JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2002,** *50,* 6663−6668 **6663**

Spectrophotometric Determination of Yellow Pigment Content and Evaluation of Carotenoids by High-Performance Liquid Chromatography in Durum Wheat Grain

VERENA HENTSCHEL,[†] KATJA KRANL,[†,‡] JÜRGEN HOLLMANN,[†] MEINOLF G. LINDHAUER,[†] VOLKER BÖHM,*[,‡] AND ROLAND BITSCH[‡]

Federal Centre for Cereal, Potato and Lipid Research, D-32756 Detmold, Germany, and Institute of Nutrition, Friedrich-Schiller-University Jena, D-07743 Jena, Germany

The so-called "yellow pigment" content of durum wheat has been used for a long time as an indicator of the color quality of durum wheat and pasta products. For decades the chemical nature of these pigments has been assigned to carotenoids, mainly to the xanthophyll lutein and its fatty acid esters. The chemical composition of the yellow pigments of eight German durum wheat cultivars was studied. Grains were milled on a laboratory mill. Pigment extraction of millstream fractions was performed according to the optimized ICC standard method 152 procedure, and the chemical composition of the extract was analyzed by isocratic reversed phase high-performance liquid chromatography. *all-trans*-Lutein ranged from 1.5 to 4 mg kg^{-1}, and zeaxanthin was found in traces. No lutein esters and carotenes were detected. Surprisingly, the fraction of carotenoids of the complete yellow pigment content amounted to only 30−50% of the yellow pigment quantities, so there are still compounds in durum wheat not yet identified that contribute considerably to the yellow color of the grain extracts. The isolation and chemical identification of those pigments are under investigation.

KEYWORDS: Yellow pigment; ICC standard method 152; carotenoids; lutein; durum wheat

INTRODUCTION

The color of durum wheat (*Triticum durum*) semolina is an important quality criterion with regard to pasta production. A yellow to amber color is generally preferred by consumers rather than a brown or cream one. The degree of yellowness is influenced by several factors such as the milling diagram, the extraction rate, and the chemical nature of the pigments as well as origin, growing conditions, and varieties. In 1912, Monier-Williams (*1*) incorrectly assigned the yellow color of wheat flour to the presence of carotenes by comparing the 440 nm light absorption of wheat extracts to that of carotene from carrots. In 1935, Markley and Bailey (*2*, *3*) showed that the predominant carotenoid pigments in Naphtha extracts of durum wheat grains are xanthophylls, and in 1938, Munsey (*4*) confirmed that durum wheat grains contain only a small proportion of carotene but a relatively large quantity of xanthophylls. In 1940, Zechmeister and Cholnoky (*5*) confirmed the preponderance of xanthophylls, mainly lutein, behind the yellow pigments in wheat flour. They did not discover any β-carotene in the flour. In more recent investigations in 1968, Lepage and Sims (*6*) analyzed durum wheat extracts and found that free lutein accounts for ∼84.8% of the yellow pigments, lutein monoesters for 9.8%, and lutein diesters for 5.3%. Wildfeuer and Acker (*7*) analyzed 39 samples of durum semolina for their carotenoid content and found a total carotenoid content average of 5.3 mg kg^{-1} with ∼1% carotenes. Cumulative data on the yellow pigment composition of several durum wheat varieties, collected since 1912, showed that >90% of the pigments of durum wheat consist of lutein and lutein esters. β-Carotene amounts to ∼1%. To our knowledge, these data have never been questioned since 1968 and have never been checked by modern analytical methods available today.

Durum wheat yellow pigments are analyzed by using ICC standard method 152 (ICC 152) or AACC standard method 14-50 (*8*, *9*). Both are based on the extraction of pigments from durum wheat semolina, flour, or pasta products with water-saturated *n*-butyl alcohol and subsequent spectrophotometrical measurement using a wavelength of 440 nm with pure β-carotene as standard. The butyl alcohol-extractable content of yellow pigments in the samples is expressed as parts per million of β-carotene per sample, the so-called carotene value. These methods have found widespread acceptance, although having the inherent shortcoming that there is only little if any β-carotene in durum wheat. HPLC is widely accepted as an accurate and sensitive technique in carotenoid analysis (*10*). The literature related to the HPLC analysis of carotenoids in cereals is scarce: there is only one publication reporting data of Finnish cereal products (*11*) and another on carotenoids of Australian wheat flour (*12*).

