RESEARCH ARTICLE

In vitro antioxidant activity of tocopherols and tocotrienols and comparison of vitamin E concentration and lipophilic antioxidant capacity in human plasma

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A comparative study investigated four tocopherols, four tocotrienols, and α-tocopheryl acetate on their antioxidative activities in five different popular assays, which were adapted to non-polar antioxidants. α-Tocopherol, used as calibration standard, showed the highest ferric reducing antioxidant power. Greater ring methyl substitution not only led to an increase of scavenging activity against the stable 2,2-diphenyl-1-picrylhydrazyl radical, but also to a decrease in oxygen radical absorbance capacity. Regarding α-tocopherol equivalent antioxidant capacity no significant differences in the antioxidant activity of all vitamin E isoforms were found. In contrast, a significantly lower peroxyl radical-scavenging activity of α-tocochromanols was determined in a chemiluminescence assay. Except oxygen radical absorbance capacity, no significant differences of the antioxidant activity related to the side chain could be detected. The data show that the reducing ability and radical chain-breaking activity of the several vitamin E forms depends on the circumstances under which the assays are performed. In our opinion, the used lipophilic methods can be useful for estimating the antioxidant activity of strong non-polar antioxidants, *e.g.* carotenoids, too. Furthermore, we could show a significant correlation between the total tocopherol content in human plasma and the lipophilic antioxidant capacity measured by α-tocopherol equivalent antioxidant capacity and 2,2-diphenyl-1-picrylhydrazyl.

Keywords:
Antioxidant capacity / Radical-scavenging activity / Reductive activity / Tocopherol / Tocotrienol

1 Introduction

The term vitamin E summarizes four vitamers with a saturated side (phytyl) chain, called tocopherols, and four vitamers with unsaturated side chain, called tocotrienols (T3), only synthesized by plants and cyanobacteria [1], and therefore essential for human and animal nutrition. In all vitamin E forms, the chromanol ring is substituted with methyl groups in different positions (α-, β-, γ-, and δ-) (Fig. 1). The molecules have three chiral carbons at 2-, 4′-, and 8′-position of the side chain. The natural tocopherol isomers have an R-configuration in all the three positions. The identification of the 6-hydroxy chroman ring system in all compounds with vitamin E activity led to the designation of the term ''tocochromanols'', a group encompassing all tocopherols and tocotrienols [2].

α-Tocopherol (α-T) is the most abundant form of tocopherols in green leaves of many plant species, whereas γ-T is predominant in most seeds [3]. Some plant seeds contain mainly tocopherols, in some others tocotrienols are predominant, *e.g.* rice bran is a distinguished source [4]. Due to the differences in the affinity of different vitamin E forms to the α-T transfer protein, expressed in liver and brain, α-T is the predominant tocochromanol in human metabolism, red

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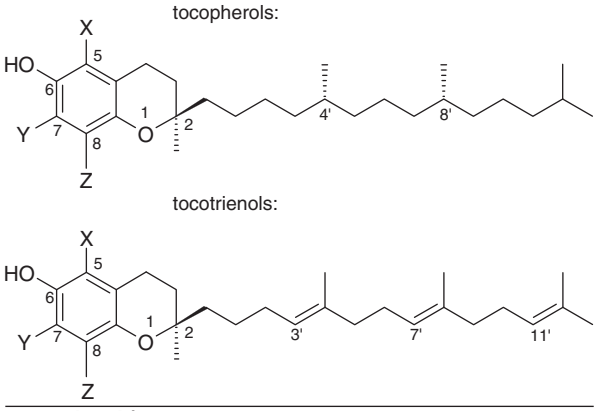
Abbreviations: **α-T,** α-tocopherol; **α-TE,** α-tocopherol equivalents; **αTEAC,** α-tocopherol equivalent antioxidant capacity; **AAPH,** 2,2′-azobis-(2-amidinopropane) hydrochloride; **ABTS,** 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; **AMVN,** 2,2′-azobis-(2,4-dimethylvaleronitrile); **AUC,** area under the curve; **BHT,** 2,6-di-*tert*-butyl hydroxytoluene; **CL,** chemiluminescence; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl; **ET,** electron transfer; **FRAP,** ferric reducing antioxidant power; **HAT,** hydrogen atom transfer; **ORAC,** oxygen radical absorbance capacity; **RMCD,** randomly methylated β-cyclodextrin; **TPTZ,** 2,4,6-tripyridyl-s-triazine



tocopherol / tocotrienol	X	Y	Z
α-	CH_3	CH_3	CH_3
β-	CH_3	H	CH_3
γ-	H	CH_3	CH_3
δ-	H	H	CH_3

Figure 1. Structures of tocopherols and tocotrienols.

blood cells and plasma (20.5–26.8 μmol/L), respectively, with other forms of vitamin E being present only in low amounts (γ-T: 1.5–3 μmol/L) [5–7]. The protective effect of α-T on LDL oxidation is related to its lipid solubility and its concentration in the lipid fraction of LDL. One LDL particle contains between 5 and 12 molecules of α-T, whereas the concentrations of γ-T, β-carotene, and other antioxidants are less than one molecule *per* LDL particle [8].

Tocochromanols belong to the class of lipid antioxidants. The ability to protect double bonds of unsaturated fatty acids in oils against oxidative damage was discovered by Cummings *et al.* [9]. Vitamin E, especially α-T, is known as a strong chain-breaking [10], peroxyl radical-scavenging [11] antioxidant, due to its 6-hydroxychroman structure [11]. The tocopherol reacts with peroxyl radicals to tocopheroxyl radicals very fast, before they can abstract hydrogen from a target [1, 12], and after further reaction with lipid peroxides and hydrolysis, yields in tocopheryl quinone [13]. The function to quench singlet oxygen ($^1O_2$) is another important role of tocopherols in antioxidant processes in plants and animals [12]. Chemically, T and T3 are closely related, however, it has been observed that they have widely varying degrees of biological effectiveness [14]. The *in vivo* antioxidant activities of tocopherols against lipid peroxidation are α-T>β-T>γ-T>δ-T [15–18], depending on the degree (α>β=γ>δ) and pattern of methylation. Some scientists have reported that α-T is *in vitro* more effective than β-T and γ-T [1, 11, 19], others controvert them [3, 18]. The antioxidant activity of tocotrienols compared to tocopherols is also discussed controversially. α-T3 seemed to be several times more active than α-T in lipid peroxidation in membranes [20], because T3 are more mobile within the membrane bilayer and less restricted in interaction with lipid radicals due to their unsaturated side chain [21]. Furthermore, in a system with multi-lamellar vesicles, T3 were more active against the typical synthetic radicals 2,2′-azobis-(2-amidinopropane) hydrochloride (AAPH), AMVN, and SIN-1 in the order α>β>γ>δ [22]. Other articles showed that the relative effectiveness depends on the experimental conditions [14, 20, 23–25].

