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Bioaccessibility of Carotenoids and Vitamin E from Pasta: Evaluation of an in Vitro Digestion Model

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ABSTRACT: The present investigation aimed to expand the knowledge of bioaccessibility of carotenoids, tocopherols, and tocotrienols from cereal products such as pasta. Because most of the published approaches assessing the bioaccessibility of lipophilic micronutrients dealt with fruits and vegetables, a prevalent in vitro digestion procedure was modified. Additionally, several digestion parameters were evaluated with regard to their impact on the bioaccessibility of carotenoids and vitamin E from pasta. Overall, the estimated values were highly dependent on the amount of bile extract present in the digestive medium and to a lesser extent on the simulated gastric pH and the incubation time with digestive enzymes. The bioaccessibility of carotenoids and vitamin E from durum wheat pasta was quite high (71 ± 5 and 70 ± 4%, respectively), whereas these micronutrients were considerably less accessible from pasta containing 10% eggs (57 ± 1 and 49 ± 5%, respectively).

KEYWORDS: Lutein, tocopherols, tocotrienols, durum wheat pasta, micellarization, lipid digestion

INTRODUCTION

Cereal products have been an important part of the human diet for thousands of years. Wheat has become one of the leading grains used for human consumption. Today, the main wheat variety grown is bread wheat (*Triticum aestivum*). Durum wheat (*Triticum durum*) represents about 10% of the total wheat production worldwide. The major use of durum wheat is for pasta production, particularly in Europe and North America. Whereas Italian pasta is almost exclusively pure durum pasta, in Germany about 80−85% of total pasta production is represented by egg pasta. The increasing popularity of pasta products is partly due to their taste, convenience, and nutritional value.[1] Pasta is a low-fat food that contains high amounts of complex carbohydrates. Pasta products are also a good source of many essential micronutrients.

Carotenoids and vitamin E are lipophilic micronutrients with potential health benefits in humans. Epidemiological studies have shown associations between intake of fruits and vegetables rich in carotenoids and reduced risks of different forms of cancer, cardiovascular diseases, and age-related macular degeneration (AMD).[2] Since the 1930s it has been known that the predominant carotenoid pigments in durum wheat are xanthophylls,[3] with a preponderance of lutein.[4,5] Especially lutein and zeaxanthin play important roles in the prevention of frequently occurring eye diseases such as AMD, cataract, and retinitis pigmentosa.[6] Even though cereal grains contain far fewer carotenoids than most vegetables and fruits, they are consumed frequently in considerable amounts.

Vitamin E, which is the general term for tocopherols and tocotrienols, is the most important chain-breaking antioxidant in the lipophilic environment. It is able to protect biological membranes from lipid peroxidation. Thus, vitamin E is believed to prevent or modulate diseases associated with oxidative stress, such as cardiovascular diseases, cancer, chronic inflammation, and neurologic disorders.[7] Some physiological properties of vitamin E seem to involve the modulation of signal transduction and gene expression.[7,8] For instance, the cholesterol-lowering activity of tocotrienols is a nonantioxidant function.[9] Although cereal grains have relatively low amounts of tocopherols, their content of tocotrienols is higher than in many other foods. The vitamin E content of wheat comprises >60% tocotrienols.[10] The amount of vitamin E obtained from cereals is important because they are consumed at high levels. Together with palm oil products, wheat and other cereals are the major sources of tocotrienols in the human diet.[10]

For nutritional evaluation of a certain foodstuff as well as for comparison with other foods, not only the amount of nutrients but also their bioavailability has to be considered. Bioavailability is defined as the fraction of an ingested nutrient that reaches the systemic circulation.[11] In contrast, bioaccessibility describes the fraction of a nutrient that is available for absorption into enterocytes. For absorption of lipophilic micronutrients, such as carotenoids and vitamin E, their release from the food matrix and their transfer into emulsified lipid droplets in the stomach as well as their subsequent incorporation into mixed micelles along with products of lipolysis in the small intestine are required. Formation of mixed micelles is necessary to move the nonpolar lipids across the unstirred water layer adjacent to the mucosal cells. Micellarization is a process that depends on adequate bile and pancreatic secretions because mixed micelles are formed by the action of bile salts, phospholipids, and the hydrolysis products of dietary lipids.[12] In vitro methods that mimic gastrointestinal digestion and/or absorption into enterocytes are being extensively used at present. In comparison to human studies, they are rapid and safe as well as less time-consuming, labor intensive,

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and costly. The in vitro digestion coupled with the Caco-2 cell model provides a tool for screening the effects of cultivars, food processing and other dietary factors on bioaccessibility as well as for investigating mechanisms in digestion and intestinal cell uptake. The results provide testable hypotheses of the appropriate design of human studies.[13]

Whereas many studies on bioaccessibility of carotenoids from fruits and vegetables were conducted in recent years, only a few papers on the bioaccessibility of vitamin E are available, and tocotrienols were usually disregarded. Literature data on bioaccessibility of carotenoids and vitamin E from foods other than fruits and vegetables are scarce. Thus, the present investigation mainly aimed at expanding the knowledge on bioaccessibility of carotenoids, tocopherols, and tocotrienols from cereal products such as pasta. Pure durum wheat pasta and pasta containing 10% eggs were compared regarding bioaccessibility of these lipophilic micronutrients. Most of the published procedures for measuring the bioaccessibility of carotenoids and vitamin E were used for in vitro digestion of fruits and vegetables and are based on the method of Garrett et al.[14] Reboul et al.[15] modified this procedure regarding pH and incubation times by taking data on lipid digestion and carotenoid processing in vivo[16] into account. Our previous investigations on durum wheat and products thereof have shown a poor chemical extractability of carotenoids from wheat and pasta in comparison to vegetables,[5] which may be ascribed to the inclusion of lipophilic micronutrients into the complex network of protein and starch. Thus, in vitro digestibility of pasta may also differ from that of fruits and vegetables, and digestion procedures described in the literature have to be adapted to the food matrix of cereal products. In the present investigation, the method of Reboul et al.[15] was evaluated by measuring the release of amino acids, fatty acids, and sugars during the simulated digestion of pasta. Many published in vitro approaches for measuring the bioaccessibility of lipophilic micronutrients vary significantly in pH values during in vitro digestion, incubation times, concentrations of digestive enzymes, and the method of isolating mixed micelles. Therefore, the impact of these parameters on the bioaccessibility of carotenoids and vitamin E from pasta was also examined to evaluate the comparability to literature data.

