**The establishment of tocopherol reference intervals for Hungarian adult population using a validated HPLC method**

Gábor Veres1,2 | László Szpisjak1 | Attila Bajtai1 | Andrea Siska3 | Péter Klivényi1 | István Ilisz4 | Imre Földesi3 | László Vécsei1,2 | Dénes Zádori1

1 Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Szeged, Hungary
2 MTA-SZTE Neuroscience Research Group, Szeged, Hungary
3 Department of Laboratory Medicine, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Szeged, Hungary
4 Department of Inorganic and Analytical Chemistry, Faculty of Science and Informatics, University of Szeged, Szeged, Hungary

**Correspondence**
Dénes Zádori, Department of Neurology, Faculty of Medicine, Albert Szent-Györgyi Clinical Center, University of Szeged, Semmelweis u. 6, H-6725 Szeged, Hungary. Email: zadori.denes@med.u-szeged.hu

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**Abstract**
Evidence suggests that decreased α-tocopherol (the most biologically active substance in the vitamin E group) level can cause neurological symptoms, most likely ataxia. The aim of the current study was to first provide reference intervals for serum tocopherols in the adult Hungarian population with appropriate sample size, recruiting healthy control subjects and neurological patients suffering from conditions without symptoms of ataxia, myopathy or cognitive deficiency. A validated HPLC method applying a diode array detector and rac-tocool as internal standard was utilized for that purpose. Furthermore, serum cholesterol levels were determined as well for data normalization. The calculated 2.5–97.5% reference intervals for α-, β-/γ- and δ-tocopherols were 24.62–54.67, 0.81–3.69 and 0.29–1.07 µmol/L, respectively, whereas the tocopherol/cholesterol ratios were 5.11–11.27, 0.14–0.72 and 0.06–0.22 mmol/mmol, respectively. The establishment of these reference intervals may improve the diagnostic accuracy of tocopherol measurements in certain neurological conditions with decreased tocopherol levels. Moreover, the current study draws special attention to the possible pitfalls in the complex process of the determination of reference intervals as well, including the selection of study population, the application of internal standard and method validation and the calculation of tocopherol/cholesterol ratios.

**KEYWORDS**
cholesterol, HPLC, human samples, reference interval, tocopherol

1 | INTRODUCTION

Most of the deleterious effects of pathological processes in the human organ are mediated by the formation of reactive species (RS; Szalárdy, Zádori, Klivényi, Toldi, & Vécsei, 2015). The synthesis and toxic effects of RS are ameliorated by a complex system of antioxidative machinery, including enzymatic (e.g. superoxide dismutase, catalase and glutathione peroxidase; Sies, 1997) and nonenzymatic F1 mechanisms (Figure 1).

The latter group consists of numerous chemical compounds, such as vitamin E, vitamin C, coenzyme Q10, β-carotene, glutathione and flavonoids with proved antioxidant properties (Sies, 1993). In addition to the capability of these agents to react with RS, their sufficient tissue concentrations and their suitability for regeneration are desirable properties as well for efficient antioxidant protection (Rose & Bode, 1993).

**Abbreviations used:** α-TTP, α-tocopherol transport protein; ACN, acetonitrile; BHT, butylated hydroxy toluene; OND, other neurological disease; RI, reference interval; RS, reactive species; THF, tetrahydrofuran