* Address correspondence to this author at the Institute of Nutrition, Friedrich-Schiller-University Jena, Dornburger Str. 25-29, 07743 Jena, Germany [telephone +49-(0)3641-949633; fax +49-(0)3641-949632; e-mail Volker.Boehm@uni-jena.de].

[†] Federal Centre for Cereal, Potato and Lipid Research.

[‡] Friedrich-Schiller-University Jena.

10.1021/jf025701p CCC: $22.00
Published on Web 10/04/2002

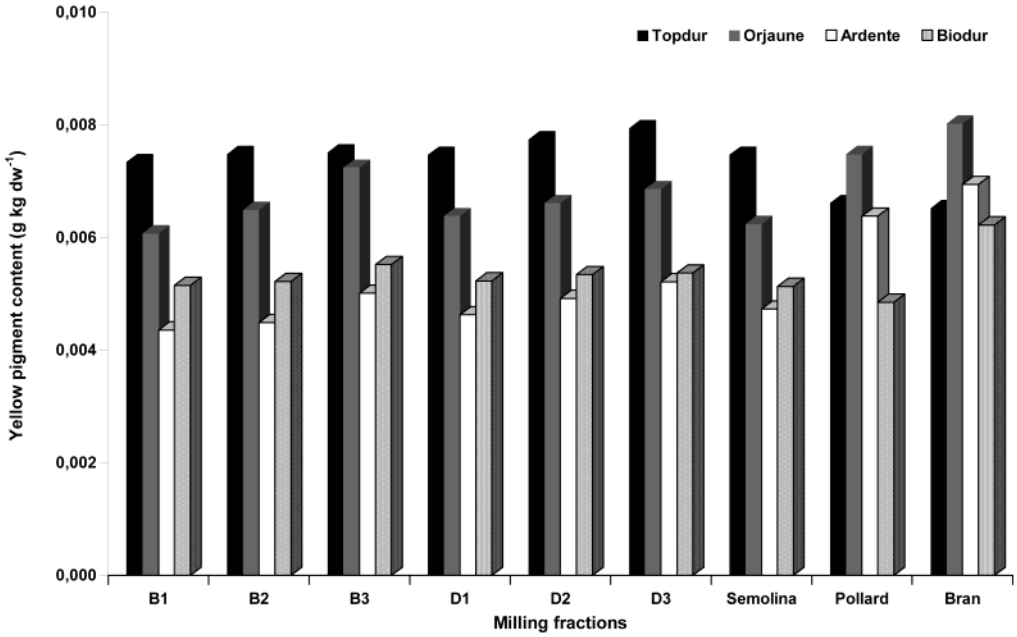

Figure 1. Yellow pigment content (mean of three replicates) of the milling fractions of the durum wheat cultivars Topdur, Orjaune, Ardente, and Biodur.

