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Carotenoids and tocols of einkorn wheat (*Triticum monococcum* ssp. *monococcum* L.)

Alyssa Hidalgo[a], Andrea Brandolini[b,*], Carlo Pompei[a], Roberta Piscozzi[a]

[a]*DISTAM–Dipartimento di Scienze e Tecnologie Alimentari e Microbiologiche, University of Milan, Via Celoria 2, 20133 Milan, Italy*
[b]*CRA-Istituto Sperimentale per la Cerealicoltura, S. Angelo Lodigiano section, Via Mulino 3, 26866 S. Angelo Lodigiano (LO), Italy*

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Abstract

The nutritional properties of einkorn and its potential as a donor of useful traits to cultivated wheat prompted the survey of carotenoid and tocol content of 54 accessions of einkorn originating from different eco-geographical areas, by HPLC analysis. Eight *Triticum turgidum* and seven *Triticum aestivum* cultivars were analysed in parallel. Carotenoids, mostly lutein, averaged 8.41 μg/g dm, 2–4 times more than non-einkorn wheats, with a maximum of 13.4 μg/g dm. Several accessions showed significant amounts of carotenes (above 25% of total carotenoids), sometimes together with high lutein contents. Tocols averaged 77.96 μg/g dm, significantly exceeding the non-einkorn wheats, with a maximum of 115.85 μg/g dm. The most abundant tocol was β-tocotrienol (48.22 μg/g dm), followed by α-tocotrienol (12.77 μg/g dm), α-tocopherol (12.18 μg/g dm) and β-tocopherol (4.79 μg/g dm); the mean tocotrienol/tocopherol ratio was 3.68. Einkorns from different geographical areas had diverse average total carotenoid and tocol values; two geographic gradients were observed, possibly reflecting the original routes of spread of einkorn into Europe from the Middle East.

Keywords: Antioxidants; Lutein; Tocopherol; Tocotrienol; *T. monococcum*

1. Introduction

Grains, fruits and vegetables contain a broad variety of phytonutrients, including antioxidants, which show a significant effect on the reduction of the incidence of aging-related and chronic diseases (Anderson and Hanna, 1999; Jacobs et al., 1998). Among the numerous antioxidant compounds present in these foods two specific groups, the carotenoids and the tocols, play an important role in disease prevention (Andlauer and Fürst, 1998; Halliwell et al., 1995; Palozza and Krinsky, 1992).

Carotenoids are lipid-soluble antioxidants produced by most photosynthetic organisms and are responsible for the yellow, orange and red colours in many flowers, fruits and bird feathers. Two classes of carotenoids are recognised: the carotenes, which are tetraterpenoid hydrocarbons, and xanthophylls, which are carotenoids with one or more oxygenated functions present in the molecule (Van den Berg et al., 2000). Their biological properties are related to their structures. The single and double bonds repeats in the polyenic chain determines their antioxidant properties, while the presence of polar groups influences their interaction with cellular membranes (Britton, 1995). In plants, they function both as light collectors and protectors against photosensitization in chloroplasts. Animals cannot synthesise carotenoids and thus must obtain them from foods. In humans, carotenoids are involved in several functions. Particularly significant is the role of α- and β-carotenes in the biosynthesis of vitamin A, an essential factor for cellular reproduction, normal development of embryo and foetus, visual functions, etc. (Zile, 1998). Another function of carotenoids related to human health is

Abbreviations: α-T, α-tocopherol; α-T3, α-tocotrienol; ANOVA, analysis of variance; β-T, β-tocopherol; β-T3, β-tocotrienol; BHT, butylatedhydroxytoluen; CV, coefficient of variation; dm, dry matter; HPLC, high performance liquid chromatography; LSD, Fisher's least significant difference; NP-HPLC, normal phase high performance liquid chromatography; RP-HPLC, reverse phase high performance liquid chromatography; SD, standard deviation; s.e., standard error; THF, tetrahydrofuran; WSB, water-saturated 1-butanol.

*Corresponding author. Tel.: +39 371 211261; fax: +39 371 210372.
E-mail address: brandolini@iscsal.it (A. Brandolini).

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their antioxidant activity, which protects cells and tissues from free radicals and singlet oxigen. Lutein and zeaxanthin, in particular, have a fundamental role in the protection of the macula region of the retina and in the prevention of the cataracts; other beneficial actions include enhancement of the immune response, protection against solar radiation, inhibition of some cancers and prevention of degenerative and cardiovascular diseases (Krinsky, 1994; Van den Berg et al., 2000).

Tocols are lipid-soluble antioxidants, produced by photosynthetic organisms and classified as tocopherols (saturated phytyl group) or tocotrienols (triunsaturated phytyl group). Each tocol class includes four derivatives (α, β, γ, and δ), differing in the number and position of the methyl groups on the chromane ring. In plants, tocols fulfil several purposes, related to both photosynthetic and non-photosynthetic activities, because of their ability to reduce free radicals: in the chloroplasts they are involved in protecting the photosynthetic apparatus from lipid peroxidation (Yamauchi and Matsushita, 1979), while in other tissues they shield the polyunsaturated fatty acids from oxidation (Goffman and Bohme, 2001). The tocols are essential dietary components as they are not synthesised by animals. In animals, tocols have the ability to quench free radicals in cell membranes, thus protecting the membrane polyunsaturated fatty acids from damage. Tissue damage from free radicals is considered to be a cause of chronic diseases such as cardiovascular and neurological disorders, cancer, cataracts and inflammatory diseases (Bramley et al., 2000). Although α-tocopherol has the highest vitamin E activity, other tocols have similar or better antioxidant activity (Miller et al., 2000; Yoshida et al., 2003). Recent evidence suggests that tocotrienols may be even more efficient than tocopherols in preventing both cancer and cardiovascular disease (Theriault et al., 1999).

Interestingly, natural antioxidant activity of carotenoids and tocols might complement their positive functional characteristics in maintaining freshness and shelf life of food products: thus are a natural alternative to synthetic antioxidants (Shahidi, 2000).

Wheat is a basic human food staple supplying significant amounts of dietary carbohydrate and protein, and is also a useful source of antioxidant compounds (Andlauer and Fürst, 1998; Baublis et al., 2000; Miller et al., 2000). In bread wheat, however, the concentration of carotenoids is low (from 0.1 to 2.4 μg/g dm) but they are more abundant in durum wheat (1.5 to 4.0 μg/g dm) (Panfili et al., 2004; Zandomeneghi et al., 2000) where the yellow colour of the semolina, and the derived pasta, is perceived as an important quality trait. Tocols, in contrast, are abundant both in bread wheat (74.3 μg/g dm) and durum wheat (60.6 μg/g dm) (Panfili et al., 2003).