MATERIALS AND METHODS

Chemicals. All chemicals were of analytical grade. Solvents for chromatography were of HPLC quality. All solutions were prepared by using HPLC grade water (18 MΩ) from a Milli-Q purification system (Millipore, Schwalbach, Germany). Carotenoid standards (97−99%) were purchased from CaroteNature (Lupsingen, Switzerland). Pure tocopherols (>95%) and tocotrienols (>97%) were obtained from Calbiochem (Darmstadt, Germany) and Davos Life Sciences (Singapore, Singapore), respectively. DL-α-Tocopheryl acetate (>96%) was purchased from Sigma-Aldrich (Taufkirchen, Germany). α-Amylase from *Aspergillus oryzae* (208 U/mg protein), pepsin, pancreatin from porcine pancreas, porcine bile extract, 2,4,6-trinitrobenzenesulfonic acid (TNBS), L-glutamic acid, cholesterol (95%), margaric acid (99%), glyceryl tripalmitate (90%), palmitic acid methyl ester (99%), and cholesteryl palmitate (91%) were also obtained from Sigma-Aldrich. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (99%) was purchased from Larodan Fine Chemicals (Malmö, Sweden), and 2′,7′-dichlorofluorescein was from Fluka (Buchs, Switzerland). The maltose/sucrose/D-glucose test kit was obtained from R-Biopharm (Darmstadt, Germany).

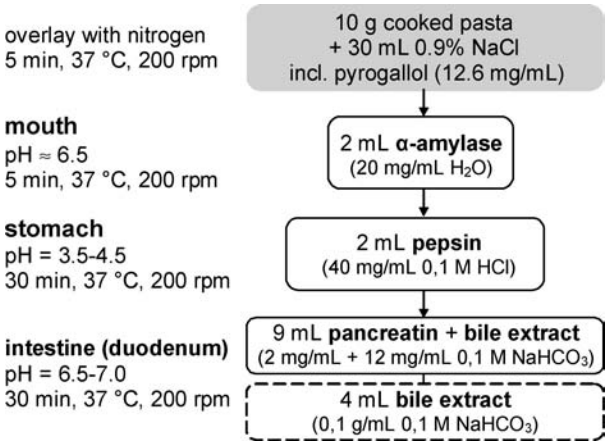

Figure 1. In vitro digestion procedure.

Pasta samples. One commercial pasta without eggs (pasta 1) as well as three different egg pastas (egg pastas 1−3), all containing 10% eggs, were purchased in local supermarkets. Four different batches of pure durum wheat pasta (pastas 2−5) without eggs were directly received from a German pasta manufacturer. Prior to in vitro digestion, all pasta products were cooked without adding salt to the cooking water using different cooking times according to the manufacturers' instructions (5−9 min). Afterward, they were crushed by using a laboratory mill type Grindomix GM 200 (Retsch, Haan, Germany) to mimic mastication.

In Vitro Digestion. The procedure described by Reboul et al.[15] was extended by an oral phase. About 10 g of pasta was weighed into 100 mL Erlenmeyer flasks and digested in the dark following the scheme in Figure 1. First, the digestion was conducted excluding the additional amount of bile extract (broken line in Figure 1) and named the initial method. Afterward, some modifications, namely, the additional amount of bile extract, a reduced pH during gastric digestion (pH 2 instead of pH 4), and longer incubation times for gastric and intestinal digestion (1 h instead of 30 min in each case) as well as the addition of 10% rapeseed oil, were evaluated with regard to their impact on bioaccessibility. For studying modifications of the digestion procedure, pasta 1 was used for all experiments.

Isolation of Micellar Fraction. After in vitro digestion had been completed, the total digesta was transferred into a 90 mL centrifuge tube and brought to a volume of 50 mL by washing the Erlenmeyer flask with an appropriate amount of water. The aqueous fraction was separated from the solid residue by using two centrifugation steps. First, samples were centrifuged for 20 min at 4500 rpm (∼3900 g) at 10 °C. Afterward, aliquots in 1.5 mL tubes were again centrifuged for 5 min at 14000 rpm (∼15800*g*) at room temperature. These centrifugation procedures were compared to ultracentrifugation procedures at 167000*g* (∼40000 rpm) for 95 min at 4 °C [14] and at 20000 rpm (∼43000*g*) for 18 h at 10 °C,[15] respectively. The micellar fraction was obtained by passing the aqueous supernatant through a 0.22 μm polypropylene filter (VWR, Darmstadt, Germany). Aliquots of filtrates and digestion residues were stored at −25 °C under a blanket of nitrogen until analysis (within 3 days).