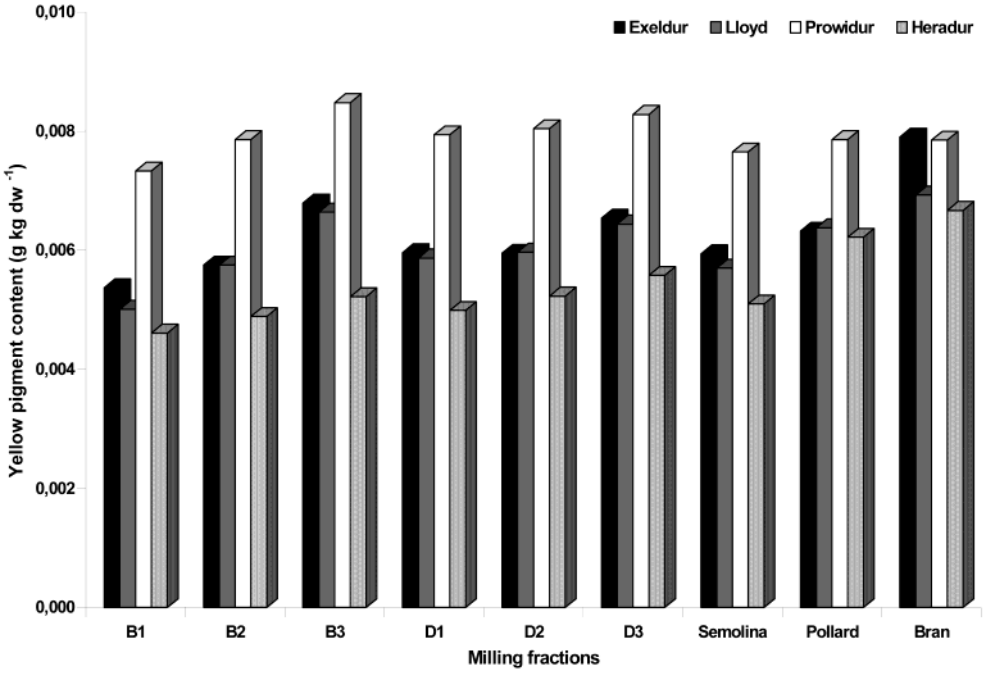

Figure 2. Yellow pigment content (mean of three replicates) of the milling fractions of the durum wheat cultivars Exeldur, Lloyd, Prowidur, and Heradur.

The aim of this study was to determine the chemical composition of the yellow pigments of different German durum wheat cultivars. Besides ICC method 152, carotenoids were analyzed by using HPLC. On the basis of the published data, this publication focuses primarily on carotenoids and especially on xanthophylls. Due to the inherent weakness of the standard method for spectrophotometrical determination, we modified the ICC 152 procedure to measure yellow pigment content.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade quality. Solvents for the HPLC mobile phase were of HPLC quality. Standard solutions in cyclohexane/toluene/(8:2, v/v) contained 2−10 mg L^{-1} lutein, zeaxanthin, β-apo-8′-carotenal, and echinenone (all gifts from Hoffmann-La Roche, Basel, Switzerland). To prepare the working solutions, these standard solutions were diluted daily 1:10 with the mobile phase. Twenty microliters of these working solutions was injected for HPLC analysis. They were stored in brown vials in the dark at −18 °C. The concentrations of the stock standard solutions and their purities were measured before new working standards were used.

Preparation of Samples. Experiments were carried out using eight durum wheat cultivars harvested in 1998: Biodur (obtained from Saatgut- und Agrarservice Beesenstedt, Germany); Exeldur, Lloyd, and Orjaune (from Landhandel Wilhelm Fromme, Aschersleben, Germany); Topdur and Prowidur (not yet commercially available); Heradur (from Probstdorfer Saatzuchtgesellschaft, Germany); and Ardente (from Semences de Provence S.A., Arles, France).

Grain was conditioned to 18% humidity and each sample milled on a Buehler laboratory mill according to a published procedure (*13*). Three millstream fractions (particles <250 μm) B1−B3, three fractions with attached bran (particles <250 μm) D1−D3, semolina (particles >250 μm), pollard, and bran were collected. Ash was determined according to ICC standard method 104. Millstream fractions were analyzed for

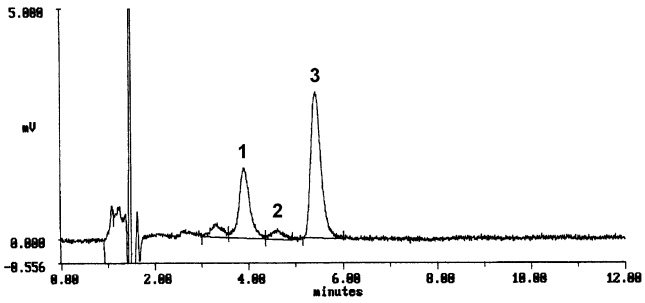

Figure 3. High-performance liquid chromatogram of carotenoids extracted from the durum wheat cultivar Prowidur (1998) on a C_{18} column; for further chromatographic conditions, see the text. Peaks: 1, *all-trans*-lutein; 2, zeaxanthin; 3, β-apo-8′-carotenal (internal standard).

yellow pigments or carotenoids immediately after preparation or stored frozen in airtight bottles under nitrogen at −18 °C until analysis.

