and isomerized standards of lutein (B) and zeaxanthin (C). 1, (all-*E*)-lutein; 3, (all-*E*)-zeaxanthin; 2, 5, and 6, tentatively identified as (*Z*)-lutein-isomers; 4, tentatively identified as (*Z*)-zeaxanthin-isomer; 7, β-apo-8′-carotenal (internal standard).

based on an extraction using water-saturated 1-butanol have existed for a long time. Water-saturated 1-butanol was suggested to be the best solvent for extracting wheat pigments in a comparative study of 60 organic solvents (*18*). Thus, this solvent is prescribed in the standard methods of the ICC and AACC. Also, for extracting carotenoids from wheat grains, water-saturated 1-butanol seemed to be more efficient than other organic solvents such as tetrahydrofuran (*11, 19*), methyl *tert*-butyl ether, 80% aqueous ethanol, and 80% aqueous methanol (*11*). Previous investigations in our laboratory have also shown that mixtures of methanol/tetrahydrofuran (1/1, v/v) and methanol/methyl *tert*-butyl ether (1/1, v/v), respectively, are less effective than water-saturated 1-butanol in extracting yellow pigments (unpublished results).

At room temperature, 1-butanol is theoretically able to dissolve nearly 20% of water. Thus, for the analysis of carotenoids according to the procedure described here, water-saturated 1-butanol is not suitable because it is neither compatible with the mobile phase used for chromatographic separation nor can it be rotary-evaporated. For extracting carotenoids there are no universally accepted methods. In practice, several organic solvents or solvent mixtures are in use (*20*). Scott (*21*) tested various solvents for their effectiveness in extracting carotenoids from vegetables. Methanol was the best solvent for dry materials, while for liquid materials, THF was most effective. Thus, a combination of both solvents appeared to result in efficient extraction and solubilization of carotenoids. This procedure was further developed by Hart and Scott (*22*) and tested on a wide range of vegetable materials. In an interlaboratory study conducted by 17 European laboratories, this extraction procedure using methanol/tetrahydrofuran (1/1, v/v), named "common procedure", was compared to different "in-house" procedures (*23*). The common extraction method tended to result in higher concentrations of carotenoids, especially of xanthophylls, in the extracts, while the in-house procedures showed higher variations (34%) than the common procedure (21%). Thus, methanol/tetrahydrofuran (1/1, v/v) containing 0.1% BHT for stabilization of carotenoids was chosen as extraction solvent.

Durum wheat samples were extracted until the solvent remained colorless, which can usually be seen as an indication of a complete extraction from the food matrix. Using this procedure, carotenoids (lutein + zeaxanthin) amounted to approximately 40% of yellow pigments in durum wheat grains and 45% in commercial durum wheat semolina, which confirmed the observations of Hentschel et al. (*14*). In contrast to other carotenoid-containing foodstuffs, the solid residue of durum wheat samples still showed a yellow color after this solvent extraction. Contrary to the suggestion of Hentschel et al. (*14*) that substances other than carotenoids may contribute to the yellow color of durum wheat, the following data demonstrate that a quantitative extraction of carotenoids from the food matrix did not take place with the employed extraction procedure.

Extraction Time. Due to the susceptibility of carotenoids to light, heat, air, and active surfaces, their isolation and analysis may be accompanied by degradation, structural rearrangement, formation of stereoisomers, and other physicochemical reactions (*22*). Therefore, rapid and gentle extraction procedures are usually preferred. However, for determination of yellow pigments according to the ICC standard method, an extraction overnight is required. Hence, the extraction time for analysis of carotenoids was also increased. In fact, it was observed that leaving the samples with methanol/tetrahydrofuran (1/1, v/v, +0.1% BHT) for some hours resulted in higher contents of carotenoids in the obtained extracts. To ascertain the optimal extraction time, the proportion of carotenoids extracted with

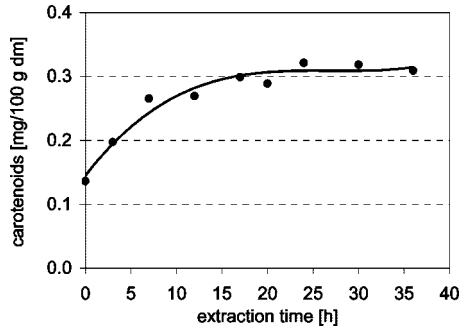

Figure 3. Content of carotenoids (lutein + zeaxanthin) in durum wheat cultivar Prowidur depending on extraction time at room temperature.