Extraction of Carotenoids and Vitamin E. Both micronutrients were extracted simultaneously by using the method of Balz et al. [17] with some modifications. All operations were performed under subdued light. Filtered supernatants (5 g), digestion residues (3 g), and cooked pasta (2 g) were weighed into centrifuge tubes. After the addition of 2 mL of ethanol and 1 mL of water to the solid samples, they were homogenized for 30 s by using an ultraturrax T25 with dispersing element S25N-8G (IKA, Staufen, Germany) to ensure a quantitative extraction. Afterward, 20 μL of β-apo-8′-carotenal and 20 μL of α-tocopheryl acetate were added as internal standards to all samples. Filtrates were directly extracted without prior homogenization. Extrac-

Table 1. Absorption Characteristics of Carotenoids in Ethanol

carotenoid	λ_{max} (nm)	$E^{1\%}_{1\ cm}$
(*all-E*)-lutein	445	2550
(*all-E*)-zeaxanthin	450	2540
(*all-E*)-canthaxanthin	466	2220
(*all-E*)-β-apo-8′-carotenal	457	2640

tion was performed by vortexing for 30 s after stepwise addition of 1 mL of ethanol, 1 mL of methyl *tert*-butyl ether (MTBE), and 1 mL of petroleum ether. Tubes were centrifuged at 5000 rpm (~3900 g) for 3 min at room temperature. The upper phase was collected, and the extraction with MTBE and petroleum ether was repeated at least twice. Combined extracts were rotary-evaporated under reduced pressure at 30−35 °C. The dried residues were dissolved in 2 mL of ethanol using an ultrasonic bath. After centrifugation (14000 rpm, 5 min), carotenoids were analyzed by using HPLC. Another 500 μL of this solution was dried under a stream of nitrogen at 30 ± 1 °C. The residue was dissolved in 500 μL of *n*-hexane/MTBE (98:2, v/m) and centrifuged (14000 rpm, 5 min) prior to HPLC analysis of vitamin E.

Analysis of Carotenoids. Analysis was performed by reversed-phase HPLC using a Jasco system with diode array detection (Jasco, Gross-Umstadt, Germany). Chromatographic separation was achieved within 45 min at 20 ± 1 °C using a PEEK-C_{30} column (250 mm × 4.6 mm, 5 μm; Trentec, Rutesheim, Germany), preceded by a VYDAC C_{18} guard column (201 GD54SP; 10 mm × 4.0 mm, 5 μm; MZ-Analysentechnik, Mainz, Germany). The mobile phase was methanol/water (97:3, v/v) at a flow rate of 1.3 mL/min. Carotenoids were detected at 450 nm and identified by comparison with external standards. (*Z*)-Isomers of lutein and zeaxanthin were tentatively identified by comparison of retention times and DAD absorbance spectra[5] as well as mass spectral data (not shown) with those of isomerized standard solutions and quantified using (*all-E*)-lutein and (*all-E*)-zeaxanthin, respectively, considering the recovery of the internal standard. (*Z*)-Isomers of lutein and zeaxanthin were obtained by iodine-catalyzed photoisomerization of the (*all-E*)-carotenoid standards according to the method of Zechmeister.[18] Stock solutions of carotenoid standards (50−100 μg/mL ethanol) were stored at −25 °C. Concentrations of stock solutions were calculated periodically using their absorption maxima and appropriate extinction coefficients (Table 1). For daily preparation of working solutions, stock solutions were diluted 1:100 with ethanol.

Analysis of Vitamin E. Tocopherols and tocotrienols were analyzed by normal-phase HPLC by means of a Merck-Hitachi system (Merck, Darmstadt, Germany) with fluorecence detection (excitation, 292 nm; emission, 330 nm). Chromatographic separation was achieved within 45 min at 35 ± 1 °C using a Eurospher-100 DIOL column (250 mm × 4.0 mm, 7 μm), preceded by a Eurospher-100 DIOL guard column (5 mm × 4.0 mm, 7 μm) (both from Knauer, Berlin, Germany). A mixture of *n*-hexane/MTBE (98:2, v/m) was used as mobile phase at a flow rate of 1.5 mL/min. Individual tocochromanols were identified by comparing their retention times with those of external standards and quantified as described for carotenoids. Stock solutions of tocopherols and tocotrienols (~1 mg/mL ethanol) as well as of α-tocopheryl acetate (~5 mg/mL ethanol) were stored at −25 °C. Concentrations of stock solutions were calculated periodically using their absorption maxima and appropriate extinction coefficients (Table 2). Calibration curves were created in the range of 0.2−2.0 μg/mL. The internal standard was diluted 1:100 with the mobile phase.

Calculations. In the present study, bioaccessibility was defined as the efficiency of micellarization, that is, as the amount of carotenoids or vitamin E that was transferred from the digesta to the micellar aqueous fraction. The stability of carotenoids and tocochromanols during simulated digestion was calculated by the contents of substances in the digesta (solid residue + supernatant) in relation to contents in the undigested food. This recovery was considered for calculation of bioaccessibility.

Table 2. Absorption Characteristics of Tocochromanols in Ethanol

tocochromanol	λ_{max} (nm)	$E^{1\%}_{1\ cm}$
α-tocopherol	292	75.8
β-tocopherol	296	89.4
γ-tocopherol	298	91.4
δ-tocopherol	298	87.3
α-tocopherol	292.5	91.0
β-tocopherol	294	87.3
γ-tocopherol	296	90.5
δ-tocopherol	297	88.1
α-tocopheryl acetate	285	44.0

Amino Acids. Released amino acids were determined in the supernatant after in vitro digestion (initial method) in comparison to a control sample, which was incubated solely with the saline solution. After reaction with TNBS, amino acids were determined photometrically according to the method of Sashidhar et al.[19] L-Glutamic acid (glu) solutions (2−20 μg/mL ethanol/0.1 M HCl (1:1, v/v)) were used for calibration. To 1 mL of supernatant were added 1 mL of 4% $NaHCO_3$ (pH 8.5) and 1 mL of 0.01% of freshly made TNBS. The incubation at 40 ± 1 °C for 2 h in a water bath was followed by the addition of 0.5 mL of 1 M HCl and 1 mL of 10% sodium dodecyl sulfate. The absorbance of sample solutions and blank was monitored at 335 nm using a Jasco spectrophotometer type V-530.