In summary, there is only a marginal and controversial knowledge on the antioxidant activity of vitamin E active substances [26], especially tocotrienols, when reacting in *in vitro* methods. There is only one comparative study in literature available obtaining results of *in vitro* antioxidant assays with all eight vitamin E homologues [14]. No study exists using current popular assays to assess the antioxidant activity of T and T3. Most comparisons of the antioxidant activity of fat soluble compounds, *e.g.* natural ones like carotenoids, lipoic acid or ubiquinones and synthetic antioxidants like 2,6-di-*tert*-butyl hydroxytoluene (BHT), 2-*tert*-butyl-hydroxy-anisol (BHA) and hydroxyltyrosol derivatives, were done only with tocopherols or exclusively with α-T [25, 27–33]. The aim of this study was to compare the different forms of vitamin E concerning their antioxidant activity. To deepen our understanding of whether or not there is any significant difference between T and T3 isoforms in the reactivities towards radicals and metal ions, the authors performed various lipophilic assays currently used in literature for single compounds, food, and biological samples. Previous hydrophilic assays were adapted to these non-polar compounds, too.

A wide range of one-dimensional assays to detect the antioxidant activity *in vitro* was investigated, using a high variation of conditions, oxidants, and methods to measure end-points of oxidation. Three single electron transfer (ET) based assays were used to detect the reducing ability of the eight vitamin E stereoisomers against oxidizing agents like ferric ions (ferric reducing antioxidant power, FRAP) or synthetic dyes like 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) (αTEAC) and 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$). The widely accepted oxygen radical absorbance capacity (ORAC) assay, a classical hydrogen atom transfer (HAT) based assay [34], was used to investigate the activity of the tocochromanols against peroxyl radicals, the primary products of lipid peroxidation [1]. A chemiluminescence (CL) assay, based on luminol luminescence, also initiated by peroxyl radical reaction, was applied for comparison with ORAC results.

In addition, the vitamin E concentrations in human plasma samples were compared to the lipophilic antioxidant capacity of the plasma measured by DPPH and αTEAC assay.

2 Material and methods

2.1 Chemicals

All chemicals used were of analytical grade. 2,4,6-tripyridyl-s-triazine (TPTZ), potassium peroxodisulfate ($K_2S_2O_8$), ABTS, AAPH, DPPH, HCl, and BHT were purchased from Sigma-Aldrich (Taufkirchen, Germany). Acetone, ethanol, *n*-hexane, $CH_3COONa \cdot 3H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 2H_2O$, NaCl, and NaOH were obtained from

VWR (Darmstadt, Germany). Luminol (3-aminophthal hydrazide) was from Fluka (Buchs, Switzerland). Ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and di-Natriumtetraborat ($NaB_4O_7 \cdot 10H_2O$) were from Roth (Karlsruhe, Germany). Acetic acid (CH_3COOH), boric acid (H_3BO_3), and manganese dioxide (MnO_2) were purchased from Merck KGaA (Darmstadt, Germany). Fluorescein and DMSO were purchased from Riedel-de Häen (Seelze, Germany). Methyl *tert*-butyl ether was purchased from Carlo Erba Reactifs-SDS (Val de Reuil, France). Randomly methylated β-cyclodextrin (RMCD; CAVASOL® W7 M) was obtained from Wacker (Burghausen, Germany). HPLC grade water (18 MΩ) was prepared using Millipore Milli-Q purification system (Millipore, Schwalbach, Germany).

DL-α-T, β-T, γ-T and δ-T were purchased from Calbiochem (Darmstadt, Germany). According to the manufacturer's instructions, the tocopherols were shown by GC to be at least 95% pure except for α-T at 100%. α-Tocotrienol (α-T3), β-tocotrienol (β-T3), γ-tocotrienol (γ-T3), and δ-tocotrienol (δ-T3) were obtained from Davos Life Sciences (Singapore) with a purity higher than 97% by HPLC. DL-α-tocopheryl acetate (α-TA) was purchased from Sigma-Aldrich, with purity higher than 96% by HPLC.

2.2 *In vitro* antioxidant activity of tocopherols and tocotrienols

All five *in vitro* methods used to assess the antioxidant activity were calibrated with α-T as standard, because α-T is considered to have the highest biopotency. All compounds were compared to α-T. Therefore, the results of all tocochromanols were calculated compared to the results of α-T.

2.2.1 FRAP assay

A newly developed FRAP assay measures the reducing potential of lipophilic antioxidants. The performance of this FRAP assay is based on the application of the hydrophilic FRAP reagent as described in the classical FRAP assay [35], but uses the antioxidants dissolved in *n*-hexane. Consequently, when combining both solutions, a two-phase system was applied. All T and T3 were dissolved in *n*-hexane at a concentration of *ca.* 25 μmol/L. The exact concentration of each working solution was derived from the molar absorptivity at the specific absorbance maximum of each vitamer (Table 1). One hundred microliters of each vitamer were mixed with 600 μL daily fresh prepared FRAP reagent, which consisted of ten volumes of 300 mmol/L acetate buffer (pH 3.6), one volume of 20 mmol/L $FeCl_3$ and one volume of 10 mmol/L TPTZ in 40 mmol/L HCl. This ferric TPTZ complex is reduced to its iron (II) form by reducing agents like antioxidants. After 6 min of mixing (1000 rpm) on a thermo shaker (Eppendorf, Hamburg, Germany) at 25°C, the solutions were transferred completely into half-micro cuvettes (Brand, Wertheim, Germany), followed by centrifugation of the cuvette (30 s, 1200 rpm) to separate the phases. Exactly 8 min after starting the mixing, the absorbance of the lower phase was measured at 595 nm in a V-530 spectrophotometer (Jasco, Gross-Umstadt, Germany). *n*-Hexane acted as blank. α-T standard solutions (*ca.* 4.5–114 μmol/L) were used to construct a linear regression line. Fresh working standards were prepared daily by diluting an ethanolic α-T stock solution (*ca.* 2.3 mmol/L, stored at −24°C) with *n*-hexane after blowing off the ethanol with nitrogen at 30 ± 1°C. The exact concentrations of the α-T standards were determined in the same way as for all vitamers. Each experiment with the vitamers was done in triplicate. Antioxidant activity of the vitamers was calculated as μmol α-T equivalents (α-TE)/μmol vitamer.

