

Synergism between DL- α -tocopherol and ascorbyl palmitate at various ratios in binary antioxidant compositions

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The effect of various concentrations (0.1 – 10.0 mM) of DL- α -tocopherol (TOH) and L(+)-ascorbic acid (AscAH)/ascorbyl-palmitate (AscPH) on their antioxidant activity as individual components during bulk lipid autoxidation is presented in this study. AscAH and AscPH didn't manifest any inhibitory effect in all the studied concentrations, i.e. they are not able to ensure oxidation stability of lipid substrate. An increase of the inhibitory effect of TOH was obtained by increasing its concentration from 0.1 up to 1.0 mM.

The strongest antioxidant activity was observed for TOH in the range 1.0 mM - 2.5 mM. However, further increasing of TOH concentration (from 2.5 to 10.0 mM) reduces its antioxidant activity. Consequently, it can be determined 2.5 mM as critical concentration of TOH during TGSO autoxidation from the results obtained.

Synergism for all antioxidant compositions of TOH + AscPH in different ratios (1:1, 1:5 and 1:10) at concentration 1.0 mM of TOH was obtained. The highest synergism for the ratio 1:10 (55.4%) was observed followed by the ratio 1:5 (47.6%) and 1:1 (42.4%). For the lower (0.1 mM) concentration of TOH only equimolar (1:1) antioxidant composition showed synergism (38.1%). Reaction mechanisms, which can explain the effects observed, were presented.

The results obtained are of importance for the practice and proved the significant role of TOH regeneration.

Keywords: Antioxidant potential, binary mixtures, α -tocopherol, ascorbic acid, ascorbyl palmitate, synergism.

1. INTRODUCTION

DL- α -tocopherol (TOH) and L(+)-ascorbic acid (AscAH) are considered to be one of the most important lipid- and water-soluble antioxidants respectively [1-5]. Their antioxidant mechanisms of action against free radicals have been widely studied [6-21] not only because of their potential as individual compounds but also in mixtures [22-30]. Both could be used as a strong base for further development of multi-component antioxidant and bio-antioxidant compositions [31-36] with plausible applications (as food additives and in cosmetics). Commonly used combinations of antioxidants include preventive antioxidants (metal chelators, singlet oxygen quenchers, or ROS-detoxicators) and chain-breaking antioxidants [37]. Despite numerous studies in this area there are many contradictions in the literature especially concerning the chain-breaking activity of ascorbic acid [25, 26, 38-40] due to the different conditions being used.

A characteristic feature of TOH is that increasing the concentration leads to inversion in its action. According to Niki et al. [41] TOH can act as a prooxidant when the rate of chain initiation is quite low and the concentration of

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TOH is quite high at the same time. Many studies, in which the prooxidant effect of TOH is reported, are described by Denisov & Afanasév [42]. Terao and Matsushita [43] have shown that TOH enhanced autoxidation of linoleic acid. Stocker and coworkers [44] studied in detail the prooxidant effect of TOH on LDL oxidation. It was suggested that α -tocopheroxyl radical TO^{\bullet} is able to abstract a hydrogen atom from unsaturated fatty acids and initiate lipid peroxidation. The mechanism of prooxidant effect of α -tocopherol in aqueous lipid dispersions such as LDLs has also been studied by Ingold et al. [45]. It has been found by Weinberg et al. [46] that TOH enhanced in vivo lipid peroxidation in cigarette smokers consuming a high polyunsaturated fat diet. Similar to TOH, both antioxidant and prooxidant activities have been found for its model compound Trolox [47]. This water-soluble analog of vitamin E stimulated or inhibited copper-initiated LDL oxidation depending on the time of Trolox addition, but its effect was always antioxidative when oxidation was initiated by peroxy radicals.

The antioxidant and prooxidant potential of AscAH depends on the conditions and particularly on the presence of transition metal ions. An interesting fact is that the effectiveness of AscAH in the treatment of scurvy is due to its prooxidant effect. The synergistic role of ascorbate with TOH is no less important. As early as 1941 it was found by Columbic & Mattil [22] that the presence of AscAH increased the inhibiting capacity and the induction period of TOH. Tappel [23] proposed the hypothesis for synergism between both components and described conjectural mechanism according to which TOH is the main antioxidant in the system and tocopheryl radical TO^{\bullet} can be regenerated from the molecule of AscAH. Packer proved this hypothesis eleven years later [24], he applied the pulse radiolysis technique. In recent years, the aspirations of manufacturers of cosmetics are directed to improvement of the stability and efficacy of formulations containing low concentrations of several antioxidants instead of higher concentrations of one ingredient, which may cause unwanted prooxidant effects [48]. Development of synergistic antioxidant compositions enables a reduction in the concentration of the antioxidants [49]. On the other hand, it was found that the presence of co-antioxidant, i.e. synergist inverted the prooxidant effect into antioxidant [50, 51]. DL- α -tocopherol (TOH) and L(+)-ascorbic acid (AscAH) are most intensively studied "tandem" and the synergistic effect between them has been proven in different model systems. Nevertheless, how the concentration influences the synergistic effect between the antioxidant and the synergist (TOH and AscAH), respectively, is not well understood. Our previous studies [52] proved synergism between TOH and AscPH in equimolar binary mixtures and 1.0 mM

concentrations. However, it is important to study which ratios and concentrations of both components in the binary mixture will ensure the maximal synergistic effect.