Determination of the Yellow Pigment Content. For the determination of yellow pigments according to the ICC standard, the absorbance of *n*-butyl alcohol extracts of the samples was measured at the absorption maximum of lutein in *n*-butyl alcohol at 447 nm. A UV−vis spectrophotometer Specord 200 from Analytik Jena AG, Jena, Germany, was used. Pigment content was calculated using the formula given in ICC method 152 [mg of β-carotene = $(a \times 5 \times 10)/1000 \times 100/(100 - b) = (a \times 5)/(100 - b)$, where $a = \mu$g of β-carotene in 10 mL of extract (= 2 g of sample) and b = humidity in %].

Analysis of Carotenoids. Magnesium carbonate and the solution of the internal standard (C_{18}, apo-8′-carotenal; C_{30}, echinenone) were added to samples of ∼2 g. Afterward, the samples were extracted with methanol/tetrahydrofuran (1+1, v/v) by homogenization for 5 min using the ultra-turrax. This extraction was repeated until a colorless residue remained. The combined organic phases were rotary evaporated under reduced pressure at 30 °C until dryness. The residue was redissolved with methanol/tetrahydrofuran (1+1, v/v), using an ultrasonic bath. Twenty microliters of this solution was injected into the HPLC system.

A Merck (Darmstadt, Germany) HPLC system (pump model L-6200, sampler model AS-2000, and diode array detector model L-4500) and a cooling system consisting of a tempered column holder (Krannich, Göttingen, Germany) and a cooling bath model Frigomix S (using ethanol) with thermostat model Thermomix UM (both Braun, Melsungen, Germany) were used. Separation was done on a 250 × 4.6 mm Vydac 201TP54 column (Promochem, Wesel, Germany), particle diameter = 5 μm, using a column temperature of 9 ± 2 °C and 2.0 mL min^{-1} of methanol/acetonitrile/2-propanol (54+44+2, m/m/m) as mobile phase (*14*).

A second separation was done on a 250 × 4.6 mm YMC C_{30} column (YMC, Schermbeck, Germany), particle diameter = 5 μm, pore diameter = 200 Å, using a column temperature of 23 ± 1 °C, preceded by a 10 × 4.0 mm ProntoSil 120-5-C18 H guard column (Bischoff, Leonberg, Germany), particle diameter = 5 μm, pore diameter = 120 Å. As mobile phase methyl *tert*-butyl ether (solvent A) and methanol (solvent B) were used at a flow rate of 1.3 mL min^{-1}. The gradient procedure was as follows: (1) initial conditions of 10% solvent A and 90% solvent B, (2) a 35-min linear gradient to 45% solvent A, (3) a 10-min linear gradient to 60% solvent A, (4) 60% solvent A and 40% solvent B for 15 min, and (5) a 5-min linear gradient to 10% solvent A (*15*).

Statistical analysis of the data was done by using the SPSS 9.0 Windows Package, SPSS Inc., Chicago, IL. Yellow pigment determinations were carried out in triplicate and carotenoid determinations in duplicate.

RESULTS

The pigment content of the millstream fractions of the durum wheat cultivars, milled on a Buehler laboratory mill, were determined. We modified the ICC method 152 standard protocol by using lutein as standard, taking into account that this xanthophyll is supposed to be the dominant carotenoid in durum wheat. Absorbance was recorded at 447 nm, where lutein shows its maximum light absorbance in *n*-butyl alcohol (data not shown).

Figures 1 and **2** show the total yellow pigment content of different millstream fractions of various cultivars. The standard deviation (SD) of the mean ($n = 3$) for all pigment values shown was <2%. The staircase-like course of the contents was verified by an ANOVA test ($p < 0.05$) for all cultivars and all milling fractions. The figures demonstrate that the outer layers (B3 and D3) of the kernels contain more pigments than the inner layers (B1 and D1). The highest pigment values throughout all millstream fractions were found for the cultivar Prowidur, which is a winter durum wheat; Ardente had the lowest values.