The vitamin E group includes four tocotrienols and four tocopherols as lipid soluble antioxidants. Their molecular structure comprises a chromanol ring with an aliphatic side chain, unsaturated for tocotrienols and saturated for tocopherols. Depending on the number and position of methyl groups on the chromanol ring, α-, β-, γ- and δ-tocotrienols and tocopherols can be distinguished (Hacquebard & Carpentier, 2005). The bioavailability of tocotrienols is inefficient and physiological serum concentrations are low (O’Byrne et al., 2000), suggesting that under normal circumstances their role as antioxidants is negligible. However, increasing α-tocotrienol concentration by its external supplementation can ameliorate the symptoms caused by α-tocopherol deficiency (Sen, Khanna, & Roy, 2007). α-Tocopherol has the highest concentration and biological activity from the vitamin E group, while the other tocopherols have a less expressed role in antioxidant protection (Hacquebard & Carpentier, 2005). Tocopherols are taken up by the enterocytes from the intestines and they are incorporated into the secreted chylomicrons and transported to the liver (Cooper, 1997). In the liver, the cytosolic α-tocopherol transport protein (α-TTP)
recognizes and binds \( \alpha \)-tocopherol with good selectivity, and the majority of the other forms of tocophers are rapidly excreted in the bile. \( \alpha \)-TTP is responsible for the secretion of \( \alpha \)-tocopherol into plasma and very-low-density lipoprotein particles (Arita, Nomura, Arai, & Inoue, 1997). Very-low-density lipoprotein is catabolized on the periphery by lipoprotein lipase, localized at the endothelium, and the formed high-density lipoprotein and low-density lipoprotein particles become responsible for the distribution of \( \alpha \)-tocopherol to extracellular tissues (Mardones & Rigotti, 2004). With regard to the other forms of vitamin E, there is no discrimination between them and \( \alpha \)-tocopherol during absorption from the intestines; however, in the liver there is a preferential secretion of \( \alpha \)-tocopherol into the blood, as well as a preferential metabolism of the other forms (Traber, 2013). Accordingly, cholesterol levels and metabolism may considerably influence the bioavailability of tocophers (Schmölz, Birringer, Lorkowski, & Wallert, 2016).

The major causes of severe tocopherol deficiency are malabsorption disorders with decreased intestinal lipid uptake (e.g., cholestatic, cystic fibrosis, short bowel syndrome), abetalipoproteinemia and ataxia with vitamin E deficiency where the underlying pathological alteration is the decreased activity of \( \alpha \)-TTP caused by mutations in the TTPA gene (Morley, et al., 2004).

Decreased vitamin-E levels are associated with several neurological symptoms, such as cerebellar ataxia, peripheral neuropathy and myopathy (Muller, 2010; Ueda et al., 2009), as the nervous system is particularly sensitive to oxidative damage resulting from reduced antioxidant capacity (Zádori, Klivényi, Plangár, Toldi, & Vécsei, 2011; Zádori et al., 2012). Accordingly, in addition to the above-mentioned disorders with their characteristic symptoms, some other neuropsychiatric conditions, such as Alzheimer’s disease (Lopes da Silva et al., 2014) and the exacerbation of multiple sclerosis (Karg et al., 1999) can also be accompanied by significantly reduced serum or plasma tocopherol levels. Therefore, the measurement of serum tocopherol levels is advised to be part of the differential diagnostic process in certain neuropsychiatric conditions. The establishment of unbiased reference values for the targeted population is essential for that purpose.

The aim of the current study was to develop an unbiased method for tocopherol (\( \alpha \), \( \beta \), \( \gamma \) and \( \delta \)-tocopherol) measurements in human serum samples and to hereby establish reference intervals (RI) via the participation of 120 individuals for the adult Hungarian population based on the pooling of the values of control subjects without any chronic disease and neurological patients suffering from conditions without symptoms of ataxia, myopathy or cognitive deficiency (the so-called control group of other neurological diseases: OND).

2 | MATERIALS AND METHODS

2.1 | Materials

The investigated reference compounds \( [\alpha , \gamma , \delta \text{-tocopherol; (2R)}-2,5,7,8\text{-tetramethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol, (2R)-2,7,8\text{-trimethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol, (2R)-2,8\text{-dimethyl-2-[(4R,8R)-(4,8,12-trimethyltridecyl)]-6-chromanol, respectively, ammonium acetate, ascorbic acid ([5R]-[1S]-1,2\text{-dihydroxyethyl}-3,4-dihydroxyuran-2(SH)-one], butylated hydroxytoluene (BHT), dioxane and methanol were purchased from Sigma-Aldrich (Saint Louis, MO, USA), rac-toc-tol [2-methyl-2-(4,8,12-trimethyltridecyl)]-6-chromanol, as internal standard] was purchased from Mateyra LLC. (Pleasant Gap, PA, USA), and acetonitrile (ACN), absolute ethanol, n-hexane and tetrahydrofuran (THF) were purchased from VWR International (Radnar, PA, USA).