The aim of this study is to establish the critical concentration of TOH after which its efficiency decreased, and also to get information which is/are the most effective binary mixture/mixtures of TOH and AscAH/AscPH by studying different ratios between the individual components at various concentrations.

2. EXPERIMENTAL PART

2.1. CHEMICALS

DL- α -tocopherol and ascorbyl palmitate were purchased from Sigma-Aldrich and used without further purification. All solvents were of HPLC grade purity.

2.2. CHAIN-BREAKING ANTIOXIDANT ACTIVITY

2.2.1. Lipid samples

Triacylglycerols of commercially available sunflower oil (TGSO) were cleaned from pro- and antioxidants by adsorption chromatography [53] and stored under nitrogen at -20°C . Fatty acid composition of the lipid substrate was determined according to Christy [54] by GC analysis of the methyl esters of the total fatty acids obtained with a GC-FID Hewlett-Packard 5890 equipment (Hewlett-Packard GmbH, Austria) and a capillary column HP INNOWAX (polyethylene glycol mobile phase, Agilent Technologies, USA) 30 m \times 0.25 mm \times 0.25 mm. The temperature gradient started from 165°C increased to 230°C with $4^{\circ}\text{C}/\text{min}$ and held at this temperature for 15 min; injection volume was 1 μl . Injector and detector temperatures were 260 and 280°C , respectively. Nitrogen was the carrier gas at a flow rate 0.8 ml/min. The analyses were performed in triplicate. Different fatty acids were present in TGSO: 10:0 - 0.2%; 14:0 - 0.2%; 16:0 - 7.4%; 16:1 - 0.3%; 18:0 - 2.6%; 18:1 - 29.1%; 18:2 - 59.1%; 18:3 - 0.7%; 20:0 - 0.3%. Solvents were removed under a nitrogen flow.

2.2.2. Lipid autoxidation

The process was carried out at 80°C ($\pm 0.2^{\circ}\text{C}$) by blowing air (2.0 ml, at a rate of 100 ml min^{-1}) through the samples in special vessels. The process was monitored by withdrawing samples at measured time intervals and subjecting them to iodometric determination of the primary products (hydroperoxides, LOOH) concentration, i.e. the peroxide value (PV) [55]. All kinetic data are expressed as the average of two independent measurements which

were processed using the computer programs Origin 8.1 and Microsoft Excel-2010.

2.3. DETERMINATION OF THE MAIN KINETIC PARAMETERS OF THE STUDIED COMPOUNDS [20]

2.3.1. Antioxidant efficiency

refers to the increase in the oxidation stability of the lipid sample by blocking the radical chain process and can be expressed by the following kinetic parameters:

Induction period (IP) i.e.: the time, in which the concentration of antioxidant is fully consumed, and can be determined as a cross point for the tangents to the two parts of the kinetic curves of lipid autoxidation; IP_A - in the presence of antioxidant and IP_C for the control lipid sample without antioxidant.

Protection Factor (PF): means as to how many times the antioxidant increases the oxidation stability of the lipid sample and can be determined as a ratio between the induction periods in the presence (IP_A) and in the absence (IP_C) of the antioxidant, i.e., $PF = IP_A / IP_C$.

2.3.2. Antioxidant reactivity

expresses the possibility of an antioxidant to take a part in side reactions of the oxidation process, i.e., to change the initial oxidation rate and can be represented by the following kinetic parameters.

Initial rate of lipid autoxidation (R_C in the absence and R_A in the presence of antioxidant): it can be found from the tangent at the initial phase of the kinetic curves of hydroperoxides accumulation.

Inhibition degree: (ID) is a measure of the antioxidant reactivity, i.e., as to how many times the antioxidant shortens the oxidation chain length ($ID = R_C/R_A$).

2.3.3. Antioxidant capacity

can be presented with the following kinetic parameters.

Main rate of antioxidant consumption (R_m) means the rate of inhibitor consumption during the induction period:

$$R_m = [AOH]/IP_A$$

Relative main rate of antioxidant consumption (RR_m) shows how many times R_m differs from R_A :

$$RR_m = R_m/R_A$$

Synergism, additivism or antagonism. If two or more antioxidants are added to oxidizing substrates, their combined inhibitory effect can be synergistic, additive or antagonistic [14].

Synergism (positive effect) occurs when the combined inhibiting effect of the mixture is higher than the sum of inhibiting effects of the individual com-

ponents, i.e. $IP_{A1+A2} > IP_{A1} + IP_{A2}$. We applied the equation of Frankel [56] for determination of the synergism and calculation of % of it:

$$\% \text{ Synergism} = 100 \{ [IP_{A1+A2} - (IP_{A1} + IP_{A2})] / (IP_{A1} + IP_{A2}) \}.$$

Additivism (summary effect) exists when the antioxidant mixture ensures the same inhibiting effect as the sum of the inhibiting effects of the individual components, i.e.

$$IP_{A1+A2} = IP_{A1} + IP_{A2}$$

Antagonism (negative effect) takes place when the combined inhibiting effect of the antioxidant mixtures is weaker than the sum of those of the individual components, i.e.

$$IP_{A1+A2} < IP_{A1} + IP_{A2}$$

2.3.4. Statistical analysis

Ten independent experiments were carried out in association with previous results on inhibited oxidation and the standard deviations (SD) at different values of the induction periods (IP) and of peroxide values (PV) are obtained [57]. The R_A and R_C were quite constant varying by less than 2%.