Table 1. Molar absorption coefficients and absorbance maxima of tocopherols and tocotrienols in ethanol, modified from [43]

Vitamer	Molar weight (g/mol)	Absorbance maximum λ (nm)	Molar absorption coefficient ε ($mol^{-1}\,cm^{-1}$)
α-T	430.7	292	3259
β-T	416.7	296	3719
γ-T	416.7	298	3802
δ-T	402.6	298	3509
α-T3	424.7	293	3858
β-T3	410.6	294	3579
γ-T3	410.6	296	3711
δ-T3	396.0	297	3489
α-TA	472.8	285	2077

2.2.2 α-TE antioxidant capacity assay

A lipophilic α-TE antioxidant capacity (αTEAC) assay was performed by using $ABTS^{\bullet+}$ according to the TEAC assay published by Miller *et al.* [36], slightly modified in accordance to Böhm *et al.* [37] and calibrated with α-T instead of Trolox. $ABTS^{\bullet+}$ was preformed by passing a 5 mmol/L aqueous stock solution of ABTS through a filter paper (Merck-Schuchardt, Hohenbrunn, Germany), which was coated with manganese dioxide. This stock solution was filtered by passing it through a 0.25 μm syringe filter (Schleicher & Schuell, Dassel, Germany). An $ABTS^{\bullet+}$ working solution was prepared daily by diluting the $ABTS^{\bullet+}$ stock solution with PBS (75 mmol/L, pH 7.4) to an absorbance of 0.70 ± 0.05 at 734 nm and preincubated at room temperature for 2 h. All T and T3 were dissolved in *n*-hexane at a concentration of *ca.* 25 μmol/L. The exact concentration of each working solution was derived from the molar absorptivity at the specific absorbance maximum of each vitamer (Table 1). One hundred microliters of each T and T3, respectively, were mixed with 600 μL $ABTS^{\bullet+}$ working solution. After being shaken in a tube for 30 s, the solutions

were transferred completely in the half-micro cuvette, followed by centrifugation of the cuvette (30 s, 1200 rpm) to separate the phases. Two minutes after starting the mixing, the absorbance of the lower phase was measured at 734 nm in the V-530 spectrophotometer. The α-T standard solutions (*ca.* 4.5–114 μmol/L), already used in FRAP assay, were used to construct a linear regression line. The working standards were prepared daily as described above. *n*-Hexane acted as blank. Each experiment was repeated three times. Antioxidant capacity of the vitamers was calculated as μmol α-TE/μmol vitamer.

2.2.3 DPPH-free radical-scavenging activity assay

The lipophilic DPPH assay was done according to the procedures of Liu *et al.* [28]. DPPH is one of a few stable and commercially available organic nitrogen radicals, soluble in organic media [38]. Upon reduction, color intensity fades. As in the FRAP and αTEAC assays described above, all vitamers were dissolved to a concentration of *ca.* 25 μmol/L, however, this time in ethanol/*n*-hexane, 1+1 v/v. One milliliter vitamer solution was mixed with 0.5 mL of 0.3 mmol/L ethanolic DPPH stock solution (stored in fridge) in a reaction tube. After 15 min of shaking (1000 rpm) on the thermo shaker at 25 °C, the solutions were transferred completely into macro cuvettes (Ratiolab, Dreieich, Germany), followed by measuring the absorbance at 515 nm in the V-530 spectrophotometer. The ethanol/*n*-hexane solvent mixture acted as blank. α-T standard solutions (*ca.* 4.5–114 μmol/L) were used to construct a linear regression line. Fresh working standards were prepared daily as described above. The exact concentrations of the α-T standards were determined as explained above. Each experiment was done in triplicate. Antioxidant capacity of the vitamers was calculated as μmol α-TE/μmol vitamer.

2.2.4 CL oxygen radical-scavenging activity assay

A lipophilic CL assay was developed based on the luminescent light emission formed in the reaction of luminol with oxygen radicals generated by degradation of AAPH [39]. A 10 mM luminol solution was freshly prepared daily in a mixture of borax buffer (20 mmol/L, pH 9.5) and DMSO (20+80, v/v) for use as chemiluminescent probe. Fifty microliters of each vitamer dissolved in DMSO to a concentration of approximately 200 μmol/L (exact concentration was analyzed as in the other assays before), 100 μL DMSO-borax buffer mixture, and 50 μL luminol solution were pipetted into the wells of white 96-well microplates (Greiner Bio-One, Frickenhausen, Germany) and the plate was pre-warmed for 10 min at 37°C. DMSO acted as blank and control. Daily freshly prepared α-T standard solutions (*ca.* 12–240 μmol/L) were used for calibration as described above, however this time in DMSO. By addition of 150 μL freshly prepared and ice cooled 60 mmol/L AAPH solution in freshly prepared solvent mixture of PBS (75 mmol/L, pH 7.4) and DMSO (20+80, v/v), the reaction was started and the CL intensity was measured every second minute for 90 min at 37°C. Wells for controlling the stability of luminol without radical impact of AAPH were filled up with the same volume of PBS-DMSO mixture. To calculate the CL value of the samples, first the AUC (area under the curve) of each well of samples, blank, standard, and control was calculated. Thereafter, the AUC_{net} was calculated by subtracting the AUC of sample and standard from that of the blank, respectively (Eq. 1).

$$AUC_{net} = AUC_{blank} - AUC_{sample/standard} \quad (1)$$

By using a regression equation between AUC_{net} and α-T concentration, the final CL value for the analyzed compounds was expressed as μmol α-TE/μmol vitamer. Blank, each vitamer and each standard was measured on the microplate in quadruplicate. Each experiment was repeated three times.