Although environmental factors play an important role in carotenoid and tocols concentration in cereals, the genetic component is predominant with heritability values that, for yellow pigment, a trait strictly related to carotenoids, in wheats range from 0.85 to 0.97 (Clarke et al., 1998; Elouafi et al., 2001; Parker et al., 1998), and for tocols in maize, oats and barley range from 0.73 to 0.93 (Peterson and Qureshi, 1993; Wong et al., 2003).

Einkorn (*Triticum monococcum* ssp. *monococcum* L.) is a diploid ancestral wheat, related to durum (*T. turgidum* ssp. *durum*) and to bread (*T. aestivum* ssp. *aestivum*) wheats. Einkorn was instrumental in the rise and spread of agriculture and a significant food source for several thousand years, before it was replaced by the more productive polyploid wheats during the Eneolithic period (Nesbitt and Samuel, 1996). The renewed nutritional interest in this cereal is related to its high protein and yellow pigment contents (Abdel-Aal et al., 1995; Borghi et al., 1996; Corbellini et al., 1999; D'Egidio et al., 1993), as well as its putative low allergenicity (De Vincenzi et al., 1996; Molberg et al., 2005). Einkorn may also be an excellent source of traits such as disease resistance, yellow pigment content, etc, for durum and bread wheats. The yellow pigments, as in other wheats, consist mostly of lutein (Abdel-Aal et al., 2002); in whole einkorn flours, lutein content is around 8.5 mg/kgdm, with an average value four times higher than bread wheat (Abdel-Aal et al., 2002). No information is available for tocols in einkorn.

Here we report HPLC analysis of the content of carotenoids, tocopherols and tocotrienols in 54 einkorns from different geographical regions, and several durum and bread wheats. Extraction and analysis methods for both classes of antioxidants were compared.

2. Experimental

2.1. Samples

Fifty-four accessions of cultivated einkorns (Table 2), kindly provided by several gene banks and researchers (see acknowledgements), were cropped during the 2004–2005 growing season in the Po plain, at S. Angelo Lodigiano (Italy), using cultural practices described by Castagna et al. (1995). Their origin can be traced to eight different eco-geographic areas (Table 2): Turkey (the original source of einkorn), Greece, Eastern Europe (Bulgaria and Rumania), the Balkans (Albania, Bosnia Herzegovina, Macedonia and Serbia), Italy, Germany, the northern side of the Alps (Austria and Switzerland) and Western Europe (Spain and Morocco). Eight *Triticum turgidum* and seven *Triticum aestivum* accessions (Table 3), belonging to different subspecies, were also grown as controls. After manual harvesting, samples were dried at ca. 10% humidity, dehulled in a M3B micro-thresher (Co.Mi.L, Rome, Italy) and ground in a Cyclotec 1093 laboratory mill (FOSS Tecator, Denmark) to a particle size < 200 μm. The resulting whole flour was stored under vacuum at -20 °C for a maximum of 24 h before analysis.

2.2. Extraction and analytical methods

To select the most efficient extraction method for carotenoids and tocols, three (water-saturated 1-butanol,

hot saponification, and tetrahydrofuran) and four (water-saturated 1-butanol, hot saponification, methanol and room temperature saponification) protocols, respectively, were tested on four replicates of the same einkorn sample, as follows:

2.2.1. Water-saturated 1-butanol (WSB)

The extraction was made according to AACC method 14-50 (AACC, 1994), with slight modifications: whole flour (1.5 g), accurately weighed in 10 ml plastic test tubes, and 10 ml of water-saturated 1-butanol were magnetically stirred for ca. 1 h; additionally, the samples were mixed on a vortex mixer for 1 min every 10 min. After centrifugation at 4024 *g* for 10 min at 8 °C using a Centrikon T-42 K centrifuge (Kontron Instruments, Bletchley, UK), the supernatant was filtered through a 0.22 μm PTFE membrane (Diana Beck Scientific, Angera, Italy). For carotenoid analysis, samples were immediately injected onto HPLC system. For tocol analysis, the supernatant was evaporated under vacuum at 60 °C in a Rotavapor (model VV 2000, Heidolph, Milan, Italy) and dried under a stream of nitrogen. The residue was dissolved in 2 ml hexane: 2-propanol (99:1, v/v) and filter through a 0.22 μm PTFE membrane (Diana Beck Scientific, Angera, Italy).

2.2.2. Hot saponification

The extraction was performed using the method of Fratianni et al. (2002). Exactly 2 g of whole flour was weighed into a screw-capped tube and saponified under nitrogen for 45 min at 70 °C, in 5 ml of ethanolic pyrogallol (60 g/l) as antioxidant, 2 ml of ethanol (95%), 2 ml of sodium chloride (10 g/l) and 2 ml of potassium hydroxide (600 g/l). During the saponification, the tubes were mixed on a vortex mixer every 5–10 min. Afterwards, they were cooled in an ice bath and 15 ml of sodium chloride (10 g/l) were added. The suspension was then extracted twice with 15 ml of hexane:ethyl acetate (9:1, v/v). The organic layer was collected and evaporated under vacuum, followed by nitrogen drying; the residue was dissolved in 2 ml of methanol:THF (95:5, v/v) for carotenoid analysis and in 2 ml hexane:2-propanol, (99:1 v/v) for tocol determination, and filtered through a 0.22 μm PTFE membrane (Diana Beck Scientific, Angera, Italy).

2.2.3. Tetrahydrofuran (THF)

Carotenoids were extracted using the method of Riso and Porrini (1997), with slight modifications: to 5 g of whole flour accurately weighted in 50 ml centrifuge test tubes were added 3 ml of methanol, and 15 ml of THF stabilised with 1% butylatedhydroxytoluene (BHT). The sample was mixed on a vortex mixer for 1–2 min and subsequently centrifuged at 21,900 *g* for 10 min at 5 °C. The residue was submitted to two additional cycles of THF extraction and centrifugation. The supernatants from each extraction were pooled, made up to 50 ml with stabilised THF and, after adding 20 ml of 20% NaCl, extracted three times with 50, 25 and 15 ml of petroleum ether 1% stabilised with BHT. The petroleum ether was evaporated under vacuum at 30 °C, nitrogen-dried, resuspended in 5 ml of methanol:THF (95:5, v/v) and filtered through a PTFE membrane.

2.2.4. Methanol

Tocols were extracted following the procedure described by Pinzino et al. (1999), with slight modifications: 0.5 g of whole flour accurately weighted in 10 ml polypropylene test tubes plus 5.5 ml of methanol were stirred for 2.5 h using a magnetic mixer and then centrifuged at 447 *g* for 8 min at 12 °C. The extraction was repeated after adding 5 ml of the same solvent to the residue. The two supernatants were combined in a 250 ml boiling flask, evaporated at 38 °C under vacuum and nitrogen-dried. The sample was then resuspended in 2 ml of hexane:2-propanol (99:1, v/v) and filtered through a PTFE membrane (Diana Beck Scientific, Angera, Italy).