3. RESULTS AND DISCUSSION

3.1. CHAIN-BREAKING ANTIOXIDANT ACTIVITY OF INDIVIDUAL COMPOUNDS

Chain-breaking antioxidant activity of DL- α -tocopherol (TOH) and ascorbic acid (AscAH) or ascorbyl-palmitate (AscPH) as individual components at various concentrations (0.1 - 10 mM) is presented on Figure 1 (A and B). Table I summarizes the main kinetic parameters obtained.

It is seen (Fig. 1, Tab. I) that oxidation stability of lipid substrate increases when the concentration of TOH grows up to 1.0 mM (416.7 ppm). The antioxidant efficiency (PF) is 2- and 3-fold higher in 0.5 mM and 1.0 mM concentration of TOH respectively, compared to those obtained for 0.1 mM. Increasing the TOH concentration from 1.0 mM to 2.5 mM (416.7 ppm ÷ 1041.6 ppm) ensures the best (maximal) oxidative stability of lipid substrate. Besides, the antioxidant efficiency of TOH is similar within this concentration range (Fig. 1B). It can be concluded that 2.5 mM is the critical concentration of TOH as far as further increasing leads to a significant decrease of its antioxidant activity. However, TOH doesn't act as a prooxidant even in the highest concentration used 10 mM (4166.7 ppm). These results agree with those reported in other studies [17, 58] in which triacylglycerols (TG), not methyl esters, are used as oxidizable substrates.

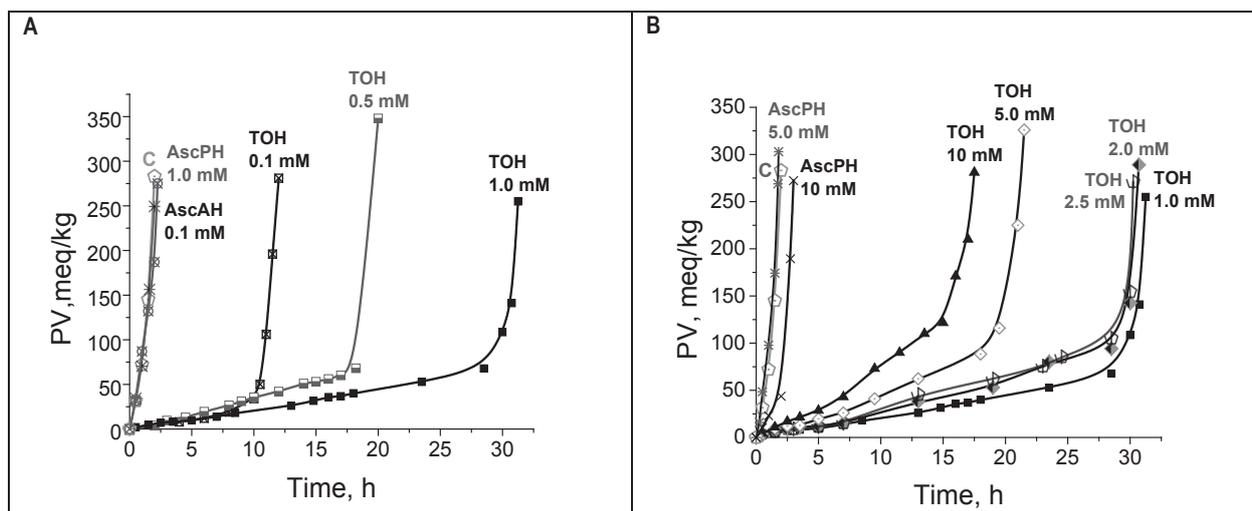


Figure 1 - Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in absence (control sample C) and in presence of studied compounds in concentration range 0.1 mM - 1.0 mM (A) and 1.0 mM - 10 mM (B).

Table I - Main kinetic parameters, characterizing TGSO autoxidation at 80°C in presence of individual compounds: DL- α -tocopherol (TOH), ascorbic acid (AscAH), and ascorbylpalmitate (AscPH) in a concentration range between 0.1 ÷ 10 mM. For control sample $IP_C = (1.3 \pm 0.2)$, h; $R_C = 8.8 \pm 0.5 \cdot 10^{-6}$, M s⁻¹.