2.2.5 ORAC assay

ORAC assay was performed following the procedures reported by Huang *et al.* [40] with slight modifications. Fluorescein dissolved in PBS (75 mmol/L, pH 7.4) was used as fluorescent probe. A 1.2 μmol/L working solution, freshly prepared from a 120 μmol/L fluorescein stock solution stored in the fridge, was used for analysis. As fluorescent filters in the Optima FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany) 485 nm (excitation) and 520 nm (emission) were used. Reactions were carried out in 75 mmol/L PBS (pH 7.4) on a 96-well quartz glass micro plate (Hellma, Müllheim, Germany). All T and T3 solutions were prepared from an ethanolic stock solution (*ca.* 2.3 mmol/L) as described above. After blowing off the ethanol under nitrogen at 30±1°C, the vitamers were mixed with a definite volume of a 7% RMCD solution in acetone–water mixture (1+1, v/v) to a concentration of *ca.* 50 μmol/L for 30 min (1400 rpm) on the thermo shaker at 25°C. A total volume of 50 μL vitamer solution, 100 μL PBS, and 25 μL fluorescein working solution were pipetted into microplate wells and pre-warmed for 10 min at 37°C. RMCD solvent of 7% acted as blank and control. Daily freshly prepared α-T standard solutions (*ca.* 12–240 μmol/L) were used for calibration as described above, however, this time in 7% RMCD. The exact concentrations of each standard and vitamer were analyzed as reported in the other assays above. By addition of 150 μL freshly prepared and ice cooled 129 mmol/L AAPH solution in PBS the reaction started, and the fluorescence intensity was measured by the plate reader each minute for 4 h at 37°C. Wells for controlling photostability of fluorescein were filled up with the same volume of PBS. To calculate the ORAC value of the samples, first all measurements were expressed relative to the initial

reading. Afterwards, the AUC of each well was calculated (Eq. 2),

$$\mathrm{AUC} = 1 + \left(\frac{f_1 + f_2 + f_3 + \cdots + f_i}{f_0}\right) \quad (2)$$

where f_0 is the relative fluorescence at 0 min and f_i is the relative fluorescence at time i. The $\mathrm{AUC_{net}}$ of the samples were obtained by subtracting the AUC of the blank from that of sample and standard, respectively (Eq. 3).

$$\mathrm{AUC_{net}} = \mathrm{AUC_{sample/standard}} - \mathrm{AUC_{blank}} \quad (3)$$

After using a regression equation between $\mathrm{AUC_{net}}$ and α-T concentration of the standard, the final ORAC values of the analyzed vitamin E forms were expressed as μmol α-TE/μmol vitamer. Blank, each standard and each vitamer was measured on the microplate in quadruplicate. Each experiment was done in triplicate.

2.3 Vitamin E content and lipophilic antioxidant capacity in human plasma

The plasma was sampled after the approval (ethical vote no. 2377–09/08) by the Ethical Committee of the Friedrich Schiller University Jena at the Medical Faculty (Bachstrasse 18, 07743 Jena, Germany). The plasma was collected into Li-heparin Monovettes (Sarstedt, Nümbrecht, Germany) and was stored at −80°C until analysis.

2.3.1 Concentrations of tocopherols in plasma

After precipitation of plasma proteins by ethanol, the quantification of tocopherols in plasma started by extraction with *n*-hexane containing 0.1% BHT. The extract was analyzed by HPLC on a Eurospher 100 DIOL (250 × 4.0 mm, 7 μm) column (Knauer, Berlin, Germany) by using *n*-hexane-methyl *tert*-butyl ether (98+2, v/m) as mobile phase with a flow of 1.5 mL/min at a column temperature of 35 ± 1°C with fluorescence detection (excitation: 292 nm, emission: 330 nm) [41–43]. All samples were analyzed in triplicate. α-Tocopheryl acetate acted as internal standard for calculating recovery. Plasma tocopherol concentration (sum of all detectable tocopherols) was calculated by means of peak areas of the respective standards α-, β-, γ- and δ-T and expressed as micromole total tocopherols/100 mL. The exact concentrations of all standards were determined using the molar absorptivity at the specific absorbance maximum (Table 1).

2.3.2 αTEAC assay of plasma

The lipophilic αTEAC assay of plasma was performed as previously described as TEAC by Böhm *et al.* [44] with some slight modifications. Instead of Trolox α-T standard solutions (*ca.* 4.5–114 μmol/L) were used to calculate a linear regression line, as described in the αTEAC for the vitamers above. First, 500 μL plasma and 500 μL ethanol were mixed for 30 s. After addition of 500 μL *n*-hexane, the mixture was shaken for 30 s, placed 10 s in an ultrasonic bath, shaken for 30 s, and centrifuged at 1000 rpm for 2 min. The extraction with *n*-hexane was repeated three times. The combined organic phases were evaporated to dryness under N_2 at 30 ± 1°C, and the residue was dissolved in 400 μL *n*-hexane by shaking and using ultrasonic bath. One hundred microliters of the plasma extracts were analyzed in this assay as described above for the vitamin E homologues. The α-T standard solutions (*ca.* 4.5–114 μmol/L) were used to construct a linear regression line. The working standards were prepared daily as described above. *n*-Hexane acted as blank. Each plasma extract was measured twice and each plasma sample was analyzed in triplicate. Antioxidant capacity of the vitamers was calculated as μmol α-TE/100 mL of plasma.

2.3.3 DPPH-free radical-scavenging activity of plasma

The plasma samples were extracted as described above (αTEAC of plasma), however, the resulted residue was dissolved in 1 mL ethanol/*n*-hexane 1+1 v/v. The complete plasma extract was mixed with 500 μL 0.3 mmol/L ethanolic DPPH solution and shaken (1000 rpm) at 25°C for 15 min on the thermo shaker. Afterwards, the shaken solutions were transferred completely into macro cuvettes, and the absorbance was measured at 540 nm in the V-530 spectrophotometer. The ethanol/*n*-hexane solvent mixture acted as blank. α-T standard solutions (*ca.* 4.5–114 μmol/L) were used to construct a linear regression line. Fresh working standards were prepared daily by diluting an ethanolic α-T stock solution (*ca.* 2.3 mmol/L, stored at −24°C) with ethanol/*n*-hexane mixture (1+1, v/v) after blowing off the ethanol with nitrogen at 30 ± 1°C. The exact concentration of the α-T working solutions was derived from the molar absorptivity at the specific absorbance maximum (Table 1). Each plasma sample was analyzed in triplicate. Antioxidant capacity of the plasma samples was calculated as μmol α-TE/100 mL plasma.

2.4 Statistical analysis

All experiments with tocopherols, tocotrienols, and plasma samples were done in triplicate. Data were expressed as means ± SD. For comparison of means of vitamer analysis one-way ANOVA with Student–Newman–Keuls *post-hoc* test was used, after checking the homogeneity of variances by Levene statistic, performed using SPSS 17.0 (SPSS, Chicago, IL, USA). A difference was considered statistically significant at $p < 0.05$. Correlations between tocopherol

concentrations and lipophilic antioxidant capacity of the plasma samples were done by bivariat Pearson procedure with $p < 0.01$.