2.2.5. Room temperature saponification

Tocols were extracted by the method of Konopka et al. (2004): whole flour (5 g) was accurately weighed into 50 ml volumetric flask and 7.5 ml of a solution of hexane:acetone:ethanol:toluene (10:7:6:7, v/v/v/v) and 1.5 ml of 40% KOH in methanol were added. After manual stirring, the samples were saponified for 16 h in the dark, supplemented with 7.5 ml of hexane and made up to 50 ml with 10% Na_2SO_4. The upper phase was collected and the lower phase was extracted three times, each with 2.5 ml of hexane. All supernatants were evaporated at 38 °C under vacuum, dried under nitrogen, dissolved in 3 ml hexane: 2-propanol (99:1, v/v) and filtered through a 0.22 μm PTFE membrane.

2.2.6. Carotenoid chromatography

Carotenoids separation was performed by RP-HPLC, by the method of Riso and Porrini (1997). The reverse-phase approach was selected to discriminate between α- and β-carotenes: they are direct precursors of vitamin A (Van den Berg et al., 2000) and precise measurements of the content of each compound are needed to better assess einkorn potential as vitamin A source. The filtered solution (20 μl) was injected in the HPLC system under the following operating conditions: column GRACE-Vydac 201TP54 C18, 250 × 4.6 mm, 5 μm (Hesperia, CA, USA); precolumn LiCHrospher WP 300 RP-18, 5 μm (Merck, Darmstadt, Germany); mobile phase, stabilised methanol:stabilised THF (95:5, v/v); flow rate, 1 ml/min; pump Waters 510 (Millipore, Milford, MA, USA). Carotenoids were detected at 445 nm, using a Waters 996 series photodiode array detector (Millipore, Milford, MA, USA), controlled by the software Millenium[32] Cromatography Manager (Waters Chromatography Division, Millipore, Milford). The wavelength range used was 200–600 nm.

2.2.7. Tocopherol and tocotrienol chromatography

Tocols were assessed by NP-HPLC by the method of Shin and Godber (1993), as modified by Panfili et al. (2003). Fifty ml of filtered extract was injected in a HPLC system including: an Alltima SI column, 250 × 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); an Alltima SI guard column 7.5 × 4.6 mm, 5 μm (Alltech Associates Inc., Deerfield, IL, USA); mobile phase, hexane:ethyl acetate:acetic acid (97.3:1.8:0.9, v/v/v); flow rate, 1.6 ml/min; a Multisolvent Delivery Unit Waters 600 (Millipore, Milford, MA, USA); with continous degassing with helium; fluorimetric detector Jasco 821 FP Intelligent Spectrofluorometer (Japan) at excitation-emission wavelengths of 290 and 330 nm, respectively; connected to a Hitachi D-7500 integrator (Merck, Darmstadt, Germany). After every 8–10 injections, the column was reactivated with hexane:2-propanol (90:10, v/v) for 30 min at a flow rate of 0.4 ml/min.

For peak quantification, carotenoid calibration curves were constructed using eight different concentrations (between 0.08 and 4.00 mg/l) of lutein standard (Fluka BioChemika, Buchs, Switzerland), eight different concentrations (between 0.02 and 1.18 mg/l) of α-carotene (LGC Promochem, Teddington, UK) and eight different concentrations (between 0.04 and 1.18 mg/l) of β-carotene (Sigma-Aldrich, Steinheim, Germany) in methanol:THF (95:5, v/v).

Tocol standard curves were constructed using eleven different concentrations (between 0.40 and 109.73 mg/l) of α-tocopherol standard (Fluka BioChemika, Buchs, Switzerland), and using 13 different concentrations (between 0.38 and 72.20 mg/l) of β-tocopherol standard (Supelco, Bellefonte, PA, USA) in hexane:2-propanol (90:10, v/v). The standard stock solutions concentrations were confirmed spectrophotometrically using the known absorption coefficient of each compound (Podda et al., 1996; Rodriguez-Amaya and Kimura, 2004). Tocotrienols were quantified using the standard curves of their corresponding tocopherol, as indicated by the official method (AOCS Ce 8-89, 1997). On the basis of the calibration curves, the detection limits were computed as the intercept value of the regression line plus three times the standard error of the estimate (Miller and Miller, 1988). Lutein, α-carotene, β-carotene, α-tocopherol, and β-tocopherol calibration curves were linear ($r^2 = 1$; $p \leqslant 0.001$) in the concentration ranges considered with detection limits in the standard solutions of 0.09, 0.04, 0.04, 0.39, and 0.80 mg/l. Carotenoid and tocol content (μg/g dm) are averages of two measurements.

The repeatability of the selected extraction method of carotenoids (WSB) and tocols (hot saponification) was assessed by performing, in each case, eight replicate measurements on the same einkorn sample. The results are expressed in terms of standard deviation (SD) and coefficient of variation (CV). The tocol recovery was assessed by adding to a replicate flour sample, 100 μl of a solution containing α-T (109.73 mg/l) and 100 μl of a solution containing β-T (95.01 mg/l). Four replicates of the spiked sample were extracted and quantified by HPLC.

2.3. Statistical analysis

Analysis of variance (ANOVA) was performed on all traits, first considering as main factors wheat species (*T. monococcum vs. T. turgidum vs. T. aestivum*) and, then, the geographic origin of the different einkorns. A significant number of samples had no detectable α- or β-carotene levels, which suggested the use of the nonparametric Kruskal–Wallace test for assessing the existence of dissimilarities among groups for these two parameters.

When significant differences ($p \leqslant 0.05$) were detected, Fisher's least significant difference (LSD) or standard errors (s.e.) were computed. Both ANOVA and LSD were determined using the statistical programme STATGRAPHICS plus (version 4); the Kruskal-Wallace test was performed with MSTAT-C software. Pearson's correlations of the means were calculated using the SYSTAT for Windows software (version 5).