Concentration		Antioxidant efficiency		Antioxidant reactivity		Antioxidant capacity		Refs.
ppm	mM	IP_{AOH} h	PF	$R_{AOH} \cdot 10^{-6}$, M s ⁻¹	ID	$R_m \cdot 10^{-8}$, M s ⁻¹	$RR_m \cdot 10^{-3}$, -	
DL-α-tocopherol TOH								
41.7	0.1	10.5 ± 0.8	8.1	0.3 ± 0.2	29.3	0.26	8.7	[20, 21]
208.3	0.5	19.0 ± 2.1	14.6	0.3 ± 0.2	29.3	0.73	24.3	tw
416.7	1.0	29.5 ± 2.5	22.7	0.3 ± 0.2	29.3	0.94	31.3	[52]
833.3	2.0	29.5 ± 2.5	22.7	0.4 ± 0.2	22.0	1.88	47.0	tw
1041.6	2.5	29.5 ± 2.5	22.7	0.4 ± 0.2	22.0	2.35	58.8	tw
2083.3	5.0	19.5 ± 2.0	15.0	0.6 ± 0.2	14.7	7.12	129.5	tw
4166.7	10	16.0 ± 1.7	12.3	0.9 ± 0.2	9.8	17.4	204.7	tw
Ascorbic acid (palmitate), AscAH (AscPH)								
41.7	0.1	1.3 ± 0.2	1.0	8.8 ± 0.5	1.0	21.4	24.3	tw
	0.5 ÷ 5.0	1.3 ± 0.2	1.0	8.8 ± 0.5	1.0	21.4	24.3	tw
4166.7	10.0	2.3 ± 0.2	1.8	2.8 ± 0.2	3.1	120	428.6	tw

tw: this work;

It is obvious (Fig. 1, Tab. I) that at much higher concentrations (5 and 10 mM) TOH still remains inhibitor of lipid autoxidation process but much less efficient in comparison to its activity at lower concentrations (up to 2.5 mM). This means that in our experimental conditions a prooxidant effect of TOH is not found even at concentrations, much higher than that reported by other authors [15, 59, 60]. It can be explained with the difference in methods and models applied from various authors. Remorova & Roginsky [59] proved that the prooxidant effect of TOH exists at 1.0 mM concentration during methyl linoleate oxidation at 37°C because of reaction $TO^{\bullet} + LH \rightarrow TOH + L^{\bullet}$. Kortenska et al [60] found the maximal inhibitory effect of TOH at 700 ppm concentration during methyl linoleate autoxidation at 50°C by ki-

netic modelling (computer study). Kasaikina et al [15] presented data comparing the antioxidant potential of three main groups of phenolic antioxidants (depending on their sterical hindrance) by kinetic modelling of initiated and autocatalytic lipid (methyl linoleate and TGSO) oxidative process. Data presented proved that the regeneration of TOH by disproportionation reaction of its phenoxyl radicals is a reason for the highest antioxidant potential of TOH as chain-breaking antioxidant.

It is known that AscAH is a singlet oxygen quencher but there are many contradictory results in the literature about radical-scavenging and chain-breaking antioxidant activity of ascorbic acid (vitamin C) and its ester forms [25, 38-40, 52, 61]. The results obtained showed, that in contrast to TOH, AscAH

and its ester AscPH didn't manifest any inhibitory effect in all studied concentrations. It means that they cannot increase the oxidation stability of lipid substrate, i.e. AscAH and AscPH are not chain-breaking antioxidants. This fact is in agreement with those reported by Mäkinen et al. [65], who monitored the primary products of lipid autoxidation process in presence of AscAH, i.e. lipid peroxides (LOOH). It is important to note that the oxygen uptake method was used in cases in which it has been noticed that AscAH inhibits the oxidation chain process [25, 38, 40]. In cases in which a certain product (LOOH) of the lipid autoxidation process was monitored [52, 61], it has been proved that AscAH is not a chain-breaking antioxidant but only a synergist. Watanabe et al [39] studied the autoxidation stability of linoleic acid (LA), monitoring the unoxidized LA, in presence of different concentrations of AscAH and its lipid soluble esters including the palmitate AscPH in a wide range of temperatures (35°C, 50°C, 60°C and 80°C). Authors observe a significant oxidation stability of the substrate in the presence of all the studied compounds. Furthermore, the inhibition periods of lipid soluble esters of AscAH were almost 2-fold longer than that of AscAH. The reason for the effects observed could be the much higher concentrations towards the lipid substrate being used, exceeding above 3.5 times the highest concentration (10 mM) of palmitate in our study.

Figure 2A presents the effect of TOH concentrations on one of the main kinetic parameters of TGSO autoxidation – antioxidant efficiency (as protection factor, PF).

Based on data presented (Fig. 2A), we may notice the following tendencies:

a) Three areas can be selected for PF at different TOH concentrations:

- *First area: 0.1 - 1.0 mM TOH* – antioxidant efficiency increases;
- *Second area: 1.0 - 2.5 mM TOH* – maximal antioxidant efficiency, retained for all area;
- *Third area -2.5 – 10 mM TOH* – antioxidant efficiency decreases;

b) The absence of linear dependence of PF on the TOH concentration (Fig. 2A) is due to its participation in side reactions, other than the main reaction (I) of chain termination (Scheme 1). Therefore, the following relation between the main rate of antioxidant consumption R_m and its concentration exists in this case according to Denisov & Afanasév [42]:

$$R_m = R_{in} / f + K_{eff} [InH]^n$$

Where R_{in} is the initiation rate of inhibited oxidation and f - the stoichiometric coefficient of inhibition (i.e. the number of radicals which are trapped from one molecule inhibitor InH). There is no linear dependence in the whole concentration range 0.1 - 10 mM

(41.7 - 4166.7 ppm) at $n = 1$ but for the range between 0.5 mM and 5.0 mM (208.3 - 2083.3 ppm) at $n = 2$ - the plot is linear (Fig. 2B). These results agree with the data published by Marinova, Kamal-Eldin and others [66] for TOH at concentrations above 200 ppm. This supports the thesis that TOH participates in side reactions IV and V (Scheme 1) when presents at concentrations above 0.5 mM. The effective rate constant K_{eff} determined from the slope is $0.3 \cdot 10^{-2} \text{ M}^{-1}\text{s}^{-1}$.