3 Results and discussion

3.1 Method validation of the FRAP assay

3.1.1 Linearity

The linear relationship between absorption and concentration of the antioxidant was evaluated using α-T. Table 2 summarizes correlation coefficient, slope, and intercept of the α-T standard curves. All analyzed samples demonstrate a good linear relationship between absorption and concentration. α-T was used as a calibration standard. An acceptable correlation coefficient (r^2) was ≥ 0.99.

3.1.2 Precision and accuracy

Table 3 summarizes precision and accuracy of the FRAP assay. The precision, which is expressed as % RSD for all quality control samples, was within $\pm 15\%$. The accuracy of the method varies from 98 to 110% within individual batches and from 99 to 107% between the batches.

3.2 Method validation of the CL assay

3.2.1 Linearity

α-T was used to calculate the linear regression between the net AUC and concentration of the antioxidant. Figure 2 shows the CL progression curves in the presence of α-T and AAPH, and depicts the linear response between concentration and the net AUC for α-T. Table 4 summarizes correlation coefficient (r^2), slope, and intercept of the α-T standard curves. A good linear relationship between absorption and concentration ($r^2 \geq 0.99$) was determined in all analyzed samples.

Table 2. Summary of α-T calibration curve[a)] in the FRAP assay

Run	R^2	Slope (*a*)	Intercept (*b*)
1	0.9975	0.0064	0.0218
2	0.9986	0.0071	0.0190
3	0.9977	0.0060	0.0041
4	0.9988	0.0069	0.0174
5	0.9986	0.0056	0.0211
6	0.9993	0.0070	0.0116
7	0.9939	0.0073	0.0069
8	0.9993	0.0074	0.0054
Average	0.9979	0.0067	0.0134
Accepted criteria	$\geqslant 0.9900$	n. a.	n. a.

a) [*Y* [μM] = *b*+*aX* (net absorbance)]; n.a., not applicable.

3.2.2 Precision and accuracy

Table 5 summarizes precision and accuracy in the CL assay using α-T as candidate compound. Again, the precision is expressed as % RSD for all quality control concentrations, and ranged within $\pm 15\%$. The accuracy of the method ranged from 96 to 107% within individual batches and from 100 to 102% between all batches.

3.3 Antioxidant activity of tocopherols and tocotrienols

The antioxidant activities of all tocochromanols investigated are shown in Fig. 3. The hydroxyl group in C-6 of the chromanol ring is very important for the radical-scavenging activity, as known from other phenols [11]. The phenolic hydrogen is donated to lipophilic free radicals [45, 46]. Hence, by esterification the antioxidant activity gets almost lost [47]. We were able to show this relationship for α-TA in all five *in vitro* assays (Fig. 3). α-TA is popular as additive in the industry. Indeed, neither oxygen nor UV radiation has any destructive effect on this compound [48]. As mentioned, α-TA did not show activity as antioxidant in our assays and therefore it is not useful as antioxidant in food and cosmetics to avoid deterioration of the matrix by lipid peroxidation. Consequently, α-T is used in its unesterified form to stabilize *e.g.* animal fats, which are lower in vitamin E content than vegetable oils [48]. However, α-TA is useful as biological antioxidant, as this tocopheryl ester is hydrolyzed in the human organism prior to absorption [1].

In four of the five assays used (Fig. 3) we approved the perception that the side chain, physiologically used as anchor in biomembranes, does not affect the antioxidant activity *in vitro*, which is in agreement with other studies [11, 16, 21, 49, 50]. Except ORAC assay, there was no significant difference in the results of T compared to the related T3. As yet, this relation was only observed for α-T and α-T3 in systems working with organic solvents [20, 24] and against lipid peroxidation in solution and liposomal membranes [14].

3.4 Single ET-based assays

The FRAP assay as one method with ET-based reaction used in our studies, determines the ability of antioxidants to reduce ferric ions in a di-TPTZ complex. To date, there was no FRAP system investigated to measure fat-soluble compounds [38]. Therefore, we developed a new FRAP version based on a two-phase system, similar to the antioxidant capacity measurement system reported by Miller *et al.* [36], to measure lipophilic antioxidants. Both, α-T and

Table 3. Precision and accuracy of quality control samples[a)] in the FRAP assay

	QC 1	QC 2	QC 3
Nominal α-T concn (μM)	10.00	25.00	45.00
Run 1			
Intra-mean (μM)	10.34	26.45	45.34
SD	0.43	0.36	1.40
% RSD	4.11	1.36	3.10
% REC	103.4	105.79	100.75
N	3	3	3
Run 2			
Intra-mean (μM)	10.19	27.64	44.39
SD	0.09	1.07	0.19
% RSD	0.88	3.88	0.44
% REC	101.9	110.5	98.5
N	3	3	3
Run 3			
Intra-mean (μM)	9.90	25.85	44.71
SD	0.87	1.74	0.33
% RSD	8.77	6.73	0.73
% REC	99.0	103.4	99.4
N	3	3	3
Pooled runs			
Intra-mean (μM)	10.14	26.65	44.81
SD	0.23	0.91	0.48
% RSD	2.23	3.41	1.07
% REC	101.4	106.6	99.6
N	12	12	12

a)% REC, relative recovery.

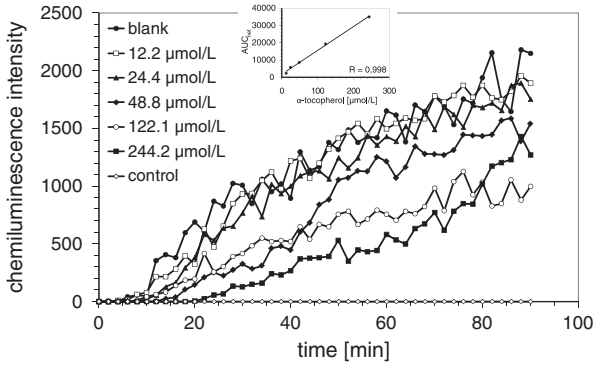


Figure 2. Effect of α-T concentration on CL progression curves induced by AAPH, and regression of the net AUC and α-T concentration in the CL assay.