2.2 | Enrollment criteria and sample preparation

The study sample population comprised 30 male (age range 21–71 years, mean age 49.50 year) and 30 female volunteer individuals (age range 25–76 years, mean age 50.03 year) without any major chronic illness and 30 male (age range 18–73 years, mean age 49.43 year) and 30 female (age range 24–78 years, mean age 49.60 year) patients with OND (main diagnoses for males were the following: ischemic stroke, 8; Parkinson’s disease, 5; epilepsy, 5; lumbar disk disorder, 3; and other, 9; main diagnoses for females were the following: multiple sclerosis in remission, 8; ischemic stroke, 6; epilepsy, 5; and other, 11) where the presence of ataxia, myopathy or cognitive dysfunction were excluding criteria. The distribution of the age of the subjects was Gaussian in all groups (\( p > 0.05 \), Anderson–Darling test) and the variances were equal (\( p = 0.98 \), Levene test). There was no significant difference between the groups (F = 0.01, p = 0.99, one-way ANOVA). The recent regular intake of antihyperlipidemic agents or any kind of drugs or food supplements containing antioxidants were exclusion criteria as well in all groups. All participating individuals were of Hungarian origin and were enrolled in the Department of Neurology at the University of Szeged. The study was approved by the Ethics Committee of the Faculty of Medicine, University of Szeged (19/ 2007). All study participants gave their written informed consent, in accordance with the Declaration of Helsinki.

Blood was collected by venipuncture into gold-top vacutainers following fasting for 12 h. The blood was immediately centrifuged at 3500 rpm for 10 min. A 200 \( \mu \)L aliquot of the supernatant serum was shot into a solution containing 200 \( \mu \)L ascorbic acid (0.085 M) and

\[ \text{ascorbic acid} \]

\[ \text{reduced glutathione} \]

\[ \text{2 reduced glutathione} \]

\[ \text{NADPH} \]

\[ \text{NADP}^+ \]

\[ \text{Glutathione reductase} \]

FIGURE 1 The schematic depiction of nonenzymatic mechanisms in antioxidant protection. NADPH, Reduced nicotinamide adenine dinucleotide phosphate; RS, reactive species.
400 μL BHT (1.14 mM) and the resulting solution was stored at −80°C until measurement, while the remaining serum was aliquoted and stored at −80°C as well.

Before high-performance liquid chromatography (HPLC) measurement, 600 μL n-hexane containing 1.14 mM BHT and rac-toc as internal standard was added to the freshly thawed serum samples treated with antioxidants (800 μL). This mixture was mixed for 1 min, then centrifuged at 3500 rpm for 5 min at 4°C. The hexane layer was transferred to a test vial and evaporated under nitrogen flow. The residue was reconstituted with 75 μL ACN and 50 μL EtOH–dioxane (1:1). The resulting solution was transferred into a 200 μL glass insert placed into an amber-colored vial for measurement.

2.3 Serum cholesterol and triglyceride measurement

Total cholesterol and triglyceride levels were determined by commercially available kits from Diasys (Diagnostics Systems GmbH, Holzheim, Germany) on Roche Modular P800 analyzer (Roche, Rotkreuz, Switzerland).

2.4 Chromatographic conditions

The concentrations of α-tocopherol, β-tocopherol, δ-tocotrienol, and γ-tocotrienol were determined with a modified Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with an UV/Vis diode array detector applying the modified method of Hess, Keller, Oberlin, Bonfanti, and Schuep (1991). Chromatographic separations were performed on an Alltech Prevail C18 column, 150 x 4.6 mm i.d., 5 μm particle size (Alltech Associates Inc., Deerfield, IL, USA) after passage through a SecurityGuard pre-column, 4 x 3.0 mm i.d., 5 μm particle size (Phenomenex Inc., Torrance, CA, USA) with a mobile phase composition of ACN–THF–MeOH–1% w/v ammonium acetate–distilled water (684:220:68:28:28) applying isocratic elution. The flow rate and the injection volume were 2.1 mL/min and 50 μL, respectively. The detector was set at 292 nm (α-tocopherol) and 297 nm (β- and δ-tocopherol, rac-tocotrienol).