Denisov & Afanasév [42] discussed the following peculiarities of the mechanism of inhibited oxidation of organic compounds:

- Reaction IV of antioxidant (TOH) with hydroperoxides LOOH (Scheme 1) is slow due to the high activation energy required. However, at elevated temperatures and with a sufficiently high concentration of antioxidant and hydroperoxides, this reaction becomes fast and can accelerate the rate of oxidation. From other side, this reaction shortens the induction period.
- Reaction of antioxidant (TOH) with dioxygen (O_2) - phenols and amines interact with O_2 at elevated temperatures according to the reaction V (Scheme 1) and the process of inhibited oxidation is characterized by increasing the oxidation rate and decreasing the induction period.
- Inhibited chain oxidation when TO^* radical propagate the chain by reaction with LH - if the radicals (TO^*) formed from the antioxidant are active toward LH, the chain termination involves the reaction VI presented.
- Inhibited chain oxidation when TO^* radical propagates the chain by reaction with hydroperoxides (LOOH) - if the oxidized LH substance is partially oxidized and contains LOOH, that is sufficiently active TO^* radicals react with LOOH according to the reversed key reaction VII, which results in chain propagation.

Niki et al., [41] called the prooxidant effect of TOH "crossover effect" that depends on the competing reactions II and VII. Furthermore, according to the authors when the ratio $[LOO^*]/[LOOH]$ is high (as the oxidation initiated), i.e. $[LOO^*] \gg [LOOH]$, TOH functions as an antioxidant, while it acts as a pro-oxidant when the ratio is low - $[LOOH] \gg [LOO^*]$.

On the other hand, if the phenoxyl radical participates in side reaction IV (Scheme 1), then the initial rate of inhibited oxidation is in a direct ratio to $[TOH]^{-0.5}$ [42, 63]

$$R_{TOH} \sim [TOH]^{-0.5}$$

If we observe a linear plot for the relation $R_{TOH} \sim [TOH]^{-1}$ - this means that the phenoxyl radical does not participate in side reaction [63].

It is seen (Fig. 2C and D) that neither of the above two relations is valid. Furthermore, there is an exponential dependency in both cases suggesting that