Figure 3 shows a chromatogram of the isocratic separation of a durum wheat extract (Prowidur, bran) on an octadecyl silica column (detection at 450 nm). Pigments were freshly extracted with a methanol/tetrahydrofuran (50:50) mixture from the

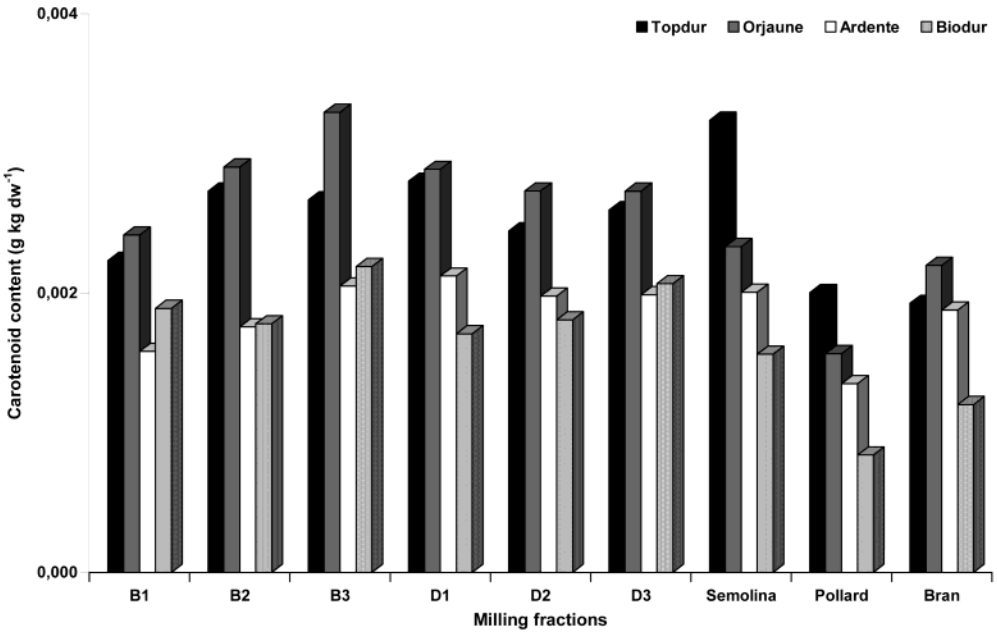

Figure 4. Carotenoid content (mean of two replicates) of the milling fractions of the durum wheat cultivars Topdur, Orjaune, Ardente, and Biodur.

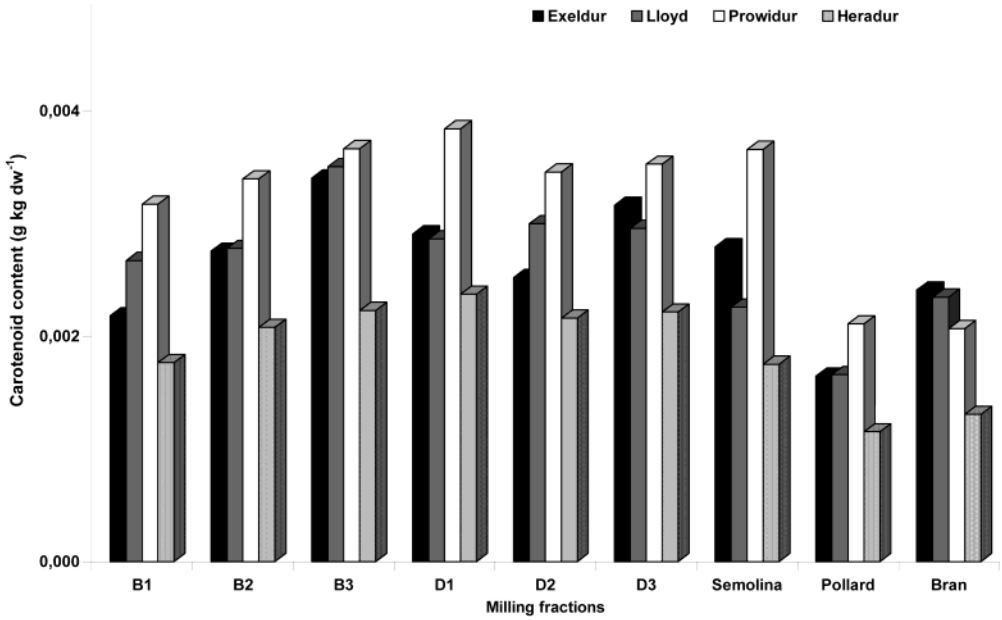

Figure 5. Carotenoid content (mean of two replicates) of the milling fractions of the durum wheat cultivars Exeldur, Lloyd, Prowidur, and Heraldur.