Separating β- and γ-tocopherol is challenging because they only differ in the position of a methyl group. With the use of a C18 column these two compounds have almost the same retention times (Saha, Walia, Kundu, & Pathak, 2013). The β- and γ-tocopherols can only be separated with the application of special columns and methods (Gornas et al., 2014; Grebenstein & Frank, 2012), the application of which may be challenging for routine clinical practice. Accordingly, only γ-tocopherol was applied as a standard compound for the establishment of the calibration curve in this study, and the concentration at the corresponding retention time includes both substances and is reported as β-γ-tocopherol.

2.5 HPLC method validation for serum samples

2.5.1 Calibration curve and linearity

Calibrants were prepared in six different concentration levels and spiked serum samples were used with concentration ranges of 0–40, 0–6, 0–6 and 0–24 μM for α-, β-, Δ-tocopheryl and rac-toc, respectively. The peak area responses were plotted against the corresponding concentration, and linear regression computations were carried out by the least square method with the freely available R software (R Development Core Team, 2002). Very good linearity (R² ≥ 0.99) was observed throughout the concentration ranges for α-, β-, γ- and δ-tocopherol and rac-toc.

2.5.2 Selectivity

The selectivity of the method was checked by comparing the chromatograms of α-β-γ-, δ-tocopherol and rac-toc for a blank serum sample and those for a spiked sample. All compounds could be detected in their own selected chromatograms without any significant interference, as can be seen in Figure 2.

2.5.3 Precision

For the determination of between-run precision five samples for four concentration levels were applied (i.e., 20 replicates altogether). This study was repeated two more times with at least one week intervals to obtain between-run precision. With regard to within-run precision the coefficients of variation of the measured concentrations were 4.53, 3.72 and 5.11% for α-, β-γ- and δ-tocopherols, respectively, whereas in case of between-run precision, they were 3.59, 5.93 and 4.76% for α-, β-γ- and δ-tocopherol, respectively.

2.5.4 Recovery

The relative recoveries were estimated by measuring spiked samples of α-, β-γ- and δ-tocopherol at two different concentrations with three replicates of each. No significant differences were observed for the lower and higher concentrations. The recoveries for the serum samples ranged from 86 to 105%, from 95 to 108% and from 116 to 124% for α-tocopherol, β-γ-tocopherol and δ-tocopherol, respectively.

![FIGURE 2](image.png)
2.6 | Statistics

All statistical calculations were performed with the use of the freely available R software (R Development Core Team, 2002) according to the International Federation of Clinical Chemistry and Laboratory Medicine and Clinical and Laboratory Standards Institute guidelines (Horowitz, 2016). Based on these guidelines, the minimum required number of individuals for the determination of RI with the bootstrap method is at least 100. First we checked the distribution of our data with the Anderson–Darling test and we also performed the Levene test for analysis of the homogeneity of variances. If the distribution proved to be Gaussian and the variances were equal, one-way ANOVA was applied to compare the groups, otherwise the Kruskal–Wallis test was utilized. To obtain the necessary quantiles and their confidence intervals for the determination of the reference intervals, the bootstrap method (1000 iterations) was applied. The correlation between the concentration of the measured compounds and the age of individuals in the sample population was examined with the nonparametric Spearman’s method (1000 iterations) was applied. The correlation between the concentration of the measured compounds and the age of individuals in the sample population was examined with the nonparametric Spearman’s test. We rejected the null hypothesis when the corrected p-values were ≤0.05, and in such cases the differences were considered significant. Data with Gaussian or non-Gaussian distributions were plotted as means (±SD) or medians (and interquartile range), respectively.

3 | RESULTS

The group-wise comparisons failed to detect any significant difference between groups regarding the concentrations of α-tocopherol (p = 0.48, χ² = 2.46; Kruskal–Wallis test), β/γ-tocopherol (p = 0.47, χ² = 2.53; Kruskal–Wallis test) or δ-tocopherol (p = 0.82, χ² = 0.94; Kruskal–Wallis test; Table 1).

Accordingly, in order to establish RI with appropriate subject numbers, the values for each measured compounds were pooled and the minimum required sample size (n = 120) was achieved. For the determination of lower (2.5%) and upper (97.5%) RI with the corresponding confidence intervals and standard errors, the bootstrap method was applied and the results are demonstrated in Table 2.