Glucose and Maltose. Both sugars were determined by using a commercial maltose/sucrose/D-glucose test kit (R-Biopharm, Darmstadt, Germany) following the manufacturer's instructions. Contents of glucose and maltose in supernatant after simulated digestion by using the initial method were compared to those of the control sample (undigested). At pH 6.6, maltose was hydrolyzed in the presence of α-glucosidase to D-glucose within 20 min at 20−25 °C. The enzyme hexokinase subsequently catalyzed the phosphorylation of D-glucose by ATP at pH 7.6 under simultaneous formation of ADP. The formed D-glucose-6-phosphate (G-6-P) was oxidized by NADP in the presence of G-6-P dehydrogenase to D-gluconate-6-phosphate with the formation of NADPH. After the completion of this reaction (10−15 min), the increase in NADPH was measured by means of its absorbance at 340 nm by using a Jasco spectrophotometer type V-530. The amount of NADPH was stoichiometric to the amount of D-glucose and to half the amount of maltose.

Free Fatty Acids. Separation of fat extracts was achieved by TLC. Cooked pasta was mixed with 10% rapeseed oil and digested by using the initial method. Both the digesta and a control sample solely incubated with the saline solution were lyophilized and subsequently extracted with petroleum ether by using a Soxhlet extractor model Soxtherm 2000 automatic (C. Gerhardt, Königswinter, Germany). The fat extract was diluted to 0.1% with chloroform. Then, 200 μL of this solution together with 5 μL of a standard mixture (1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, margaric acid, glyceryl tripalmitate, palmitic acid methyl ester, cholesteryl palmitate; 60 mg/mL chloroform) was applied on a silica gel coated TLC plate (Merck). The eluent was *n*-hexane/diethyl ether/glacial acetic acid (80:20:1, v/v/v). After 30 min, bands were visualized by UV light after the plate has been sprayed with 2′,7′-dichlorofluorescein (0.2% in ethanol).

Statistical Analysis. All determinations were conducted at least in triplicate. For comparing the bioaccessibility of pure durum wheat pasta and egg pasta, the in vitro digestion was carried out on two different days to provide a minimum of six different observations for each pasta.

Results are expressed as the mean ± standard deviation. To ascertain differences between means, Student's t test or one-way analysis of variance (ANOVA) followed by the Student−Newman−Keuls (SNK) procedure was performed using SPSS 17.0 for Windows (SPSS Inc., Chicago, IL). Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Carotenoids and Vitamin E in Pasta. Pure durum wheat pasta was composed of approximately 83% (*all-E*)-lutein, 12% (*Z*)-lutein isomers, 4% (*all-E*)-zeaxanthin, and 1% (*Z*)-zeaxanthin. Egg pasta contained slightly lower quantities of lutein (77% (*all-E*)-lutein and 11% (*Z*)-isomers), considerably higher amounts of (*all-E*)-zeaxanthin (8%), 2% (*Z*)-zeaxanthin, and additionally about 2% (*all-E*)-canthaxanthin. Pasta did not contain any xanthophyll esters. Total carotenoids of raw durum wheat pasta were in the range of 0.26−0.34 mg/100 g dm, whereas raw egg pasta had higher amounts of total carotenoids (0.49−0.55 mg/100 g dm). The content of vitamin E of pure durum wheat pasta consisted of 13% α-tocopherol, 6% β-tocopherol, 8% α-tocotrienol, and 73% β-tocotrienol. In egg pasta the portion of tocopherols was slightly higher, being 21% for α-tocopherol, 5% for β-tocopherol, and 1% for γ-tocopherol. The main vitamer was β-tocotrienol (64%). α-Tocotrienol accounted for 9% of the vitamin E content of egg pasta. Contents of carotenoids and vitamin E remained almost unchanged during cooking of pasta. Qualitative changes, that is, isomerization of carotenoids, were not observed (data not shown).

Digestion Products. The initial digestion method excluding the additional amount of bile extract (see Figure 1) was evaluated by resulting increases in degradation products of starch, protein, and lipids in supernatants after centrifugation of total digesta.

For hydrolysis of pasta starch the digestion method of Reboul et al.[15] was extended by an oral phase. Starch hydrolysis was realized by α-amylase and led to a 3-fold amount of sugars in the supernatant after simulated digestion (0.45 ± 0.03 g of glucose, 0.055 ± 0.010 g of maltose) compared to the supernatant of the control (undigested) sample (0.12 ± 0.22 g of glucose, 0.032 ± 0.005 g of maltose). Aura et al.[20] found a positive effect of pepsin, pancreatin, and bile on the liberation of glucose from wheat and rye breads. In the absence of other digestive enzymes and bile, salivary α-amylase (10−40 U/1.5 g) had little effect on the digestibility of starch. Therefore, we have chosen a higher amount of α-amylase (168 U/g of pasta) for in vitro digestion of pasta. Starch encapsulated in the protein matrix was released by the action of pepsin, making the starch accessible to α-amylase. Starch hydrolysis probably went on in the duodenal phase of the in vitro digestion because pancreatin is a complex enzyme mixture with amylolytic activity. As emulsifying agents, bile salts may enhance the interactions between starch and pancreatin.[20] Overall, the digestion procedure led to a striking degradation of pasta starch.