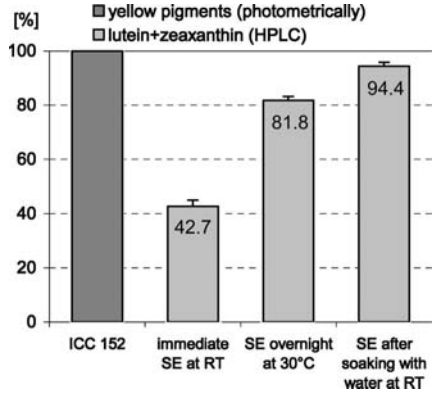

Figure 4. Comparison of different extraction procedures for carotenoids from durum wheat cultivar Prowidur in relation to yellow pigment content (100%). SE: solvent extraction. RT: room temperature.

the solvent mixture was determined depending on time at room temperature (22 ± 2 °C). As expected, the amount of carotenoids in the extract increased with the course of time. A maximum yield was achieved after 24 h, resulting in a doubling of the content of carotenoids in the extract (**Figure 3**). For such long extraction times, an addition of 0.1% BHT to the extraction solvent was proven to be necessary.

Permanent Shaking during Extraction. Parallel to the extraction at room temperature for 24 h, samples were permanently shaken with the solvent, and the content of carotenoids in the extract was analyzed hourly in the first 4 h of extraction using an ultra turrax as described in the Methods section. These results were compared to the samples only standing with solvent without shaking. The assumption that permanent shaking or stirring of samples with solvent would increase or accelerate the extraction was not confirmed (data not shown).

Extraction Temperature. Using a temperature of 30 °C, the extraction yield slightly increased in comparison to room temperature. An extraction temperature of 35 °C had no further effect. With both temperatures, the maximum carotenoid extraction yield had already been reached after 12–16 h followed by a plateau (data not shown). As a result, an extraction overnight (16–18 h according to ICC method 152) at a temperature of 30 °C was suggested as suitable at that time. At substantially higher temperatures, improved extractability of carotenoids might be accompanied by degradation processes. In the literature, contradictory findings for the influence of temperature on carotenoids are discussed. Heating of carotenoid solutions normally leads to a decrease of carotenoid concentrations dependent on temperature and heating time (*24–26*). Thermal processing of vegetables may also cause losses of carotenoids (*27–30*). In particular, xanthophylls, e.g., lutein, are found to be very sensitive to heat treatment (*29, 30*). However, stability differs in different foods even when the same processing conditions are used (*31*). While Khachik et al. (*32*) ascertained that levels of carotenoids in different vegetable samples remained unchanged under mild cooking conditions, some studies found higher contents of carotenoids in thermally processed vegetables in comparison to raw ones (*33–35*). This is usually explained by increased extractability from the food matrix after cooking, steaming, or microwave heating, which is due to the destruction of cellular structures and denaturation of carotenoid-protein complexes (*31, 32*). However, an alteration of moisture content and leaching of soluble solids during thermal processing as well as enzymatic carotenoid destruction in raw samples are usually not considered when comparing carotenoid contents of raw and cooked vegetables (*31*).

Soaking in Water. The solvent for extracting yellow pigments contained approximately 16% water. Originally, saturating 1-butanol with water aimed at clarifying the extracts because the alcohol alone showed a tendency to yield turbid extracts which could not be clarified by centrifuging (*18*). Surprisingly, our investigations have shown that water plays an essential role for the quantitative extraction of carotenoids from cereal grains. Only 5 min of soaking at room temperature (22 ± 2 °C) prior to extraction with organic solvents was sufficient for a complete extraction. Very coarse material needed a longer soaking time, e.g., 30 min. Best results were obtained with 5 mL water for 5 g of sample. Soaking in 3 mL water was presumably insufficient for a complete wetting of the sample and therefore resulted in lower extraction yields. Soaking in 7 mL water complicated the removal of water prior to analysis and yielded lower recovery of the internal standard. To remove the water prior to HPLC, samples were filtered over sodium sulfate, and approximately 2 mL ethanol was added at the end of rotary evaporation.

Soaking in water at 30 and 40 °C, respectively, had no significant impact on the extraction yield. However, soaking at 60 °C resulted in significantly ($p < 0.05$) lower content of carotenoids in comparison to the other temperatures, which might be partially explained by the heat sensitivity of carotenoids (see former section). Additionally, at this temperature an incipient gelatinization of starch created an adhesive dough which was poorly extractable with organic solvents.