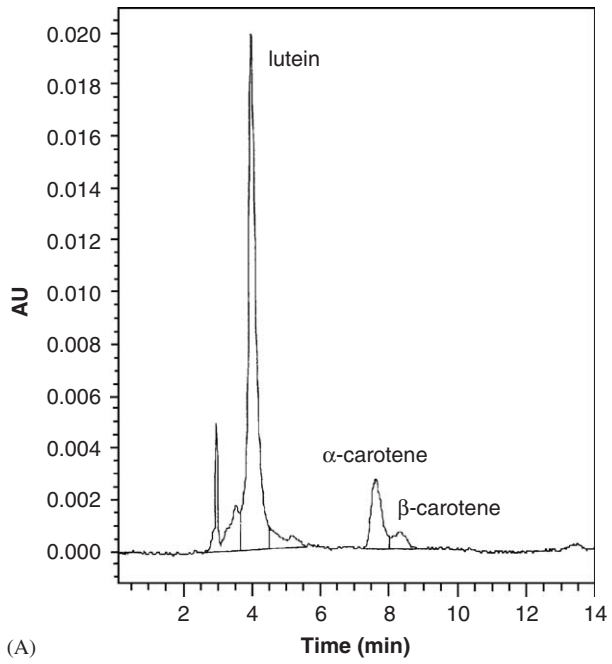
3. Results

3.1. Analytical methods

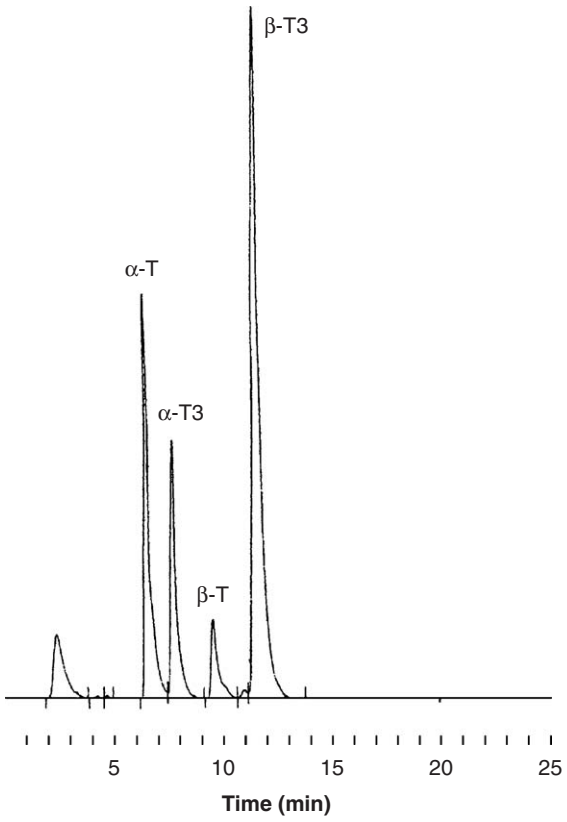
Typical chromatograms for carotenoids (A) and tocols (B) from einkorn integral flour are presented in Fig. 1. Table 1 reports the HPLC lutein and tocols contents obtained by applying different extraction protocols on the same einkorn sample. The ANOVA highlighted significant differences ($p \leqslant 0.001$) among extraction protocols, both for lutein and for tocols.

For lutein, which accounts for more than 90% of total carotenoids (but see later), the LSD test indicated that the butanol method extracts significantly better than the other two techniques. This method was therefore selected for all further analyses because, besides the higher extraction ratio, is easy and quick, thus insuring fewer analytical errors. Panfili et al. (2004), comparing several protocols for normal phase chromatography, did not detect significant differences between the hot saponification and the 1-butanol methods. However, they reported that this last approach resulted in higher CVs.

For tocols, the LSD test suggested that hot saponification and the butanol protocols have a better extraction efficiency than the others; however, because no antioxidants were used in the original protocol, the lower recovery by room temperature saponification might be due to oxidative effects. Hot saponification gave a significantly higher extraction of the α-T3 fraction and this method was selected for all further analyses. Moreover, the 1-butanol technique required an additional evaporation step (at 60 °C under vacuum for 45 min) because of the incompatibility between 1-butanol and the mobile phase required for normal phase analysis; and a longer separation time (50 vs 25 min) because of the presence of interference



(A)



(B)

Fig. 1. Typical chromatograms for carotenoids (A) and tocols (B) from einkorn integral flour.

between peak tails. Interestingly, Panfili et al. (2003), working other cereals, concluded that hot saponification was more efficient than methanol or no-saponification extraction methods.

The repeatability of the two selected methods, expressed in terms of mean ± SD (μg/g dm) and CV, was 8.27 ± 0.08 (CV = 1%) for lutein, 13.64 ± 0.28 (CV = 2.1%) for α-T, 15.11 ± 0.17 (CV = 1.1%) for α-T3, 5.55 ± 0.03 (CV = 0.6%) for β-T, 44.38 ± 0.24 (CV = 0.6%) for β-T3.

The results obtained in the recovery assay demonstrated that the hot saponification method did not degrade α-T and β-T naturally present in einkorn samples. In fact recovery percentages were of 101.8% (theoretical value 17.00 vs detected value 17.27 ± 0.55 μg/g dm) for α-T and of 103.2% (theoretical value 9.50 vs detected value 9.84 ± 0.12 μg/g dm) for β-T.

3.2. Carotenoid content

The carotenoid contents of the einkorn accessions are reported in Table 2. The total carotenoid content varied between 5.33 (ID1605) and 13.64 (ID432) μg/g dm, with an average of 8.41 μg/g dm. Lutein was the most common component (on average, 91%), but several einkorns (ID471, ID492, ID1643, ID1607, ID515, ID322) had up to 25–33% of α- + β-carotenes. The average lutein content (7.69 μg/g dm) was comparable to that reported by Abdel-Aal et al. (2002), but the range of variation was higher (from 3.92 of ID342 to 12.64 μg/g dm of ID 432 compared with 1.78 to 8.69 μg/g dm). Furthermore, while Abdel-Aal et al. (2002) found only traces of β-carotene and of some unidentified substances, in our analyses 37 out of 54 accessions (68%) contained α-carotene, averaging 0.77 μg/g dm and reaching 1.74 μg/g dm in samples ID471 and ID1643, whereas β-carotene was detectable only in 24 samples (44.4%), (mean 0.43 μg/g dm, maximum value of 0.65 μg/g dm). No other major carotenoids were detected. The accessions with high carotene levels (e.g. ID471, ID492, ID1643) had low total carotenoid (lutein) content; an interesting exception was represented by accession ID119 which showed 1.88 μg/g dm carotenes, together with 7.82 μg/g dm of lutein (total carotenoids 9.70 μg/g dm). The apparently higher total carotenoid levels reported by Borghi et al. (1996) and Corbellini et al. (1999) are not directly comparable, since their method is not valid for carotenoid quantification but only for colour measurement.