As expected, the hydrolysis of pasta proteins by using the initial digestion procedure resulted in a significant increase ($p < 0.05$) in the amount of free amino acids from 0.20 ± 0.03 g in the supernatant of the control sample to 0.34 ± 0.01 g in the supernatant of digested pasta. Proteins were predominantly degraded by pepsin. Because pancreatin also contains proteases, hydrolysis of the protein matrix might have been continued during the duodenal phase of simulated digestion. It has to be kept in mind that TNBS does not react with secondary amino groups. Thus, proline, one of the major amino acids in wheat, was not detected. However, for the purpose of evaluating the digestion procedure, a quantitative analysis of all amino acids was not required.

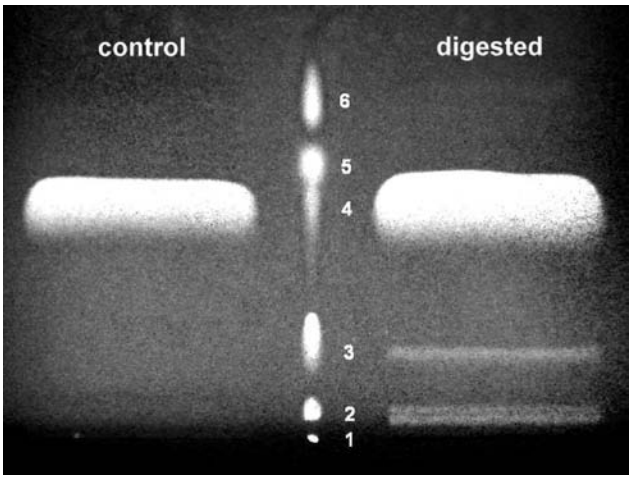

Figure 2. TLC separation of fat extracts obtained from digested pasta and control (both samples containing 10% rapeseed oil) in comparison to a standard mixture by using *n*-hexane/diethyl ether/glacial acetic acid (80:20:1, v/v/v): 1, phospholipids; 2, cholesterol; 3, free fatty acids; 4, triacylglycerides; 5, fatty acid methyl ester; 6, cholesteryl ester.

Because the addition of 10% rapeseed oil to the pasta sample had unexpectedly no impact on the bioaccessibility of carotenoids (see below), the lipolysis was checked by using TLC separation of fat extracts from pasta containing 10% oil before and after in vitro digestion. The control sample contained only triacylglycerides. After simulated digestion, two minor bands appeared on the TLC plate. However, the triacylglycerides were still the main components of fat extracts from digested pasta (Figure 2). One minor band was identified as free fatty acids by comparison to the standard mixture. The other minor band coeluted with cholesterol, which was not expected in pure durum wheat pasta without eggs and which was absent in the undigested sample. Christie[21] achieved a better separation of individual fat components by using a slightly modified eluent (*n*-hexane/diethyl ether/formic acid (80:20:2, v/v/v). Monoacylglycerols, 1,2-diacylglycerols, and 1,3-diacylglycerols eluted between phospholipids and cholesterol. In our study, a coelution of cholesterol and these products of lipolysis is suggested. Thus, the lower band was expected to be monoacylglycerol and/or diacylglycerol rather than cholesterol. Because the increase of fatty acids during simulated digestion was quite low, a limited lipolysis was assumed. As a consequence, the concentration of pancreatin should be raised for further experiments. Some authors also used other lipases in addition to pancreatin, such as pancreatic lipase, colipase, and phospholipase A_2.[13,22−25]

Reproducibility. For bioaccessibility of total carotenoids, the within-day coefficient of variation (CV) for determinations in triplicate was 4.5%. The variability of determinations on three different days was in the same range (5.5%). For vitamin E, the within-day CV was 3.6%, whereas the between-day CV was 10.1%.

Isolation of Micellar Fraction. Garrett et al.[14] as well as Reboul et al.[15] used ultracentrifugation at 167000*g* (95 min, 4 °C) and 20000 rpm (18 h, 10 °C), respectively, to separate mixed micelles from oil droplets and food particles. In our study, both methods resulted in lower amounts of carotenoids and vitamin E in filtrates compared to low-speed centrifugation (data not shown).

This loss may be due to the instability of micelles toward ultracentrifugation and the subsequent detachment of liberated micronutrients by the filtration step.[26] In contrast, Failla et al.[13] did not find differences in bioaccessibility of lutein, α-carotene, β-carotene, and lycopene between ultracentrifugation (167000*g*) and low-speed centrifugation (5000*g*). In recent years, ultracentrifugation for isolation of mixed micelles was frequently displaced by low-speed centrifugation or sedimentation overnight. A centrifugation at 5000 rpm for 20 min at room temperature was most applicable in practice and yielded the highest recovery in comparison to other centrifugation procedures.[27] Because centrifugation at 4500 rpm (~3900*g*), which was chosen in the present investigation, was insufficient in clarifying the suspension to enable a subsequent microfiltration, it was combined with an additional high-speed centrifugation step (14000 rpm, 5 min, room temperature).