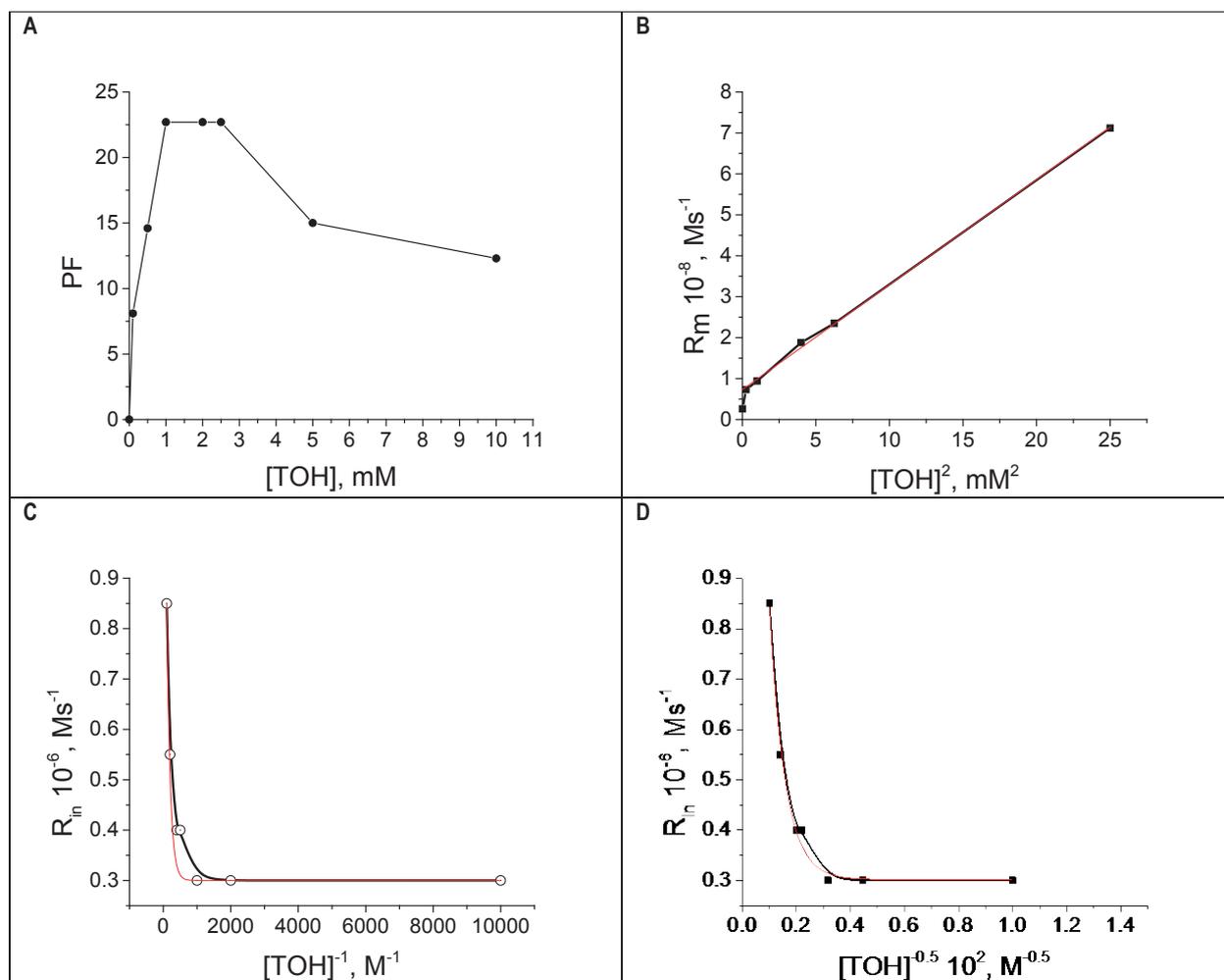


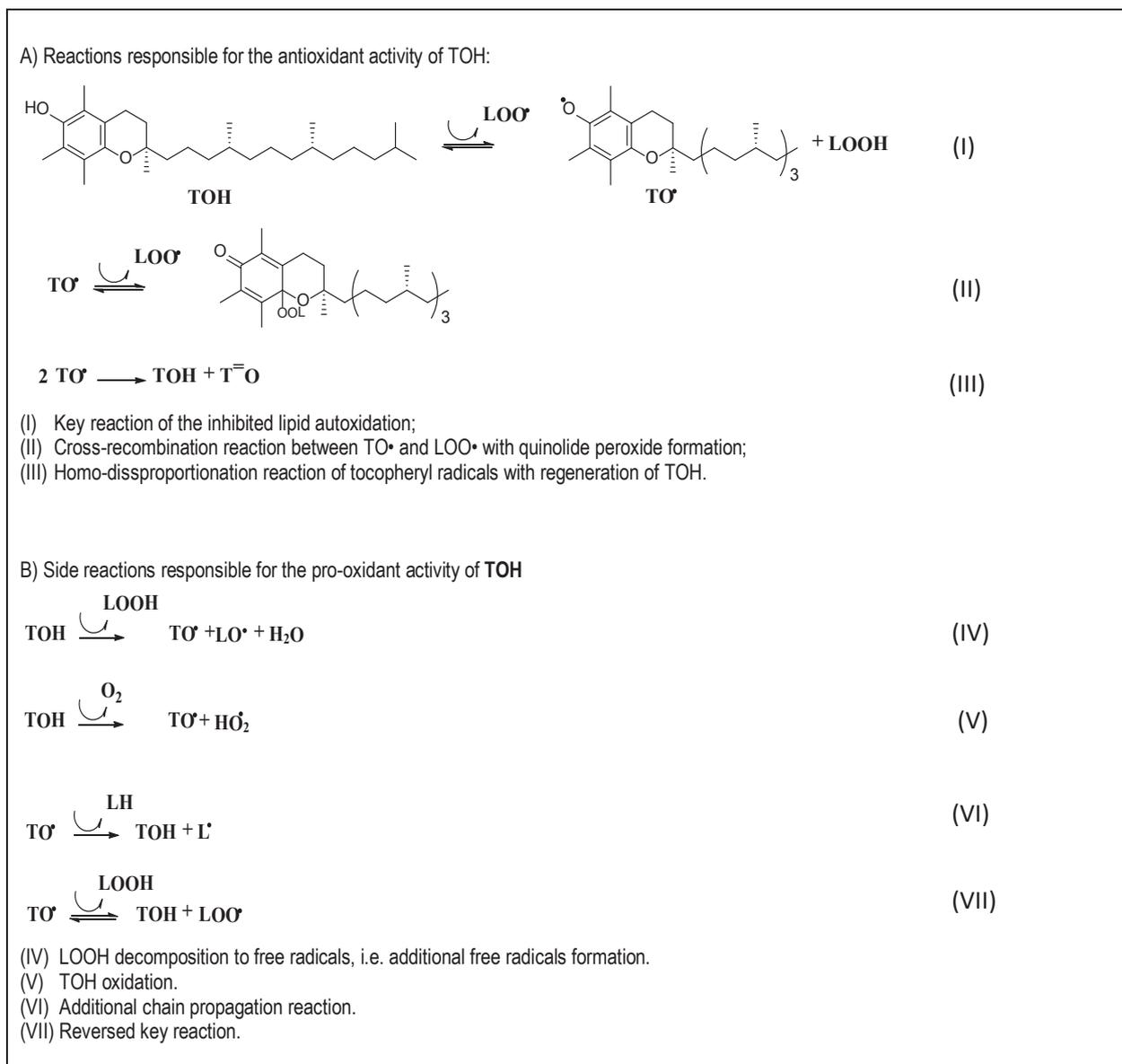
Figure 2 - Dependence of the protection factor PF (A) and the main rate of TOH consumption R_m (B) on its concentration and dependence of the initial rate of inhibited oxidation R_{TOH} on the reciprocal value of the TOH concentration (C) and the square root of its reciprocal concentration (D) during autoxidation of triacylglycerols of sunflower oil (TGSO).

tocopheroxyl radicals participate in more than one side reaction.