The values for non-einkorn *Triticum* species are presented in Table 3. In the *T. turgidum* group, α- and β-carotenes were below the detection limit in seven out of the eight cultivars, and the mean lutein content was 3.00 μg/g dm (range 1.89 to 4.79 μg/g dm). The cultivar Baio, a well-known yellow-semolina durum wheat, showed traces of β-carotene (0.21 μg/g dm) and had the highest total carotenoid content (5.00 μg/g dm). These results are consistent with those for wholemeal (Abdel-Aal et al., 2002; Fratianni et al., 2005; Panfili et al., 2004). The level of α- and/or β- carotenes reported in each of these reports and observed only in cv. Baio in the present research, are however below our stringent detection limits. Zeaxanthin, a carotenoid found in small amounts by Fratianni et al. (2005) and Panfili et al. (2004) using normal-phase chromatography, was not observed, possibly because it

Table 1
Lutein and tocols contents (μg/g dm) in an einkorn sample using different extraction methods

Extraction method	Lutein	α-T	α-T3	β-T	β-T3
WSB[a]	8.33±0.06	8.37±0.61	5.80±0.37	3.13±0.50	20.25±1.40
Hot saponification	7.89±0.06	9.09±0.20	7.57±0.20	3.25±0.06	23.24±0.83
Tetrahydrofuran	7.02±0.15				
Methanol		5.57±1.28	4.15±1.14	2.15±0.61	14.89±5.10
Room temperature saponification		6.14±1.15	4.08±0.61	2.56±0.35	18.12±2.16

[a]Water-saturated 1-butanol.

was below our detection limits and/or because of the reverse-phase approach selected for this research.

The *T. aestivum* group had a low lutein content, with a mean value of 1.90 μg/g dm (range 1.40 to 2.71 μg/g dm), but interestingly some accessions (the bread wheat Mieti and the spelts Trivento and Rouquin) showed measurable quantities of α- and β-carotene. The total carotenoids, as a consequence, are slightly higher than lutein, reaching a mean value of 2.12 μg/g dm (range 1.52–3.05 μg/g dm); similar results were reported for lutein by Abdel-Aal et al. (2002), Adom et al. (2003), Konopka et al. (2004) and Panfili et al. (2004). Small amounts of β-carotene, zeaxanthin or β-cryptoxanthin (often below our detection limit) were also reported in some these studies.

Differences in carotenoid content among the einkorns and the two groups of testers were assessed by ANOVA or by the nonparametric Kruskal–Wallace test (Section 2.3). Einkorns showed significantly higher means than *T. turgidum* and *T. aestivum* for lutein (7.69 vs 3.00 and 1.90 μg/g dm), α-carotene (0.53, nd and 0.11 μg/g dm), β-carotene (0.19, 0.03 and 0.11 μg/g dm) and total carotenoids (8.41, 3.02 and 2.12 μg/g dm). On average einkorns had four times more lutein than the *T. aestivum* controls and almost all samples recorded higher values than *T. turgidum* wheats, with some accessions having two to three times more lutein than the highest durum and Kamut wheats; these results are similar to those of Abdel-Aal et al. (2002).

3.3. Tocopherol and tocotrienol content

The tocopherol and tocotrienol content of einkorn is shown in Table 4, as well as the T3/T ratio. The only tocols detected were α-tocopherol (α-T), α-tocotrienol (α-T3), β-tocopherol (β-T), β-tocotrienol (β-T3). The predominant tocol in einkorn was β-T3 (61.9%), followed by α-T3 (16.4%), α-T (15.6%) and β-T (6.1%). The unsaturated/saturated tocol ratio (T3/T) was on average 3.68, with a minimum of 2.63 (ID1623) and a maximum of 6.34 (ID471). Comparisons with other einkorn data are not possible because, to the best of our knowledge, they are not available in literature.

The tocol values for the 15 non-einkorn wheat controls are shown in Table 3. The *T. turgidum* group contained on average 52.91 μg/g dm and the T3/T mean ratio was 2.97, while for the *T. aestivum* group the total tocol content was 62.75 μg/g dm and the T3/T ratio was very low (1.79). The predominant tocol in both the *T. turgidum* and *T. aestivum* groups was, as in *T. monococcum*, β-T3 (65.1% and 55.2%, respectively). Tocol contents and compositions for both groups of non-einkorn wheats were very similar to the values reported by Marconi et al. (2001), Panfili et al. (2003) and Piironen et al. (1986).

Differences in tocol content among einkorn and the two groups of testers were assessed by ANOVA. Einkorn showed significantly higher means than *T. turgidum* and *T. aestivum* for α-T3 β-T3, total tocols and T3/T ratio (Tables 3 and 4). On the other hand, *T. aestivum* showed the highest values for α-T and β-T. Compared with einkorn wheat, the controls showed a higher proportion of α-T (18.2% and 23.0% for durum and bread wheats, respectively) and β-T (7.8% and 13.2%), and a lower proportion of α-T3 (9.0% and 8.6%). The data suggest that einkorn is a rich source of tocols, especially of tocotrienols. The total tocol content observed in einkorn (61.80–115.85 μg/g dm), in fact, compares favourably not only with wheats but also with values for barley: Cavallero et al. (2004; 51.0–61.4 μg/g dm); Panfili et al. (2003; 74.7); Peterson and Qureshi (1993; 42.16–80.03 μg/g), and for oats: Panfili et al. (2003; 72.1 μg/g dm); Peterson and Qureshi (1993; 19.00–30.32 μg/g dm). The einkorn tocotrienol/tocopherol ratio (3.7) was superior to those (1.9–3.3) for different wheats and close to that for barley (3.8) (Panfili et al., 2003). Some *T. monococcum* accessions had T3/T ratios (5.05–6.14) in the range reported for oats (6.00) (Panfili et al., 2003). A high tocotrienol/tocopherol ratio in the diet might prove important because of the hypocholesterolemic action of the T3 (Qureshi et al., 1991).

The correlations (not shown) among the 54 einkorn accessions revealed a highly significant and positive relationship between carotenoid and lutein content ($r = 0.90$; $p \leqslant 0.001$), and a negative relationship between carotenoid and carotenes ($r = -0.61$; $p \leqslant 0.001$). Total tocol content was strongly linked to β-T3 ($r = 0.841$; $p \leqslant 0.001$), whereas correlations among single tocols, even when significant, were quite low. Finally, carotenoid and tocol contents were completely uncorrelated.