Following the centrifugal separation, bile salt micelles and unilamellar vesicles containing liquid-crystalline particles coexist in the aqueous fraction.[28] To ensure that carotenoids in the aqueous fraction were actually in micelles, Garrett et al.[14] introduced a microfiltration step (0.22 μm pores) for separation of crystals, vesicles, and oil droplets. Filtration did not alter the amounts of lutein, α-carotene, and β-carotene, whereas the quantity of lycopene in the filtrate was significantly reduced to <0.05% of that in the digesta.[14] Others found significant losses of β-carotene, too, due to microfiltration.[12] Our own experiments resulted in equal amounts of carotenoids (lutein, zeaxanthin) in the supernatant before and after filtration ($p > 0.05$, *t* test). The content of vitamin E tended to decline (~15%) by the filtration step ($p = 0.069$, *t* test).

pH Value during Gastric Digestion. In numerous in vitro studies, gastric digestion and intestinal digestion were conducted at pH 2.0−2.5 and 7.0−7.5, respectively. Such a low pH in the stomach reflects rather the fasting state than the fed state. Additionally, a pH above 7 is found rather in the lower gut (ileum) than in the duodenum, where carotenoids and tocochromanols are mainly absorbed. The results of several in vivo studies[16,29,30] led to an adjustment of pH values during simulated digestion. According to Reboul et al.[15] the pH of the gastric medium was set at 4.0 on average. An accurate adjustment of pH was impeded because both the pH of the gastric medium and that of the duodenal medium slightly increased during in vitro digestion. However, the bioaccessibility of carotenoids and vitamin E was also determined at low gastric pH. At pH 2, carotenoids were less bioaccessible by trend ($p = 0.087$, *t* test) than at pH 4. Due to their instability in acid media the loss of carotenoids during gastric digestion at pH 2 was slightly higher than at pH 4 (Figure 3). Whereas the transfer of carotenoids from the food matrix to the lipid phase may be enhanced by low pH,[31] their transfer from lipid emulsion to mixed micelles was reduced.[32] The same may apply to vitamin E because bioaccessibility significantly declined by 24% at pH 2 (Figure 4). Overall, in vitro studies using different pH conditions for gastric digestion seem to be poorly comparable. In contrast, micellarization of carotenoids was quite independent of duodenal pH in the range of 5.0 and 9.5, provided that concentrations of pancreatin and bile approximate the fed state.[33]

Incubation Time. Gastric emptying is based on the caloric content of foods and can vary from 30 min to 5 h after ingestion.[34] In the first 30 min of digestion in vivo, most lipids and fat-soluble vitamins (>75% of vitamins A and E) disappeared from the stomach. Afterward, the rate of gastric emptying was slower

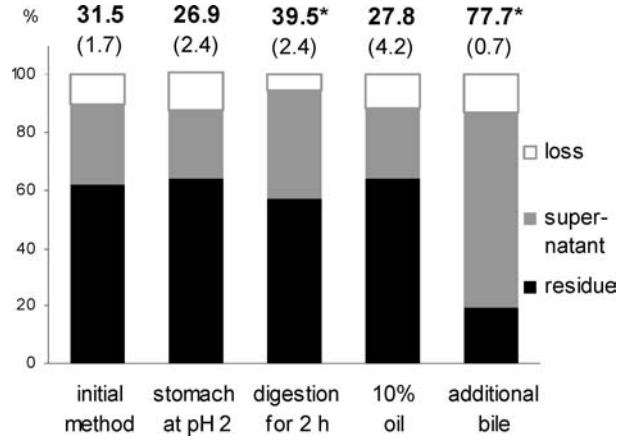

Figure 3. Influence of digestion procedure on bioaccessibility, distribution between residue and supernatant, and losses of carotenoids during digestion. Bold values above bars denote bioaccessibility of total carotenoids (%). Values in parentheses are standard deviations ($n = 3$). ∗ indicates significant difference from initial method ($p < 0.05$, *t* test).

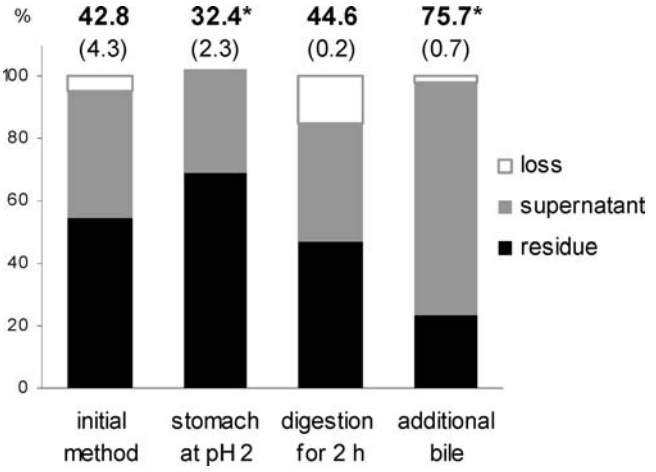

Figure 4. Influence of digestion procedure on bioaccessibility, distribution between residue and supernatant, and losses of vitamin E during digestion. Bold values above bars denote bioaccessibility of vitamin E (%). Values in parentheses are standard deviations ($n = 3$). ∗ indicates significant difference from initial method ($p < 0.05$, *t* test).

and relatively constant.[30] The incubation time at duodenal conditions was also set at 30 min to approach the digestive transit time in the human duodenum.[15] Because most of the other in vitro studies used incubation times around 2 h for the gastric as well as for the duodenal phase, the bioaccessibility of carotenoids and vitamin E was also determined by using incubation times of 1 h for both the gastric and intestinal phases of digestion. This prolongation of in vitro digestion resulted in a significant increase in the bioaccessibility of carotenoids (Figure 3), whereas the bioaccessibility of vitamin E was not changed (Figure 4). The latter observation may be due to higher losses of vitamin E (15%) in comparison to incubation times of 30 min each for the gastric and duodenal phases, which were probably caused by a longer contact of tocochromanols and oxygen. Additionally, losses of vitamin E possibly interfered with increased liberation from the food matrix and solubilization in mixed micelles due to the prolonged contact with digestive enzymes and bile salts.