α-T3 showed the highest reducing ability against the used redox couple Fe^{2+}/Fe^{3+} ($\Delta E^{o} = 0.77$ V) [34] (Fig. 3A), caused by the lowest redox potential of all used vitamers (ΔE^{o} (α-T) = 0.273 V) [18]. The activity of β-, γ-, and δ-T (~0.67) was about 20–30% lower compared to the calibration standard α-T (1.01). A slight, but not significant decrease was determined from β-T, γ-T, to δ-T (0.73, 0.67, and 0.62, respectively). This correlates with the increase of the redox potentials of these vitamers (0.343 V, 0.348 V and 0.405 V, respectively) [18], which can be explained by the special pattern of methyl groups in *ortho*- and *meta*-position at the hydroxy chroman structure. Accordingly, T3 acted alike, with a decrease of *ca.* 25% between α-T3 (1.07) and β-, γ-, and δ-T3 (0.76, 0.80 and 0.85, respectively) (Fig. 3A). Only by total methylation of the aromatic ring, the tocochromanols showed the maximal ferric reducing activity. This result was in contrast to the lipid peroxidation experiments, realized with Fe^{2+} and rat liver microsomal membrane [49].

The αTEAC assay used in this study, was described first as Trolox equivalent antioxidant capacity assay by Miller *et al.* [51]. This assay was originally used to detect the antioxidant activity of hydrophilic compounds compared to Trolox as calibration standard, based on ET-reaction, too. This early version used the radical formation from hydrogen peroxide by peroxidase active metmyoglobin to determine the ability of antioxidants to delay the formation of ABTS radicals as well as the capability to scavenge existing ABTS radicals. For carotenoids, dissolved in *n*-hexane or acetone, another version was performed based on the preformation of the ABTS radical by oxidation with manganese dioxide [36], slightly modified for the present studies according to Böhm *et al.* [37]. To avoid differences in the results caused by solvent differences between standard and samples, we substituted the water-soluble calibration standard Trolox to the fat-soluble compound α-T. In the present study, using the αTEAC assay, we detected no significant differences in the activity of all analyzed T and T3 against the redox couple $ABTS^{\bullet+}$/ABTS ($\Delta E^{o} = 0.68$ V [34]). Only a slight decrease from the α-tocochromanols (0.99, 0.97) to the β-, γ-, and δ-tocochromanols (mean = 0.89) was determined (Fig. 3B). Surprisingly, the ability to reduce the synthetic radical $ABTS^{\bullet+}$ was not influenced by degree of methylation and the related differences in the potentials. No influence of the character of the side chain was observed, too. As estimated, α-TA showed no detectable $ABTS^{\bullet+}$ reducing activity.

The scavenging ability against the stable $DPPH^{\bullet}$-free radical can be used to evaluate the antioxidant activities due to the ET reactivity [52]. In this study, we incubated a DPPH solution (0.3 mmol/L in ethanol) with different tocochromanol solutions (25 μmol/L in an ethanol/*n*-hexane mixture). As shown in Fig. 3C, after 15 min incubation in the dark at 25°C, all eight tocochromanols exhibited a scavenging effect on the $DPPH^{\bullet}$. α-T and α-T3 showed a significantly higher activity than the accordant β-, γ-, and δ-vitamers. The absence of a difference between α-T (1.01) and α-T3 (1.05) shows the independence on the constitution of the ''tail'' of the vitamer, as already observed [49]. The activity of the four di-methylated vitamers (β-T, β-T3, γ-T and γ-T3, respectively) was about 10% lower than that of the accordant α-homologues (0.91, 0.89, 0.91 and 0.90, respectively). Our studies show that the activity of the tocochromals against the redox couple $DPPH^{\bullet}$/DPPH-H, whose

Table 4. Summary of α-T calibration curve[a)] in the CL assay

Run	R^2	Intercept (*a*)	Slope (*b*)
1	0.9922	135.0	4895
2	0.9969	132.5	2085
3	0.9971	138.5	1428
4	0.9930	141.0	1867
5	0.9981	125.8	2117
6	0.9960	121.3	3285
7	0.9982	130.5	2867
8	0.9967	135.1	2456
Average	0.9960	132.5	2625
Accepted criteria	≥0.9900	n. a.	n. a.

a) [*Y* [μM] = *b*+*aX* (net AUC)]; n.a., not applicable.

Table 5. Precision and accuracy of quality control samples[a)] in the CL assay

	QC 1	QC 2	QC 3
Nominal α-T conc. (μM)	25.00	50.00	125.00
Run 1			
Intra-mean (μM)	24.57	50.45	126.84
SD	2.29	3.05	4.77
% RSD	9.33	6.06	3.76
% REC	98.3	100.9	101.5
N	3	3	3
Run 2			
Intra-mean (μM)	26.56	51.84	125.19
SD	3.05	2.35	7.89
% RSD	11.48	4.52	6.30
% REC	106.3	103.7	100.2
N	3	3	3
Run 3			
Intra-mean (μM)	25.54	47.94	123.78
SD	2.30	3.68	5.88
% RSD	9.01	7.67	4.75
% REC	102.1	95.9	99.0
N	3	3	3
Pooled runs			
Intra-mean (μM)	25.56	50.08	125.27
SD	1.00	1.98	1.53
% RSD	3.90	3.95	1.22
% REC	102.2	100.2	100.2
N	12	12	12

a) % REC, relative recovery.

formal potential is reported to be 0.5 V in methanol [53], depends on the degree of methylation in *ortho*-position of the chromanol ring, independent from the position of these methyl groups. A 20% lower antioxidant activity in this assay for the only *meta*-methylated δ-tocochromanols (δ-T: 0.74; δ-T3: 0.75) compared to the α-forms reflects this. In summary, the antioxidant activity against the $DPPH^{\bullet}$ increases with degree of methylation in the *ortho*-positions, and therefore correlates with the related decrease of the redox potential. As expected, the esterified tocochromanol (α-TA) did not show any significant scavenging activity effect on the $DPPH^{\bullet}$ under current experimental conditions, due to the esterified phenol group. The DPPH assay is also discussed on the basis of a HAT reaction [34]. Whether HAT reaction is involved or not, our results are due to relative H-atom-donating ability of the different tocols, which increases in efficiency with greater ring methylation [54].

In summary, the activity of the tocochromanols differs significantly between the three used assays, which are based on ET-reactions. The differences in the execution of the assays could have caused these effects. The FRAP assay determined the reducing activity against ferric ions, whereas αTEAC assay and DPPH assay measured the activity to reduce a stable-free radical $ABTS^{\bullet+}$, respectively $DPPH^{\bullet}$. FRAP and αTEAC were realized as a two-phase system, using buffers to dissolve the dyes ferric-di-TPTZ and $ABTS^{\bullet+}$, respectively, and *n*-hexane as upper layer to dissolve the vitamin E compounds. In contrast, the DPPH assay was completely performed in a solvent mixture of ethanol and *n*-hexane, and was the only assay, in which the reducing activity of the vitamers totally correlates with their redox potential.