3.4. Geographical distribution

An ANOVA, with geographic area of origin as the independent factor and the accessions within areas as

Table 2
Einkorn accessions, their origin and carotenoids content (μg/g dm)

Sample	Donor code	Country	Area code	Lutein	α-carotene[a]	β-carotene[a]	Total carotenoids
ID5	PI 119435	Turkey	A	8.20	0.33	0.29	8.82
ID7	PI 167615	Turkey	A	5.93	0.37	nd	6.29
ID127	AT12910/89	Turkey	A	8.54	nd	nd	8.54
ID301	BGRC43446	Turkey	A	6.97	0.53	nd	7.50
ID415	18755	Turkey	A	7.82	1.00	nd	8.82
ID492	PI 119423	Turkey	A	4.81	1.65	0.65	7.11
ID496	PI 167591	Turkey	A	6.08	1.21	nd	7.29
ID505	PI 170196	Turkey	A	7.48	1.10	nd	8.59
ID339	BGRC 43485	Greece	B	8.99	0.47	0.38	9.84
ID432	19842	Greece	B	12.64	0.58	0.42	13.64
ID515	ID515	Greece	B	5.44	1.46	0.60	7.51
ID517	ID517	Greece	B	6.92	0.48	0.33	7.72
ID322	BGRC 43468	Bulgaria	C	5.37	1.49	0.52	7.37
ID342	BGRC 43488	Bulgaria	C	3.92	1.30	0.49	5.72
ID476	90451	Bulgaria	C	6.56	0.69	0.34	7.59
ID357	22553	Romania	C	5.66	1.07	0.44	7.17
ID539	PI 306542	Romania	C	9.38	0.38	nd	9.75
ID541	PI 306544	Romania	C	7.67	nd	nd	7.67
ID1134	PI 428150	Romania	C	7.68	0.29	nd	7.97
ID529	PI 277133	Albania	D	9.78	0.37	0.26	10.40
ID1641	IKB 7535	Albania	D	8.63	0.38	nd	9.01
ID1643	IKB 7537	Albania	D	5.78	1.74	0.65	8.17
ID1650	IKB 7544	Albania	D	7.64	0.38	nd	8.03
ID471	90436	Bosnia Herzegovina	D	4.92	1.74	0.65	7.31
ID571	PI 362610	Macedonia	D	9.31	0.50	nd	9.81
ID575	PI 377666	Serbia	D	8.57	nd	nd	8.57
ID574	PI 377662	Serbia	D	8.14	nd	nd	8.14
ID1605	GM1	Italy	E	4.11	0.85	0.37	5.33
ID1606	GM2	Italy	E	5.35	1.09	0.33	6.77
ID1607	GM3	Italy	E	5.75	1.64	0.61	8.00
FAR31		Italy	E	8.01	nd	nd	8.01
ID147	BGRC 7030	Germany	F	8.32	nd	nd	8.32
ID151	BGRC 7037	Germany	F	7.74	nd	nd	7.74
ID153	BGRC 7039	Germany	F	7.41	nd	nd	7.41
ID156	BGRC 7042	Germany	F	9.00	0.33	nd	9.33
ID241	BGRC 36580	Germany	F	9.32	0.37	0.33	10.02
ID260	BGRC 37352	Germany	F	9.96	0.35	nd	10.30
ID1341	MG15543	Germany	F	7.93	0.40	0.45	8.78
ID196	BGRC 13195	Austria	G	7.80	nd	nd	7.80
ID197	BGRC 13196	Austria	G	7.43	nd	nd	7.43
ID300	BGRC 43445	Austria	G	8.55	nd	nd	8.55
ID304	BGRC 43449	Austria	G	8.43	nd	nd	8.43
ID247	BGRC 36586	Switzerland	G	8.71	0.45	nd	9.15
ID251	BGRC 36593	Switzerland	G	7.95	nd	nd	7.95
ID314	BGRC 43459	Switzerland	G	7.39	nd	nd	7.39
ID315	BGRC 43461	Switzerland	G	8.98	nd	nd	8.98
ID491	PI 94740	Spain	H	8.81	0.33	0.35	9.49
ID1157	PI 518452	Spain	H	8.09	0.36	0.29	8.75
ID1389	K20498	Spain	H	7.78	0.60	0.45	8.83
ID1623	LPCH3	Spain	H	8.86	0.51	0.37	9.74
ID118	TRI 4320/74 SKL	Morocco	H	8.58	nd	nd	8.58
ID119	TRI 4321/75 SKL	Morocco	H	7.82	1.38	0.50	9.70
ID1509	LPCH 93	Morocco	H	9.95	nd	nd	9.95
ID1511	LPCH 95	Morocco	H	8.38	0.37	0.28	9.03
Mean				7.69	0.53	0.19	8.41
SD				1.63	0.54	0.23	1.32

nd = non detectable, i.e. lower than the detection limit.
[a]Means and SD were computed considering nd as 0.

Table 3
T. turgidum and *T. aestivum* controls, their carotenoids, tocopherols (T) and tocotrienols (T3) contents (μg/g dm)

Species	Subspecies	Cultivar	Lutein	α-carotene[a]	β-carotene[a]	Total caroten.	α-T	α-T3	β-T	β-T3	Total tocols	T3/T
T. turgidum	*durum*	Baio	4.79	nd	0.21	5.00	8.34	5.35	3.70	39.89	57.27	3.76
T. turgidum	*durum*	Cappelli	2.58	nd	nd	2.58	12.55	3.13	4.51	18.69	38.87	1.28
T. turgidum	*durum*	Creso	1.96	nd	nd	1.96	8.50	4.70	4.04	36.05	53.28	3.25
T. turgidum	*durum*	Duilio	2.72	nd	nd	2.72	10.66	5.91	4.57	33.55	54.70	2.59
T. turgidum	*durum*	Simeto	3.72	nd	nd	3.72	9.92	5.20	2.77	30.46	48.35	2.81
T. turgidum		Kamut®	4.42	nd	nd	4.42	7.57	4.33	3.16	25.15	40.21	2.75
T. turgidum	*dicoccum*	Caporciano	1.89	nd	nd	1.89	7.17	4.68	3.89	46.96	62.70	4.67
T. turgidum	*dicoccum*	Agnone	1.90	nd	nd	1.90	12.24	4.74	6.26	44.69	67.92	2.67
Mean			3.00	0.00	0.03	3.02	9.62	4.75	4.11	34.43	52.91	2.97
SD			1.17	0.00	0.07	1.21	2.06	0.82	1.06	9.60	10.16	0.98
T. aestivum	*aestivum*	Bolero	1.76	nd	nd	1.76	18.15	6.42	11.89	38.48	74.94	1.49
T. aestivum	*aestivum*	Eureka	1.52	nd	nd	1.52	12.39	5.65	7.35	34.68	60.06	2.04
T. aestivum	*aestivum*	Mieti	2.51	0.31	0.23	3.05	12.99	5.49	7.95	37.28	63.71	2.04
T. aestivum	*aestivum*	Sagittario	1.40	nd	nd	1.40	14.90	4.70	6.46	29.40	55.46	1.60
T. aestivum	*aestivum*	Salmone	1.78	nd	0.25	2.04	12.07	4.53	5.96	30.61	53.16	1.95
T. aestivum	*spelta*	Trivento	2.45	0.34	0.17	2.96	16.05	5.46	10.16	37.52	69.18	1.64
T. aestivum	*spelta*	Rouquin	2.71	0.27	nd	2.98	14.35	6.19	10.05	36.89	67.48	1.77
Mean			1.90	0.11	0.11	2.12	14.42	5.38	8.29	34.66	62.75	1.79
SD			0.47	0.17	0.12	0.72	2.39	0.69	2.29	3.84	8.28	0.25

nd = non detectable, i.e. lower than the detection limit.
[a]Means and SD were computed considering nd as 0.