Different reactions (Scheme 1) can be crucial for the antioxidant mechanism if some of the conditions (temperature, concentration of TOH, type of the lipid substrate and others) should be changed. Therefore, the optimal and/or critical concentration of an antioxidant needs to be defined considering the specific properties and applications of the commercial products in which it has to be added. For the aims of this study two concentrations of TOH are chosen (0.1 mM and 1.0 mM) for which the effects between the antioxidant and the synergist AscPH are studied. We selected the lowest concentration of 0.1 mM, because it is known that the participation of the phenol and its radical in side reactions is negligible. In the second case our study proved that 1.0 mM is an optimal concentration of TOH at which its inhibitory effect is maximal and ensures the highest oxidation stability of the lipid substrate.

3.2. CHAIN-BREAKING ACTIVITY OF BINARY MIXTURES WITH LIPID SOLUBLE ALPHA- TOCOPHEROL TOH, VITAMIN C OR ITS LIPID SOLUBLE ANALOGUE ASCORBYL PALMITATE AscPH.

It is known that the binary mixtures of TOH and AscAH showed synergism between them [25, 26, 38, 52]. However, it is not clear when the strongest synergistic effect can be achieved – in case of equimolar binary mixtures or in various concentrations between the individual components. For that reason, the effects between TOH and AscPH in different ratios (1:1, 1:5, 1:10) were studied during lipid autoxidation conditions and the kinetic curves of lipid peroxide accumulation in presence of the binary mixtures are presented at Figure 3 (A and B). Although vitamin E and α -tocopherol are frequently considered to be synonyms, vitamin E is actually a name corresponding to a group of natural phenolic compounds comprising four tocopherols (α , β , γ , δ , distinguished by a number of methyl substituents)



Scheme 1 - Reaction mechanism of TOH, including: (A) reactions responsible for its antioxidant activity, and (B) side reactions responsible for its pro-oxidant activity.

and four tocotrienols. By-products of oxidation of TOH (α -tocopherolquinone and α -tocopherolhydroquinone) can also be the very effective inhibitors of lipid peroxidation. It is interesting that the biological activity of α -tocopheryl acetate is the same as that of TOH in humans but significantly lower in rats [64]. ("A man is not a rat!" Professor KU Ingold) Considering that Vitamin E is lipid soluble, while ascorbic acid (Vitamin C) is water soluble, almost all the binary mixtures are prepared with ascorbylpalmitate AscPH – the lipid soluble analogue of Vitamin C with a few exceptions (see Fig. 3A). AscPH is widespread as food additive and its E number is E 304, which is another reason to use it in our experiments. New orders of antioxidant efficiency (protection factor *PF*), antioxidant reactivity (*ID*) and capacity (R_m)

were found for individual compounds and their binary mixtures and the effects obtained according to Frankel [56] are presented in Table II:

1) In the concentration range 0.1 ÷ 1.0 mM of individual components and their mixtures:

PF: $\text{TOH}_{0.1} + \text{AscAH}_{0.1}$ (12.5) > $\text{TOH}_{0.1} + \text{AscPH}_1$ (8.7) \approx $\text{TOH}_{0.1}$ (8.1) = $\text{TOH}_{0.1} + \text{AscAH}_{0.5}$ (8.1) \gg $\text{AscAH}_{0.1}$ (1.0) = $\text{AscPH}_{0.5}$ (1.0) = $\text{AscPH}_{1.0}$ (1.0);

ID: $\text{TOH}_{0.1} + \text{AscAH}_{0.1}$ (41.9) \gg $\text{TOH}_{0.1}$ (29.3) > $\text{TOH}_{0.1} + \text{AscPH}_1$ (19.6) \gg $\text{TOH}_{0.1} + \text{AscAH}_{0.5}$ (16.0) \gg $\text{AscAH}_{0.1}$ (1.0) = $\text{AscPH}_{0.5}$ (1.0) = $\text{AscPH}_{1.0}$ (1.0);

R_m : $\text{TOH}_{0.1} + \text{AscAH}_{0.1}$ ($0.17 \cdot 10^{-8}$) < $\text{TOH}_{0.1} + \text{AscPH}_1$ ($0.25 \cdot 10^{-8}$) \approx $\text{TOH}_{0.1}$ ($0.26 \cdot 10^{-8}$) = $\text{TOH}_{0.1} + \text{AscAH}_{0.5}$ ($0.26 \cdot 10^{-8}$) \ll $\text{AscAH}_{0.1}$ ($0.21 \cdot 10^{-7}$) \ll $\text{AscPH}_{0.5}$ ($0.11 \cdot 10^{-6}$) < $\text{AscPH}_{1.0}$ ($0.21 \cdot 10^{-6}$)