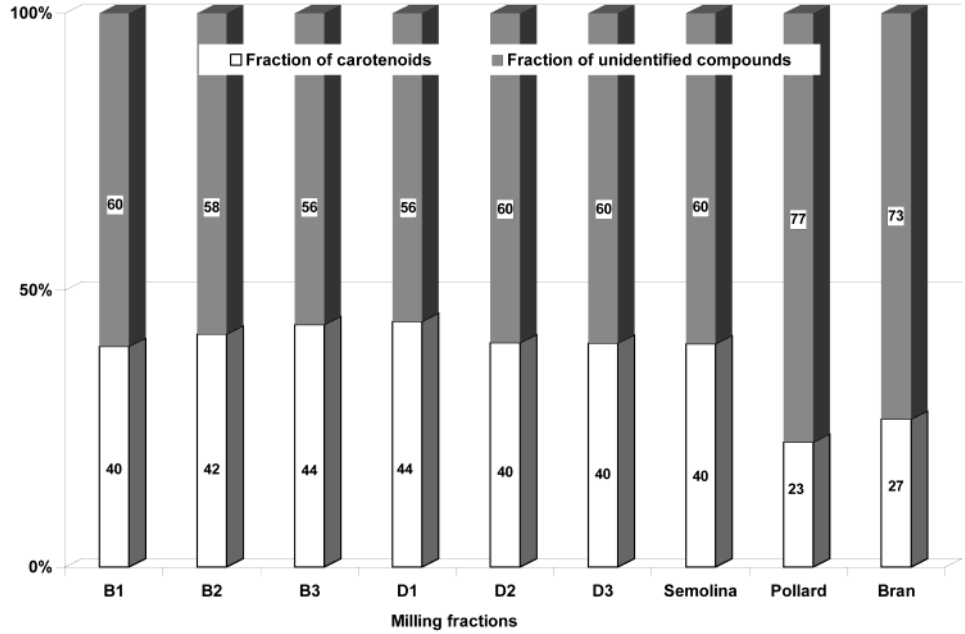

Figure 6. Relationship between carotenoids and unknown compounds within the yellow pigment fraction averaged over all wheat grain cultivars.

sample at room temperature according to a published procedure (*14*). This solvent mixture medium has been proven to be as suitable as water-saturated *n*-butyl alcohol (data not shown). Due to the higher volatility of MeOH/THF compared to butyl alcohol, the preparation of samples for HPLC analysis can be drastically sped. For the separation of all pigments a ternary mobile phase of methanol/acetonitrile/2-propanol (54:44:2) was used (*14*). β-Apo-8′-carotenal was used as an internal standard.

Features of the chromatograms are the reproducible appearance of a peak in front of the *trans*-lutein peak and a broad peak of unretarded material in the void volume of the column. The chemical nature of these compounds is unknown, but a common trait is that they absorb light at a wavelength of 450 nm just like xanthophylls of known structure. In all millstream fractions *all-trans*-lutein was detected; in pollard and bran fractions additionally zeaxanthin was found in traces. Neither carotenoid esters nor β-carotene could be detected. **Figures 4** and **5** show the cumulative results for all samples. Carotenoid content represents lutein plus zeaxanthin. Noteworthy are the small carotenoid contents of the pollard and bran fractions in relation to the other fractions. This may be an indication that the carotenoids do not follow a gradient along the kernel seen for the yellow pigments. In contrast, they seem to be more evenly distributed. Whereas all of the inner layers of the kernel contain roughly the same amount of carotenoids, outer layers have less. Comparing the yellow pigment content of the different millstream fractions with the corresponding carotenoid composition surprisingly shows that the portion of carotenoids (lutein plus zeaxanthin) amounts to ~30−50% of the yellow pigments. This was found for all durum cultivars and implies that there are unknown color-producing compounds in the durum extracts absorbing light at 447 nm. The UV−vis spectra of all extracts in *n*-butyl alcohol showed a hypsochromic wavelength shift of 7 nm compared to that of lutein (data not shown). These results indicate the presence of additional yellow compounds in the extracts. **Figure 6** presents the relationship between the propor-

tion of carotenoids to yellow pigments and the millstream fraction averaged over all durum wheat cultivars.