replicates, detected significant differences between groups of einkorns originating from different areas for all traits except α-tocopherol; the Kruskal–Wallace test confirmed the existence of statistically measurable differences also for α- and β-carotene content. Table 5 lists, for each region, the mean values and standard error (s.e.) for carotenoid and tocol content. The highest mean total carotenoid was observed for the accessions from Greece (9.68 μg/g dm) and Western Europe (9.26 μg/g dm), while the accessions from Italy showed the lowest mean level (7.03 μg/g dm). For lutein content the accessions from Northern Europe (Alps and Germany) had very low contents of α-carotene (0.06 and 0.21 μg/g dm, respectively) and β-carotene (not detected and 0.11 μg/g dm, respectively), while the areas in which the *T. monococcum* lines were richest in vitamin A precursors were Greece (0.75 and 0.43 μg/g dm) and Italy (0.90 and 0.33 μg/g dm).

As mentioned above, no significant differences were observed between geographical zones for α-T content. For α-T3, Greece showed a value (17.07 μg/g dm) well above all other areas. Italy, on the other hand, had a low content (8.99 μg/g dm), but was outstanding for both β-T (7.45 μg/g dm) and β-T3 (62.97 μg/g dm). As a consequence, einkorn from Italy had the highest total tocol content (92.02 μg/g dm), followed by the Balkans (82.56 μg/g dm), with Turkey (72.16 μg/g dm) and Eastern Europe (73.88 μg/g dm) showing the lowest values. The Balkans area was outstanding for the high T3/T ratio (4.47), while all the other regions had similar unsaturated to saturated tocol ratios (range 3.35–3.88).

When the means of carotenoids and tocols for the different geographical areas are plotted on a map of Europe, two different gradients are evident (Fig. 2). The total carotenoid content presents a consistent increase moving from the Eastern Europe (C) region to the Alps (G) and Germany (F), to Western Europe (H), whereas its value declines from East to West along the Mediterranean coast of Greece (B), the Balkans (D) and Italy (E). Carotene contents that are at the opposite ends of the range are therefore present in Western Europe (H, high) and Italy (E, low). Two gradients, exactly overlapping those of total carotenoid content, are evident also with respect to total tocopherols: their content increases only slightly along the continental route that reaches Western Europe (H), but shows a sharp rise from Greece (B) to the Balkans (D) and to Italy (E). Again, Italy (high) and Western Europe (low) are on opposite ends of the distribution. These two gradients are supported also by the individual carotenoid and tocol contents; only β-tocopherol hints at a more complex Mediterranean dispersal, with multiple local routes and/or introduction events.

Scattered *T. monococcum* ssp. *monococcum* populations, mainly used for animal feeding, persist up to now in marginal areas of their broad Eneolithic distribution. However, and different from all other wheats, during the last 4000 years *T. monococcum* has been of limited relevance for human consumption and thus einkorn most likely was not traded or improved, remaining largely unchanged both in terms of distribution and of gene pools. The spread of farming from the Fertile Crescent into Europe supposedly followed two main paths (Pinhasi et al., 2005). The first, a continental path to northern Europe, and the second, Mediterranean

Table 4
Tocopherols (T) and tocotrienols (T3) content of einkorn (μg/g dm)

Sample	Area code	α-T	α-T3	β-T	β-T3	Total tocols	T3/T
ID5	A	17.35	17.83	5.19	43.43	83.80	2.72
ID7	A	11.50	11.50	2.93	51.40	77.33	4.36
ID127	A	12.59	12.50	3.15	41.12	69.35	3.41
ID301	A	11.30	6.83	5.23	42.52	65.88	2.99
ID415	A	10.37	9.19	4.10	38.55	62.20	3.30
ID492	A	11.37	10.74	6.42	45.43	73.95	3.16
ID496	A	12.62	11.07	5.88	45.59	75.16	3.06
ID505	A	10.84	11.38	3.57	43.82	69.61	3.83
ID339	B	13.18	17.14	6.04	41.74	78.10	3.06
ID432	B	11.73	21.39	4.17	45.85	83.14	4.23
ID515	B	13.05	16.18	5.65	39.74	74.62	2.99
ID517	B	10.91	13.58	4.52	50.08	79.09	4.13
ID322	C	12.19	7.73	5.30	53.66	78.87	3.51
ID342	C	10.88	11.23	3.50	48.61	74.22	4.16
ID476	C	12.86	15.00	3.60	40.29	71.74	3.36
ID357	C	11.52	8.64	6.05	52.18	78.39	3.46
ID539	C	11.01	14.96	3.87	51.47	81.31	4.47
ID541	C	8.12	9.92	2.03	41.37	61.45	5.05
ID1134	C	12.09	12.52	5.07	41.54	71.22	3.15
ID529	D	16.94	20.27	8.14	49.01	94.35	2.76
ID1641	D	11.07	13.04	4.38	50.69	79.17	4.12
ID1643	D	10.88	9.14	5.40	61.31	86.74	4.33
ID1650	D	10.75	15.93	3.28	45.20	75.16	4.36
ID471	D	8.86	10.19	3.27	66.76	89.08	6.34
ID571	D	10.90	16.71	2.74	53.66	84.01	5.16
ID575	D	11.33	16.09	3.39	48.87	79.68	4.41
ID574	D	10.48	15.35	3.22	43.18	72.24	4.27
ID1605	E	12.35	9.22	8.90	55.36	85.82	3.04
ID1606	E	11.93	8.67	6.87	64.55	92.02	3.90
ID1607	E	12.74	7.22	5.94	48.49	74.39	2.98
FAR31	E	13.41	10.83	8.12	83.49	115.85	4.38
ID147	F	14.96	15.83	4.58	47.28	82.64	3.23
ID151	F	13.03	9.26	5.01	41.89	69.19	2.83
ID153	F	13.00	9.64	4.96	40.93	68.52	2.82
ID156	F	11.42	10.36	5.04	61.35	88.17	4.36
ID241	F	11.52	15.22	4.17	51.98	82.88	4.28
ID260	F	11.34	15.62	3.92	42.21	73.08	3.79
ID1341	F	11.09	12.44	3.30	55.22	82.04	4.70
ID196	G	13.21	15.47	3.98	42.68	75.34	3.38
ID197	G	11.50	10.58	5.34	48.24	75.66	3.49
ID300	G	16.52	24.85	3.54	44.44	89.35	3.45
ID304	G	10.46	11.32	4.14	48.56	74.48	4.10
ID247	G	10.96	8.78	6.28	49.45	75.46	3.38
ID251	G	10.57	15.48	4.18	40.79	71.02	3.81
ID314	G	9.58	8.79	3.68	43.10	65.14	3.91
ID315	G	11.78	10.93	4.76	57.35	84.82	4.13
ID491	H	14.06	12.46	5.62	46.61	78.74	3.00
ID1157	H	13.52	13.69	5.37	44.35	76.94	3.07
ID1389	H	13.28	12.72	5.17	39.08	70.25	2.81
ID1623	H	13.45	15.30	3.58	29.46	61.80	2.63
ID118	H	14.60	14.50	5.57	49.44	84.11	3.17
ID119	H	12.06	7.66	5.71	50.79	76.21	3.29
ID1509	H	14.12	13.73	5.36	51.39	84.61	3.34
ID1511	H	14.50	13.15	5.71	48.14	81.50	3.03
Mean		12.18	12.77	4.79	48.22	77.96	3.68
SD		1.81	3.71	1.39	8.44	9.19	0.74