3.5 Hydrogen atom transfer-based assays

The key of the radical chain-breaking activity of tocochromanols in the CL and ORAC assay is found in the free phenol group, too, as demonstrated by the absence of activity for α-TA (Figs. 3D–E). The antioxidant activity of T3 against peroxyl radicals in the CL assay was comparable to those of the appropriate T. Therefore, no dependence of the CL value from the character of the side chain was detected. α-T and its related T3 showed the lowest CL values (1.06 and 1.17, respectively). Significantly higher values were detected for the β- and δ-forms (β-T: 1.47, δ-T: 1.50, β-T3: 1.45 and δ-T3: 1.62, respectively) in comparison to the γ-forms (both 1.26), which resulted in only slightly higher values than the α-forms (Fig. 3D). The peroxyl radical-scavenging activity of the tocochromanols decreased with number and position of methyl groups at the chroman ring. Steric hindrance could explain this result. If the aromatic ring is totally substituted with methyl groups (α-T) the antioxidant activity is low. The elimination of the *ortho* methyl group in C-5 (γ-T) led to a slight increase of the antioxidant activity (~10%). Furthermore, with elimination of the *ortho* methyl group in C-7 (β-T, δ-T) the activity increased about 50%. The same rules could be postulated for the T3 homologues. The CL values of the tocopherols β-T = δ-T ≥ γ-T ≥ α-T determined in our peroxyl radical generating system are not in agreement with the relative reactivity *in vitro* against peroxyl radicals reported by Burton and Ingold [11] α-T>β-T = γ-T>δ-T, which correlated with the number of methyl groups at the chromanol ring. The antioxidant activity of γ-T3 and δ-T3 was determined previously in a peroxyl generating CL system

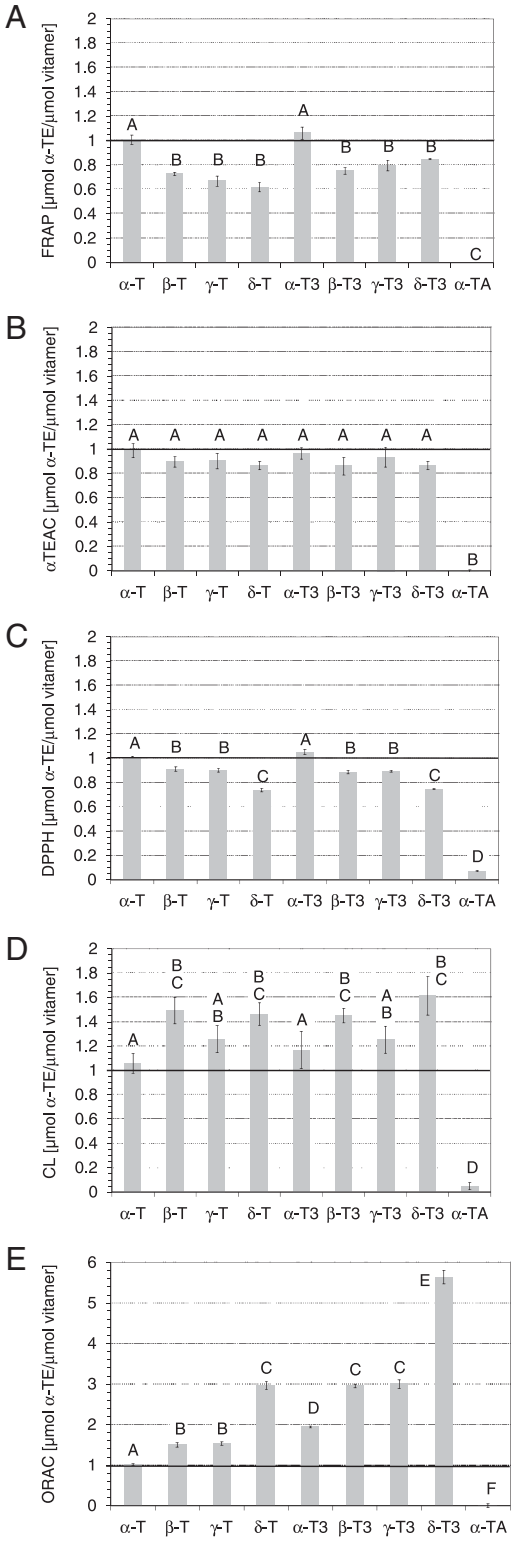


Figure 3. Antioxidant activity of tocopherols, tocotrienols and, α-T acetate measured by FRAP assay (A), αTEAC assay (B), DPPH assay (C), CL assay (D), and ORAC assay (E). Bars with different superscript letters within one assay differ significantly ($p<0.05$).

[55] to be fourfold higher compared to α-T. We found only an approximately 50% higher activity of these T3 (Fig. 3D)

The ORAC assay was often used in the last decade to determine the scavenging ability of single compounds, biological samples, and food samples. The ORAC assay was executed in accordance to the method described by Huang *et al.* [40], based on the fluorescein degradation by peroxyl radicals generated by AAPH at a physiological pH. To adapt the hydrophilic ORAC assay to the lipophilic nature of the vitamin E isomers RMCD was used as solubility enhancer. As already shown for all other assays described before, α-TA did not show any antioxidant activity. Kamal-Eldin *et al.* reported that the increase of electron-releasing substituents (like methyl groups) in *ortho*- and/or *para*-position to the hydroxyl function led to an increase of the stability of the phenoxy radical and therefore to improvement of the reactivity with peroxyl radicals [18]. In contrast, our ORAC results showed that the tri-methylated α-forms possessed the lowest antioxidant activity (α-T: 1.02, α-T3: 1.95) of all tocochromals, whereas for the δ-forms a threefold higher value (δ-T: 2.98, δ-T3: 5.65) was detected, as shown in Fig. 3E. These results are comparable to the results of the previous study on peroxyl radical-scavenging activity by T using the same reaction system [40], but in contrast to the lipophilic ORAC system using the lipophilic radical generator AMVN and pyrogallolsulfonphtalein as fluorescent dye [14]. The peroxyl radical-scavenging activity detected in our assay depends on the one hand on the degree of methylation and furthermore on the character of the side chain. As observed for α-T and α-T3 by incubation with synthetic peroxyl radical generators (AAPH, AMVN, SIN-1) by Raneva *et al.* [22] the unsaturated vitamin E forms showed a higher activity in our studies, too. The unsaturated ''tail'' of the T3 caused a doubling of the antioxidant activity. The differences in the ORAC values of the different tocopherols, respectively tocotrienols, can be attributed to the steric effect of the methyl groups in *ortho*-position to the phenolic ring. The number of methyl groups *ortho* to the hydroxyl group of the T decreases from two (α-T), one (β-T), and zero (γ-T), whereas in contrast the ORAC value increases from 1.51 (β-T) to 1.54 (γ-T), and 2.98 (δ-T). Apparently, the steric factor had a significant influence on the antioxidant activity in this assay. A less steric hindrance resulted in higher ORAC values [40]. The same relation was determined for T3. However, the effect was more pronounced.