DISCUSSION

The yellow pigment content of durum wheat semolina and pasta products has found a wide acceptance as one of several indicators of quality. Interestingly, the chemical composition of these pigments, which share as a common trait their light absorption at 435.5 nm (AACC method 14-50) or 440 nm (ICC method 152), has not been elucidated thoroughly. Several publications (*2*−*7*) claim that these pigments consist mainly of lutein and its esters (*6*, *7*). A project was started to separate the yellow pigments from different German durum wheat cultivars by HPLC and elucidate their chemical structures.

The increased interest in the possible link between fruit and vegetable consumption and a reduced risk of suffering from chronic illnesses such as arteriosclerosis or cancer initiated intensive research to collect reliable data on the carotenoid content of many foodstuffs of plant origin. Cereal products have possibly the same health-related benefits. With respect to durum wheat, the consumption of pasta products is steadily rising in the European Union (*16*). Thus, reliable data on the carotenoid composition of durum wheat cultivars and products thereof are necessary for an evaluation of whether the consumption of durum products will have any significant contribution to the proposed health-related effects of carotenoids. The distribution of compounds absorbing light at 447 nm is in accordance with results provided by Matsuo et al. (*17*) and Boyacioglu et al. (*18*). We found an uneven distribution of the pigments in the wheat kernel. The outer layers (B3 and D3) have more of them than the inner layers (B1 and D1). Therefore, in botanical terms the pericarp, testa, aleuron layer, and germ are richer in these pigments than the endosperm tissue, so fractions from the outer layer of the kernel contain a higher amount of pigments than do fraction from the inner layers.

The yellow pigments of durum wheat are assumed to consist of ~90% lutein and lutein esters. This assumption was checked by reversed-phase HPLC. To avoid problems with the low volatility of water-saturated *n*-butyl alcohol, a 1:1 THF/MeOH mixture—an excellent extraction solvent for vegetable material—was used for the extraction (*19*). Carrying out yellow pigment determination according to ICC standard method 152 and replacing the usual solvent by 1:1 THF/MeOH lead to yellow solutions with nearly identical UV spectra.

The positions of the absorption maxima are unchanged, and the whole spectrum is shifted to 10% higher absorbance values in comparison to the spectra measured in *n*-butyl alcohol. HPLC analysis detects *all-trans*-lutein in all milling passages in amounts of 1.5−4 mg kg^{-1}; lutein esters and β-carotene were not detected. In bran and semolina fractions the xanthophyll zeaxanthin was found additionally in very low amounts. The low content of carotenoids detected in semolina and bran fractions discloses a distribution along the wheat kernel similar to that demonstrated by the yellow pigments. The large difference between yellow pigment and carotenoid content is striking and cannot be explained yet. Trials to identify the chemical nature of further substances contributing to the yellow color are being done.

The presence of aurones, chalkones, anthocyanins could be excluded on the basis of light absorption characteristics, chemical tests, and shift reagents (*20*). Whether the unknown compounds belong to the class of the flavonoids, already found in wheat in 1931 (*21*), is doubtful as there is to our knowledge not any flavonoid absorbing light at 447 nm.

Using HPLC gradient separation on a YMC C_{30} column, neither flavonoids nor isomers of known carotenoids nor metabolites produced during storage or milling have been detected.

CONCLUSION

The so-called yellow pigment content as a quality parameter for durum wheat and pasta products remains an appropriate means to compare the color intensity of extracts of different whole durum wheat or durum wheat product samples. This study shows that despite the published literature the chemical composition of the yellow pigments is still not known completely. HPLC analysis of extracts of different varieties of durum wheat has shown that besides lutein (1.5−4 mg kg^{-1}) and small amounts of zeaxanthin, other substances of unknown structure contribute to the yellow color impression. Further research will be conducted to elucidate the structures of these compounds and to evaluate their nutritional function.

ABBREVIATIONS USED

ICC, International Association for Cereal Chemistry; AACC, American Association for Cereal Chemistry; HPLC, high-performance liquid chromatography; THF, tetrahydrofuran; MeOH, methanol.

ACKNOWLEDGMENT

The kind gift of the carotenoid reference materials by F. Hoffmann-La Roche, Basel, Switzerland, is gratefully acknowledged. Thanks are due to I. Schmuck (Jena) and R. Pottebaum and M. Lüders (Detmold) for technical assistance.