path through southern Europe to Spain, substantially aided by early maritime movements (Nesbitt and Samuel, 1996).

Carotenoid and tocol contents are independent traits that were not subject to conscious human selection and are selectively neutral (i.e. are unchanging as populations

Table 5
Carotenoids and tocols average content (μg/g dm ± s.e.) of einkorns per geographic area of origin

Area	Code	Lutein		α-carotene		β-carotene		Carotenoids		α-T		α-T3		β-T		β-T3		Tocols		T3/T	
		Mean	s.e.	Mean[a]	s.e.[a]	Mean[a]	s.e.[a]	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	Mean	s.e.	mean	s.e.
Turkey	A	6.98	0.45	0.77	0.20	0.12	0.08	7.87	0.34	12.24	0.78	11.38	1.11	4.56	0.46	43.98	1.34	72.16	2.42	3.35	0.18
Greece	B	8.50	1.56	0.75	0.24	0.43	0.06	9.68	1.42	12.22	0.54	17.07	1.62	5.09	0.45	44.35	2.29	78.74	1.75	3.60	0.29
East Eur.	C	6.61	0.60	0.75	1.19	0.26	0.08	7.61	0.40	11.24	0.51	11.43	0.96	4.20	0.45	47.02	1.93	73.88	2.22	3.88	0.29
Balkans	D	7.85	0.60	0.64	0.25	0.19	0.10	8.68	0.36	11.40	0.83	14.59	1.29	4.23	0.63	52.34	2.84	82.56	2.61	4.47	0.37
Italy	E	5.80	0.81	0.90	0.34	0.33	0.13	7.03	0.64	12.60	0.32	8.99	0.74	7.45	0.66	62.97	7.59	92.02	8.74	3.58	0.30
Germany	F	8.53	0.35	0.21	0.07	0.11	0.07	8.84	0.42	12.33	0.53	12.62	1.11	4.42	0.25	48.69	2.94	78.07	2.91	3.72	0.27
Alps	G	8.16	0.21	0.06	0.06	nd		8.21	0.24	11.82	0.77	13.27	1.89	4.49	0.33	46.83	1.87	76.41	2.67	3.71	0.13
West Eur.	H	8.53	0.25	0.45	0.15	0.28	0.07	9.26	0.19	13.70	0.29	12.90	0.82	5.26	0.25	44.91	2.62	76.77	2.70	3.04	0.08

nd = non detectable, i.e. lower than the detection limit.
[a]Means and s.e. were computed considering nd as 0.

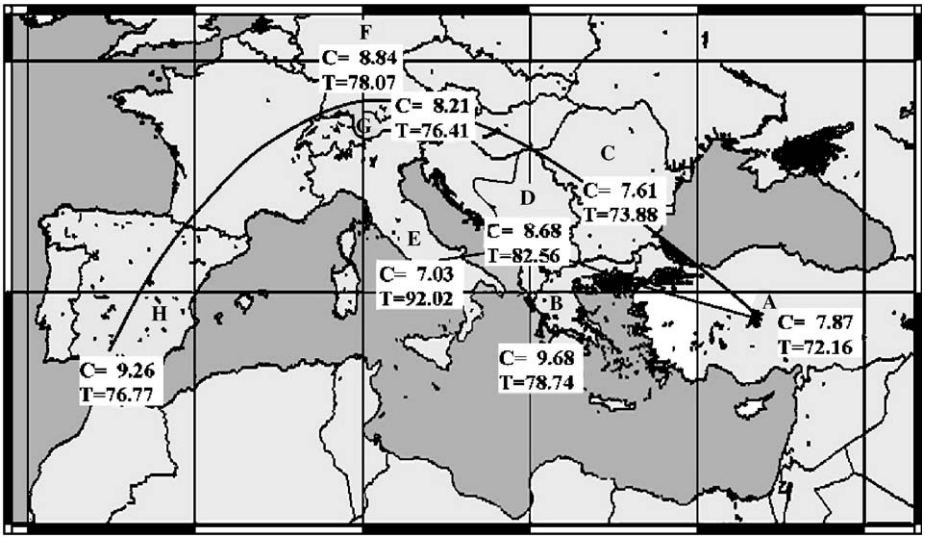


Fig. 2. Geographical variation for total carotenoids and tocols content of einkorn groups of accessions from different areas. The lines indicate the gradients observed for both classes of compounds; Turkey, the domestication place of einkorn, is the starting zone.

adapt over time to different environments). The concentration gradients observed are thus probably a trace of the human-mediated spread of einkorn into Europe.

4. Conclusions

The einkorns analysed in this survey have high carotenoid and tocol contents and demonstrate broad variations both in terms of amount and of composition. While our data support the conclusion of Abdel-Aal et al. (2002) that einkorns "may hold potential as high-lutein functional wheats", an interesting new finding is that accessions exist with significant quantities of α- and β-carotenes, indicating the possibility of selecting and/or developing new genotypes with high carotenoids levels and customised composition. The significant levels of lipophilic antioxidants, coupled with high T3/T ratios, provide more evidence that einkorn is a nutritionally outstanding cereal.

The differences in total carotenoids and total tocols observed among einkorns from different areas are probably an evidence of the prehistoric diffusion routes of *T. monococcum* into Europe: a main continental pathway that, passing north of the Alps, reaching the western end of its range (Spain and Morocco), and a Mediterranean route that reached Italy but did not proceed further.

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