Table 3. Bioaccessibility (Percent) of Carotenoids from Durum Wheat Pasta and Egg Pasta[a]

	(*all-E*)-lutein	(*all-E*)-zeaxanthin	(*all-E*)-canthaxanthin	total carotenoids
pasta 1	77.9 ± 0.8 a	87.9 ± 1.6 b		77.7 ± 0.7
pasta 2	72.3 ± 3.2 a	71.7 ± 9.0 a		72.3 ± 3.5
pasta 3	63.0 ± 4.4 a	62.6 ± 2.0 a		63.3 ± 4.0
pasta 4	73.2 ± 2.7 a	68.3 ± 13.8 a		72.0 ± 2.2
pasta 5	70.7 ± 14.7 a	71.9 ± 9.5 a		70.8 ± 14.0
egg pasta 1	58.5 ± 3.2 a	48.1 ± 21.7 a	47.7 ± 8.5 a	57.9 ± 2.2
egg pasta 2	55.7 ± 16.8 a	59.1 ± 8.6 a	52.7 ± 1.5 a	56.5 ± 14.5
egg pasta 3	57.3 ± 17.9 a	59.2 ± 11.1 a	52.3 ± 5.2 a	57.6 ± 16.5

[a] Values with different letters in a row are significantly different ($p < 0.05$, ANOVA/t test).

Addition of Oil. The impact of adding 10% refined rapeseed oil to pasta samples prior to in vitro digestion was evaluated only for carotenoids because the oil itself contained appreciable amounts of vitamin E (161 μmol/100 g), whereas carotenoids were not detected therein. Contrary to expectations, the addition of oil had no significant influence on micellarization of total carotenoids (Figure 3), which is in agreement with observations for lutein.[22] The micellarization of carotenoids is limited by the amount of micelles, which, in turn, depends on the concentration of bile salts[33] as well as on the quantity of lipolysis products.[30] The bile concentration of ~2.5 mg/mL in the initial digestion method, reflecting the fasting state, was possibly insufficient for adequate formation of mixed micelles. At low bile concentrations, more lipolysis products are required to swell the existing micelles to increase the solubilization of the aqueous phase.[33] However, lipolysis was shown to be quite low by using the initial digestion method (Figure 2). On the other hand, polar carotenoids, such as lutein and zeaxanthin, are localized at the surface of lipid droplets. Thus, they can be spontanously transferred to the aqueous phase, wheras apolar carotenes localized in the droplet's core absolutely require triglyceride lipolysis to be solubilized into mixed micelles.[35] Huo et al.[24] have also shown a higher impact of added oil on the bioaccessibility of α-carotene, β-carotene, and lycopene in comparison to lutein.

Amount of Bile Extract. With regard to the concentration of bile extract, the initial digestion method reflected the fasting state (~2.5 mg/mL), whereas the method of Reboul et al.[15] represented the fed state. Originally, they raised bile concentration in the digestive medium (~11 mg/mL) because lycopene was barely soluble in the micellar phase using the method of Garrett et al.[14] Our own experiments have also shown a remarkable increase in bioaccessibility of more polar compounds such as lutein, zeaxanthin (Figure 3), and tocochromanols (Figure 4). Garrett et al.[14] also observed a higher bioaccessibility of lutein (but not of carotenes) at a bile concentration of 3.6 mg/mL in comparison to 2.4 mg/mL. Thus, the additional amount of bile extract was used for further experiments.

Bioaccessibility of Carotenoids from Pasta. In agreement with other researchers,[14,23,27,36] carotenoids were almost stable during in vitro digestion. Recovery of carotenoids in the digesta was >83% and averaged 91%. Bioaccessibility of total carotenoids from pure durum wheat pasta amounted to 71.2 ± 5.2% on average, whereas carotenoids from egg pasta were significantly less accessible (57.3 ± 1.0%). This was unexpected because recent papers have indicated egg yolk is a source of highly bioavailable lutein. Chung et al.[37] found higher serum lutein responses in humans after egg consumption than after ingestion of spinach or lutein supplements. Lutein is located in the digestible lipid matrix of egg yolk containing cholesterol, triglycerides, and phospholipids. In contrast, in dark green leafy vegetables, being the main sources of lutein in the human diet, carotenoids form complexes with proteins in the thylakoid membranes of chloroplasts. Thus, in vitro bioaccessibility of carotenoids from green leafy vegetables was lower than from nongreen vegetables and fruits,[27,38] wherein they are accumulated in chromoplasts. During pasta processing lutein from egg yolk has presumably been incorporated into the complex network of proteins and starch, leading to its low bioaccessibility. Because carotenoids compete among themselves as well as with tocochromanols for micellarization,[39] qualitative and quantitative differences between pure durum wheat pasta and egg pasta (see above) may explain the differing bioaccessibilities. Micellarization of carotenoids and vitamin E may also be influenced by further fat-soluble compounds such as cholesterol from egg pasta. Phosphatidylcholine from egg yolk affects bioaccessibility by reducing the rates of lipolysis and micelle formation.[39] Overall, with regard to their availability in vitro pasta products appear as good sources of lutein. Indeed, they contain far less carotenoids than green leafy vegetables, such as kale or spinach, but they are consumed in substantially higher amounts.