As a result of these investigations, soaking of samples in water at room temperature prior to solvent extraction led to quantitative extraction of carotenoids and replaced the extraction overnight

Table 3. Influence of Particle Size of Ground Durum Wheat Cultivar Prowidur on Extractability of Carotenoids[A] [mg/100 g dm][a,b,c]

	solvent extraction (SE)			soaking in water prior to SE		
fraction	ultra turrax		shaker	ultra turrax		shaker
1–2 mm	0.063 ± 0.001[a]	*	0.022 ± 0.003[a]	0.263 ± 0.050[a]	*	0.136 ± 0.015[a]
0.2–0.5 mm	0.071 ± 0.000[b]		0.076 ± 0.004[b]	0.260 ± 0.008[a]		0.258 ± 0.009[b]
0.1–0.2 mm	0.106 ± 0.006[c]		0.118 ± 0.001[c]	0.266 ± 0.010[a]		0.268 ± 0.012[b]

[A] Lutein + zeaxanthin. [a,b,c] Values with different superscript letters in the same column are significantly different (ANOVA, $p < 0.05$). * Significant differences between extraction using ultra turrax and shaker (*t* test, $p < 0.05$).

with organic solvents. **Figure 4** compares the contents of carotenoids in the extracts after different extraction procedures in relation to the yellow pigment content.

Particle Size. As expected, the particle size of ground grains had a substantial influence on the extraction yield of carotenoids using common solvent extraction. With decreasing particle size, the concentration of carotenoids in the extract increased (**Table 3**). In contrast, soaking in water prior to solvent extraction using an ultra turrax resulted in the same contents of carotenoids in all three fractions. Utilization of an ultra turrax for extraction instead of shaking yielded significantly higher amounts of carotenoids ($p < 0.05$) only for very coarsely ground wheat (particles of 1–2 mm). Small particle sizes are often difficult to obtain because grains are very hard and would need intense grinding. However, this can cause heating of samples and thereby lead to isomerization or degradation of carotenoids. In general, particle sizes of $\leq$0.5 mm, which are also prescribed in the ICC standard method 152 (*5*), are suitable using an ultra turrax or a shaker during extraction. For extracting coarsely ground samples, the use of an ultra turrax is favored over shaking.

Application of Optimized Procedure. The optimized extraction procedure was tested on different durum wheat and corn samples. For whole durum grains and commercial durum semolina, carotenoids amounted to 90–95% and 100% of the yellow pigment content, respectively. Yellow pigment content of whole wheat flour determined according to ICC method 152 might be enhanced by pigments of the seed coat, which are detected photometrically but not by HPLC. This can be an explanation for greater differences between contents of carotenoids and yellow pigments within whole durum wheat in comparison to durum semolina (**Table 1**). Slight variations between these two parameters might further be due to differences in the analytical methods.

The positive impact of soaking in water on the extraction of carotenoids was also confirmed for different corn samples (whole grain, semolina, and flour). Soaking in water prior to organic solvent extraction resulted in carotenoid contents in the extracts about 1.5–2.5-fold higher than without soaking (**Table 2**).

To answer the question of whether the low moisture content of cereal products could be the reason for this phenomenon, some vegetables were analyzed as raw samples as well as freeze-dried. It was supposed that soaking in water results in better wettability of samples with the extraction solvent. However, for freeze-dried carrots and tomatoes, an incomplete extraction of carotenoids with methanol/tetrahydrofuran (1/1, v/v, +0.1% BHT) was not observed. The quantitative extraction was achieved with the organic solvent mixture within short extraction times (data not shown). Thus, the incomplete extraction of carotenoids from cereal products may be caused by their special food matrix. The location of carotenoids within the kernel or the association with special kernel structures as well as interactions with other cereal ingredients might be responsible. As already mentioned, carotenoids are often associated with proteins in the form of chlorophyll-carotenoid-protein complexes in photosynthetic membranes of chloroplasts (*36*, *37*). In chromoplasts, carotenoids are also sequestered as crystals (e.g., lycopene in tomatoes) or dissolved in oil droplets (*31*, *37*). In carrots, for example, carotenoids are present in the crystalline form which is less available than well-dispersed carotenoids (*38*). For cereal grains, such data have not been available so far. Information about interactions of carotenoids with other cereal ingredients that might influence their extractability is also lacking at present.

In conclusion, it was ascertained that carotenoids are not quantitatively extractable from some cereal grains using common solvent extraction methods. Thus, contents of carotenoids in cereals and products thereof may be underestimated in the literature. For evaluation of literature data, a critical view of the employed extraction procedures is needed. An optimization of the extraction method resulted in a complete extraction of carotenoids from the food matrix. As a result, it was ascertained that the yellow pigment content of durum wheat and its products consists only of carotenoids whose extraction might be constricted by interactions with other grain ingredients. The extractability of carotenoids from further wheat cultivars and other types of cereals as well as the cause of impeded extractability from durum wheat and corn are still under investigation.

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