To obtain cholesterol-corrected tocopherol values as well, serum cholesterol concentrations were determined for each subject [median and interquartile range 4.99 mm (4.31–5.54)] and the tocopherol/cholesterol ratios were calculated. The bootstrap method was applied again for the lipid corrected values (Table 3). To assess the incidental effect of age on measured serum lipid levels, the Spearman test was performed. The cholesterol levels positively correlated with the age of subjects (p < 0.001, Spearman’s ρ = 0.34; Figure 3).

In the case of uncorrected α- and β/γ-tocopherol concentrations, this correlation with age is present as well (α-tocopherol: p = 0.002, Spearman’s ρ = 0.28, Figure 4A; β/γ-tocopherol: p = 0.001, Spearman’s ρ = 0.29, Figure 4C) whereas δ-tocopherol levels did not correlate with age (p = 0.98, Spearman’s ρ = 0.003, Figure 4E). When tocopherol levels were normalized to cholesterol levels, all the correlations with age were eliminated (α-tocopherol: p = 0.99, Spearman’s ρ = −0.0007, Figure 4B; β/γ-tocopherol: p = 0.14, Spearman’s ρ = 0.14, Figure 4D; δ-tocopherol: p = 0.051, Spearman’s ρ = −0.18, Figure 4F).

### Table 1: Serum tocopherol concentrations of subjects belonging to the control and OND groups

<table>
<thead>
<tr>
<th></th>
<th>Controls (women)</th>
<th>Controls (men)</th>
<th>OND patients (women)</th>
<th>OND patients (men)</th>
<th>Group comparisons (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol (μM)</td>
<td>38.08 (33.70–44.10)</td>
<td>35.38 (31.35–45.83)</td>
<td>34.26 (29.40–41.60)</td>
<td>33.93 (30.54–41.08)</td>
<td>0.48 (χ² = 2.46)</td>
</tr>
<tr>
<td>β/γ-Tocopherol (μM)</td>
<td>1.83 (1.32–2.23)</td>
<td>1.68 (1.39–2.52)</td>
<td>1.57 (1.33–1.82)</td>
<td>1.75 (1.52–2.21)</td>
<td>0.47 (χ² = 2.53)</td>
</tr>
<tr>
<td>δ-Tocopherol (μM)</td>
<td>0.63 (0.52–0.86)</td>
<td>0.62 (0.53–0.78)</td>
<td>0.62 (0.55–0.82)</td>
<td>0.65 (0.57–0.75)</td>
<td>0.82 (χ² = 0.94)</td>
</tr>
</tbody>
</table>

Group-wise comparisons (Kruskal–Wallis test) of the four groups failed to detect any significant difference between serum tocopherol levels. Data are presented as median and interquartile range. OND, Other neurological disease.

### Table 2: The calculated uncorrected lower (2.5%) and upper (97.5%) reference intervals for tocopherols for the assessed Hungarian population (n = 120)

<table>
<thead>
<tr>
<th></th>
<th>2.5%</th>
<th>SE</th>
<th>CI (95%)</th>
<th>97.5%</th>
<th>SE</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol (μM)</td>
<td>24.62</td>
<td>0.76</td>
<td>23.24–26.26</td>
<td>54.67</td>
<td>4.09</td>
<td>46.88–61.84</td>
</tr>
<tr>
<td>β/γ-Tocopherol (μM)</td>
<td>0.81</td>
<td>0.13</td>
<td>0.60–1.11</td>
<td>3.69</td>
<td>0.45</td>
<td>2.71–4.55</td>
</tr>
<tr>
<td>δ-Tocopherol (μM)</td>
<td>0.29</td>
<td>0.03</td>
<td>0.22–0.32</td>
<td>1.07</td>
<td>0.13</td>
<td>0.80–1.29</td>
</tr>
</tbody>
</table>

SE, Standard error; CI, confidence interval.