There was no significant difference in bioaccessibility between (*all-E*)-lutein, (*all-E*)-zeaxanthin, and (*all-E*)-canthaxanthin (Table 3) or between different stereoisomers of lutein (data not shown). An isomerization of carotenoids during in vitro digestion was not observed. Another in vitro study has also shown no difference between lutein and zeaxanthin.[40] However, in contrast to our results, (*13Z*)-lutein was less accessible than (*all-E*)-lutein.

Bioaccessibility of Vitamin E from Pasta. The stability of vitamin E during simulated digestion was >78% and averaged 92%, which is in agreement with other in vitro studies.[23,27] The bioaccessibility of vitamin E was well correlated with the bioaccessibility of total carotenoids ($r = 0.908$, $p < 0.01$). In accordance with our observations on carotenoids, the bioaccessibility of vitamin E from pure durum wheat pasta (on average, 70.0 ± 4.2) was significantly higher than that from egg pasta (on average, 49.4 ± 5.1).

Significant differences between individual tocochromanols were hardly found. The bioaccessibility of α-tocopherol tended to be higher than that of γ-tocopherol and β-tocopherol. The bioaccessibility of β-tocotrienol was higher, by trend, than that of α-tocotrienol (Table 4). According to Reboul et al.[15] the bioaccessibility of tocopherols from different foodstuffs was highly variable. Only 0.5% of α-tocopherol was accessible from fresh apples, whereas 101% was available for absorption from

Table 4. Bioaccessibility (Percent) of Individual Tocopherols, Tocotrienols, and Vitamin E from Durum Wheat Pasta and Egg Pasta[a]

	α-tocopherol	β-tocopherol	γ-tocopherol	α-tocotrienol	β-tocotrienol	vitamin E
pasta 1	80.6 ± 0.5 a	85.1 ± 1.9 b		69.0 ± 0.0 c	73.7 ± 0.7 d	75.7 ± 0.7
pasta 2	75.8 ± 2.8 a	69.1 ± 3.7 b		68.5 ± 3.8 b	71.4 ± 3.1 b	71.9 ± 3.0
pasta 3	65.9 ± 3.0 a	64.3 ± 4.9 a		59.7 ± 3.5 a	64.5 ± 5.3 a	64.5 ± 4.2
pasta 4	70.3 ± 1.5 a	65.5 ± 6.2 a		64.6 ± 2.6 a	68.3 ± 1.8 a	68.2 ± 1.4
pasta 5	73.0 ± 10.6 a	64.1 ± 15.9 a		66.1 ± 14.3 a	69.3 ± 12.6 a	69.5 ± 12.5
egg pasta 1	60.0 ± 5.0 a	39.5 ± 4.4 b	48.0 ± 8.5 c	50.5 ± 5.1 c	53.4 ± 4.4 ac	54.3 ± 4.4
egg pasta 2	45.8 ± 10.1 a	36.9 ± 13.0 a	40.4 ± 7.9 a	41.7 ± 8.3 a	44.6 ± 11.7 a	44.2 ± 10.9
egg pasta 3	52.0 ± 14.4 a	42.0 ± 19.9 a	45.6 ± 17.9 a	48.3 ± 13.2 a	49.8 ± 15.6 a	49.8 ± 15.2

[a] Values with different letters in a row are significantly different ($p < 0.05$, ANOVA).

lettuce. For wheat bread, the bioaccessibility of α-tocopherol was very high (99%), but γ-tocopherol was hardly accessible (8%). The bioaccessibility of vitamin E from nuts and almonds, which are two of the main sources of tocochromanols, was relatively low (10−45%)[15,25] in comparison to the bioaccessibility from durum wheat pasta determined in the current study. Thus, pasta products emerge as valuable sources of vitamin E, particularly of tocotrienols.

The prevailing opinion that carotenoids are less bioavailable than vitamin E was not confirmed on the basis of in vitro data in the present study. One reason may be that carotenoids in pasta are solely xanthophylls, which have been shown to be more bioaccessible than tocopherols[27] and carotenes.[27,41] Because compounds containing hydroxyl groups are preferentially solubilized in the surface phospholipids of lipid droplets, their transfer to micelles is facilitated.[35] However, Borel et al.[35] suggested that two-hydroxy-group carotenoids, such as lutein and zeaxanthin, would transfer more rapdily between biological lipid structures than tocopherols. In the present study, the bioaccessibilities of xanthophylls and tocochromanols were in the same range.

In conclusion, differences between pure durum wheat pasta and egg pasta with regard to the bioaccessibility of carotenoids and vitamin E may result from the in vitro digestion method, which was characterized by an insufficient lipolysis. These observations may not reflect the bioavailability in vivo. Anyway, on the basis of our results pasta with and without eggs seem to be equal sources of carotenoids and vitamin E, which is due to higher contents of these micronutrients in egg pasta despite their lower bioaccessibility in vitro.

From a theoretical point of view carotenoids and vitamin E in the micellar phase are accessible in the small intestine, whereas compounds in the solid residue would reach the lower gut. A small amount of carotenoids was released from the food matrix by colonic microflora enzymes in vitro, making them available for absorption in the colon.[42] Additionally, it has to be noted that nonabsorbed carotenoids and tocochromanols play an important role in protecting the gastrointestinal tract itself from oxidative damage, thus reducing the incidence of inflammation and cancer.[43]

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ABBREVIATIONS USED

ADP,adenosine-5′-diphosphate; ATP,adenosine-5′-triphosphate; CV,coefficient of variation; dm,dry matter; glu,L-glutamic acid; HPLC,high-performance liquid chromatography; NADP,nicotinamideadenine dinucleotide phosphate; MTBE,methyl *tert*-butyl ether; PEEK,polyetheretherketone; TLC,thin layer chromatography; TNBS,2,4,6-trinitrobenzenesulfonic acid.

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