Both, ORAC and CL assay measured the activity of tocochromanols to scavenge peroxyl radicals, generated by thermal degradation of AAPH. However, in the ORAC assay we observed a reversed correlation between the pattern of methylation and the chain-breaking activity, whereas in the CL assay the methyl substitution at the chromanol ring had only slight effects. The differences in the executions of the methods can be the reason for the differences between CL and ORAC values. The CL assay was carried out in a heterogeneous lipophilic mixture of mainly DMSO, and low

ratios of phosphate and borax buffer. In the ORAC assay we used mainly phosphate buffer and a low ratio of acetone to dissolve RMCD to enhance the solubility of tocochromanols. The influence of the ''chemical nature'' of the assay on the antioxidant activity of the analyzed compounds was observed. The antioxidant activity is influenced by the kind of solvent [24, 56], chemical compounds in the vicinity to the tocochromanols, and by the oxidation conditions in general [18].

3.6 Antioxidant activity in human plasma samples

As already reported [7], α-T was the main vitamin E compound detected in the plasma samples with a relative content of approximately 95% of the total vitamin E content. α-T is known as the exclusive lipophilic antioxidant [5]. Our studies suggest a significant correlation between vitamin E content of plasma and the lipophilic antioxidant capacity measured by DPPH ($r = 0.48$, $p < 0.001$) and αTEAC ($r = 0.73$, $p < 0.001$) (Figs. 4A and B), as well as a good correlation ($r = 0.65$, $p < 0.01$) between the results of both assays (Fig. 4C).

4 Concluding remarks

Four tocopherols and four tocotrienols were tested for their antioxidant activity using FRAP assay, αTEAC assay, DPPH radical-scavenging assay, ORAC assay and CL assay. α-T is considered to have the highest biological activity and therefore it was used as standard for all comparisons. In all performed assays, we demonstrated that the phenol group is an important key for the antioxidant activity of tocochromanols. α-TA, a popular ingredient in vitamin E supplements, did not show any significant antioxidant activity *in vitro*. Consequently, tocopheryl esters are not useful as antioxidants to protect food and cosmetics against lipid peroxidation. Similar antioxidant activity trends between T and the related T3 were observed in all assays used in this study. An influence of the character of the side chain was only found in the ORAC assay. However, the antioxidant activity of tocochromanols depends on the conditions used for detection. Even, the results determined by the three ET-based assays were not totally comparable to each other (Fig. 3). In the FRAP assay and especially in the DPPH assay we observed a correlation between antioxidant activity and redox potential related to the structural differences of the several vitamin E forms. Though, in αTEAC assay there was no difference between any of the tocochromanols. In the two HAT-based assays (ORAC, CL) the α-forms showed the lowest peroxyl radical-scavenging activity. However, a significant correlation between the degree of methylation and the radical chain-breaking activity was only found for the ORAC assay, and in a reversed order. The reported observation, that the peroxyl radical-scavenging activity of α-T3 was higher than that of α-T [57], could only be shown in the ORAC assay, but not in the CL system. In both assays, the activity of the analyzed compounds to scavenge peroxyl radicals was measured. But the differences in the used solvents and dyes could have caused these differences in the total activity of the respective tocochromanol and in the order of activity between all eight vitamers.

Our studies support the assumption that the antioxidant activity of tocochromanols depends on the circumstances under which the assay is accomplished. The results of our study show that *e.g.* the extent of differences between the tocochromanols depends on the used solvents. In summary, the results determined in *in vitro* assays are mostly not comparable to those in *in vivo* experiments, published in literature. Additionally, our studies show that the lipophilic antioxidant capacity of plasma correlates well with the vitamin E content.

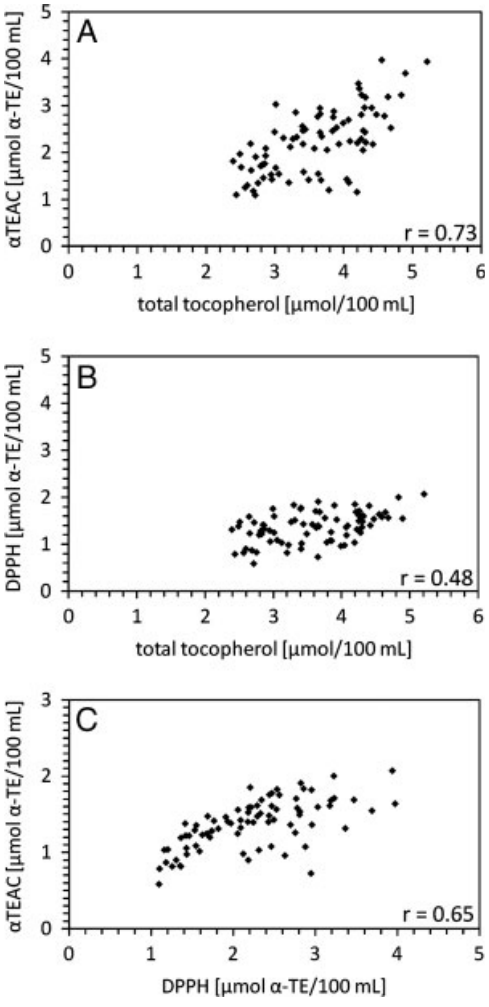


Figure 4. Pearson correlations ($p < 0.001$) of vitamin E content and lipophilic antioxidant capacity measured by αTEAC assay (A) and DPPH assay, respectively (B) of analyzed plasma samples, as well as correlation between both assays (C).

A variety of assays to detect the antioxidant activity of lipophilic compounds were used in our investigations to quantify the radical-scavenging ability of tocopherols and tocotrienols. In our opinion, these methods can be useful to determine the antioxidant activity of strong non-polar antioxidants, *e.g.* carotenoids, too.

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The authors have declared no conflict of interest.

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