### Table 3: The calculated cholesterol corrected lower (2.5%) and upper (97.5%) reference intervals for tocopherols for the assessed Hungarian population (n = 120)

<table>
<thead>
<tr>
<th></th>
<th>2.5%</th>
<th>SE</th>
<th>CI (95%)</th>
<th>97.5%</th>
<th>SE</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol (μmol/mmol)</td>
<td>5.11</td>
<td>0.14</td>
<td>4.79–5.36</td>
<td>11.27</td>
<td>0.69</td>
<td>9.91–12.82</td>
</tr>
<tr>
<td>β/γ-Tocopherol (μmol/mmol)</td>
<td>0.14</td>
<td>0.02</td>
<td>0.10–0.19</td>
<td>0.72</td>
<td>0.07</td>
<td>0.60–0.88</td>
</tr>
<tr>
<td>δ-Tocopherol (μmol/mmol)</td>
<td>0.06</td>
<td>0.01</td>
<td>0.05–0.07</td>
<td>0.22</td>
<td>0.03</td>
<td>0.16–0.27</td>
</tr>
</tbody>
</table>
The determination of exact serum tocopherol concentrations may be substantial for the diagnosis and therapeutic monitoring of certain conditions usually accompanied by neurological symptoms, such as ataxia, myopathy or cognitive deficiency (La Fata, Weber, & Mohajeri, 2014; Muller, 2010). Although the symptoms of genetically determined disorders with tocopherol deficiency usually manifest during childhood (Raizman et al., 2014), malabsorption disorders and late-onset genetically determined metabolic conditions preferentially appear in adulthood, indicating the need for tocopherol measurement in adult population as well (Ueda et al., 2009). However, these concentrations alone hold little diagnostic value, for proper evaluation physicians need a well-established RI, which can considerably vary between populations (Table S1 in the Supporting Information). The underlying cause of this variation may be multifactorial, mainly including nonstandardized patient selection criteria and some methodological issues.

**FIGURE 3** Serum cholesterol concentrations in function of age. There is a positive correlation between cholesterol levels and age ($p < 0.001$, Spearman's $\rho = 0.34$)

**FIGURE 4** Serum tocopherol concentrations and tocopherol/cholesterol ratios plotted against the age of participants. The level of $\alpha$-tocopherol positively correlates with age ($p = 0.002$, Spearman's $\rho = 0.28$; A), similarly to $\beta/\gamma$-tocopherol ($p = 0.001$, Spearman's $\rho = 0.29$; C), whereas $\delta$-tocopherol levels do not correlate with age ($p = 0.98$, Spearman's $\rho = 0.003$; E). The cholesterol corrected values of $\alpha$-tocopherol ($p = 0.99$, Spearman's $\rho = -0.0007$; B) and $\beta/\gamma$-tocopherol ($p = 0.14$, Spearman's $\rho = 0.14$; D) do not further significantly correlate with age, and the correlation of $\delta$-tocopherol levels with age also remained nonsignificant ($p = 0.051$, Spearman's $\rho = -0.18$; F)
aim of the current study was to establish RIs for the Hungarian population and to compare the method of patient selection and the analytical procedure with those of previously published studies.

The investigated population in this study is homogeneously distributed with regard to age and covers a considerably wide age range for the adult population. The selection of a homogeneous study population may have a special importance, because age distribution can considerably influence reference values in light of the fact that the levels of certain tocopherols significantly increase with age (Figure 4; Rifkind & Segal, 1983). Accordingly when the investigated reference population includes young individuals, the results may be skewed to lower levels (Ford, Schleicher, Mokdad, Ajani, & Liu, 2006; Paliakov et al., 2009; Quesada, Mata-Granados, & Luque de Castro, 2004; Zhao, Monahan, McNulty, Gibney, & Gibney, 2014). Moving on to another qualitative aspect of the composition of the study population, in addition to the involvement of subjects without any chronic illness, the group of assessed individuals also comprised patients with different neurological disorders where tocopherol levels were not previously reported to be abnormal (the establishment of the so-called control group of OND). This study setup may ensure the absence of significant alterations of tocopherol levels in neurological cases lacking the symptoms of ataxia, myopathy and cognitive deficiency, which may be important for future screening studies. Following thorough statistical assessment resulting in the lack of significant differences, these subgroups become suitable for pooling, i.e. the number of individuals in the reference population can be increased easily to the desired level.