LITERATURE CITED

(1) Monier-Williams, G. W. Reports to the Local Government Board on the nature of flour and its relationship to the process of natural and artificial bleaching. *Reports, Local Government Board (Great Britain), Public Health and Medical Subjects, New Series*; 1912; p 73.

(2) Markley, M. C.; Bailey, C. H. The nature of the pigments of the gasoline extract of wheat. *Cereal Chem.* **1935**, *12*, 33−39.

(3) Markley, M. C.; Bailey, C. H. The pigments of the dilute alcohol or acetone extract of whole wheat meal. *Cereal Chem.* **1935**, *12*, 40−48.

(4) Munsey, V. E. Application of the neutral wedge photometer to the measurement of carotenoid pigments in flour and macaroni products. *J. Assoc. Off. Agric. Chem*. **1938**, *21*, 331−351.

(5) Zechmeister, L.; Cholnoky, L. Carotenoids of Hungarian wheat flour. *J. Biol. Chem.* **1940**, *135*, 31−36.

(6) Lepage, M.; Sims, R. P. A. Carotenoids of wheat flour: Their identification and composition. *Cereal Chem.* **1968**, *45*, 600−604.

(7) Wildfeuer, I.; Acker, L. Über die Bestimmung von Carotinoiden in Teigwaren und deren Rohstoffen. *Mitt. Geb. Lebensm. Hyg.* **1968**, *59*, 392−400.

(8) Standard-Methoden der Internationalen Gesellschaft für Getreidewissenschaft und −Technologie; Vienna, Austria, 1991.

(9) *Approved Methods of the American Association of Cereal Chemists*; AACC: Minneapolis, MN, 1994; AACC 14-50 1961 (revised).

(10) Böhm, V. Antioxidativ wirksame Carotinoide und Polyphenole—Analytik, Gehalte in Lebensmitteln und intestinale Absorption. Habilitationsschrift, Friedrich-Schiller-Universität, Jena, Germany, 1999.

(11) Heinonen, M.; Ollilainen, V.; Linkola, E.; Varo, P.; Koivistoinen, P. Carotenoids and retinoids in Finnish foods: Cereal and bakery products. *Cereal Chem.* **1989**, *66* (4), 270−273.

(12) Ward, S.; Millikan, M.; Wootton, M. Examination of carotenoid pigments in wheat. *Proceedings of the 46th Australian Cereal Chemistry Conference*, Sydney; 1996; pp 145−149.

(13) Bolling, H.; Zwingelberg, H. Zur Beurteilung der Mahlfähigkeit von Durumweizen. *Getreide, Mehl Brot* **1988**, *43* (11), 330−334.

(14) Böhm, V. Effect of subambient temperature on RP-HPLC of β-carotene isomers. *Chromatographia* **1999**, *50*, 282−286.

(15) Böhm, V. Use of column temperature to optimize carotenoid isomer separation by C_{30} high performance liquid chromatography. *J. Sep. Sci.* **2001**, *24*, 955−959.

(16) Mondelli, G. Il mercato internazionale delle paste alimentari e delle macchine per la loro produzione (The international market for pasta and pasta manufacturing machines). *Tech. Molitoria* **1992**, *43* (11), 993−1010.

(17) Matsuo, R. R.; Dexter, J. E. Comparison of experimentally milled durum wheat semolina to semolina produced by some Canadian commercial mills. *Cereal Chem.* **1980**, *57*, 117−122.

(18) Boyacioglu, H. M.; Unal, S. S. Effect of particle size on mineral constitutions and pigment contents of semolina. *Pasta J.* **1992**, *74* (3), 40−44.

(19) Scott, K. J. Observation on some of the problems associated with the analysis of carotenoids in food by HPLC. *Food Chem.* **1992**, *45*, 357−364.

(20) Harborne, J. B. *Phytochemical Methods—A Guide to Modern Techniques of Plant Analysis*, 3rd ed.; London, 1998.

(21) Anderson, J. A.; Perkin, A. G. The yellow coloring matter of Khapli wheats. *J. Chem. Soc.* **1931**, 2624−2625.

Received for review May 28, 2002. Revised manuscript received September 3, 2002. Accepted September 6, 2002.

JF025701P