Several previous studies lack this study setup including well-detailed description of the health condition of reference individuals, possibly introducing a bias into the reference values (Table S1; Paliakov et al., 2009; Quesada et al., 2004). In the current study design, special attention was paid to dietary factors and to the intake of special medicines (e.g. statins) and food supplements as well, because these may considerably alter the levels of the assessed compounds (Colquhoun et al., 2005). The lack of this kind of standardization introduces another bias into the establishment of RIs (Table S1; Winbauer, Pingree, & Nuttall, 1999; Yuan et al., 2014).

With regard to the analytical procedure of the determination of tocopherol concentrations from biological matrices, several difficulties can emerge as well. While the use of serum or heparinized plasma for measurement does not affect α-tocopherol level (Table S1), the application of oxalate, citrate or ethylenediaminetetraacetic acid significantly reduces its concentration (Nierenberg & Lester, 1985). The most problematic step in the measurement process may be the sample preparation, which includes liquid-liquid extraction into n-hexane, evaporation under nitrogen flow and reconstitution in organic solvents. The emerging problems during these steps and during sample injection can considerably contribute to the overall error of the measurement. Internal standards in known quantity can be utilized to compensate for the bias between the measured and true concentrations. In the current study, rac-tocol was applied as internal standard, but several other compounds can be utilized (Table S1).

Another important matter may be the validation of the applied analytical procedure, which can provide valuable information about the robustness of the measurement and the validity of the reported values. For scientific publications at least a partial method validation is required (ICH, 1995). Without method validation the reliability of the presented data is questionable. Approximately 80% of the previous studies presenting human tocopherol concentrations applied a validation procedure (Table S1).

Tocopherol levels are often reported as tocopherol–cholesterol ratios based on the fact that there is a close relationship between the concentrations of tocopherols and lipids in the blood (Thurnham, Davies, Crump, Situnayake, & Davis, 1986). However, under special circumstances lipid-corrected tocopherol levels can be misleading, because it was reported that malnutrition and infectious diseases in children can lower the levels of circulating cholesterol and its lipoprotein carriers, which alteration can mask decreased tocopherol levels if only corrected values are reported (Das, Thurnham, & Das, 1996; Sauerwein et al., 1997; Squai Houssaini et al., 2001). Contrarily, when obese children were investigated, their α-tocopherol levels were normal while their tocopherol–cholesterol ratios were significantly lower compared with the control group (Strauss, 1999). With regard to adults, in light of the fact that lipid status can vary with aging (Figure 3; Rifkind & Segal, 1983), the application of lipid-corrected values may be necessary for the characterization of vitamin E status (Horwitt, Harvey, Dahm, & Searcy, 1972; Thurnham et al., 1986).

Nevertheless, the report of serum tocopherol concentrations with lipid ratios may be practical for the proper evaluation of tocopherol status. However, only one-third of the papers reported both of them (Table S1 in the Supporting Information).

In conclusion, the current study presents RIs for the first time for serum tocopherol concentrations and their corresponding cholesterol corrected values with regard to the adult Hungarian population. These results can facilitate the diagnostic process for certain neurological conditions, such as ataxia with vitamin E deficiency. Moreover, this paper draws attention to the importance of thorough design associated with the establishment of these RIs and the possible pitfalls in tocopherol measurements.

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DISCLOSURE OF INTEREST

The authors report no conflict of interest.
REFERENCES


**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

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- If you intend to annotate your proof electronically, please refer to the E-annotation guidelines.
- If you intend to annotate your proof by means of hard-copy mark-up, please use the standard proofing marks. If manually writing corrections on your proof and returning it by fax, do not write too close to the edge of the paper. Please remember that illegible mark-ups may delay publication.

Whether you opt for hard-copy or electronic annotation of your proofs, we recommend that you provide additional clarification of answers to queries by entering your answers on the query sheet, in addition to the text mark-up.

<table>
<thead>
<tr>
<th>Query No.</th>
<th>Query</th>
<th>Remark</th>
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<tbody>
<tr>
<td>Q1</td>
<td>AUTHOR: Please check the captured given names and family names are</td>
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<tr>
<td>Q2</td>
<td>AUTHOR: Please verify that the linked ORCID identifiers are correct for each author.</td>
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