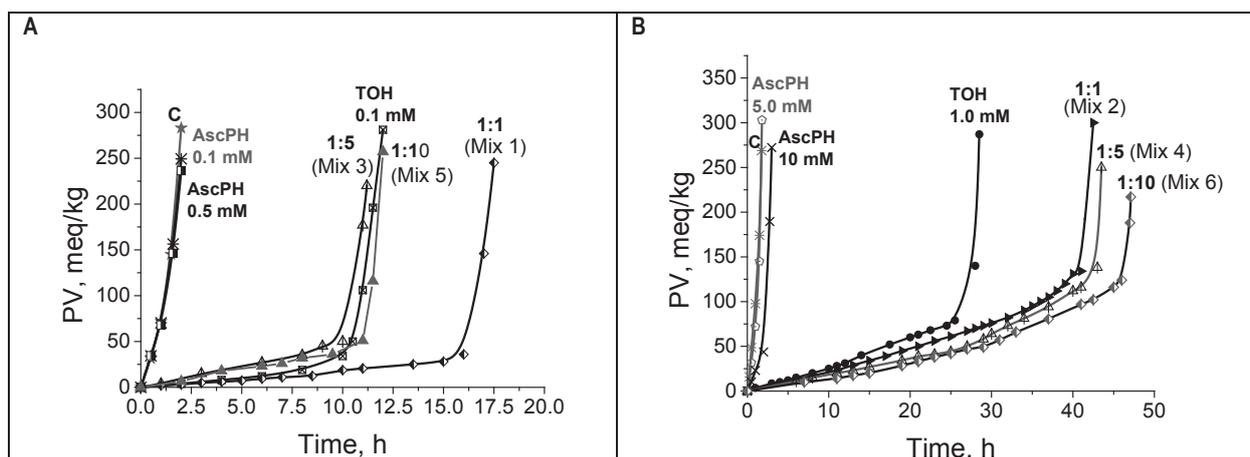


Figure 3 - Kinetic curves of lipid hydroperoxides accumulation during TGSO autoxidation at 80°C in absence (control sample C) and in presence of: (A) α -TOH (in 0.1 mM), AscAH (0.1 mM and 0.5 mM) and AscPH (1.0 mM) and their binary mixtures in ratios: 1:1, 1:5 and 1:10; (B). α -TOH (1.0 mM) and AscPH (1.0 mM, 5.0 and 10 mM) and their binary mixtures in ratios: 1:1, 1:5 and 1:10.

2) In the concentration range 1.0 ÷ 10 mM of individual components and their mixtures:

PF: $TOH_1 + AscPH_{10}$ (35.6) \geq $TOH_1 + AscPH_5$ (32.7) \geq $TOH_1 + AscPH_1$ (31.5) $>$ TOH_1 (23.5) \gg $AscPH_{10}$ (1.8) \geq $AscPH_5$ (1.0) = $AscPH_1$ (1.0)

ID: $TOH_1 + AscPH_{10}$ (44.0) $>$ $TOH_1 + AscPH_5$ (35.2) $>$ $TOH_1 + AscPH_1$ (29.3) = TOH_1 (29.3) \gg $AscPH_{10}$ (3.1) $>$ $AscPH_5$ (1.0) = $AscPH_1$ (1.0)

R_m : $TOH_1 + AscPH_{10}$ ($0.60 \cdot 10^{-8}$) \approx $TOH_1 + AscPH_5$ ($0.65 \cdot 10^{-8}$) \approx $TOH_1 + AscPH_1$ ($0.68 \cdot 10^{-8}$) \ll TOH_1 ($0.10 \cdot 10^{-7}$) \ll $AscPH_1$ ($0.21 \cdot 10^{-6}$) \ll $AscPH_5$ ($1.07 \cdot 10^{-6}$) $<$ $AscPH_{10}$ ($1.20 \cdot 10^{-6}$)

3.2.1. Equimolar (1:1) binary mixtures – concentration effects

✓ 0.1 mM TOH and AscAH (Mix 1)

Strong synergism of 38.1% was observed for the equimolar binary mixture at lowest concentration of individual components. Lipid oxidation stability is 15-fold higher in presence of this mixture compared with the control sample and AscAH and 1.7-

fold higher in comparison with individual TOH. The lipid oxidation chain length is shortened 1.5-fold.

✓ 1.0 mM TOH and AscPH (Mix 2)

Strong synergism (42.4%) was also observed in case of equimolar binary mixture in 1.0 mM concentration of individual components. There is a little bit stronger effect in comparison with the equimolar binary Mix 1. Lipid oxidative stability is 2.5-fold higher in this case, in comparison with Mix 1.

ID values of the equimolar mixture $TOH_1 + AscPH_1$ (Mix 2) and individual TOH don't differ, i.e. they are equal (29.3). This value remains constant for TOH in the concentration diapason 0.1 ÷ 1.0 mM.

3.2.2. Binary mixtures with ratios 1:5 and 1:10 – concentration effects

• Ratio TOH/AscPH = 1:5

✓ 0.1 mM TOH and 0.5 mM AscAH (Mix 3)

It is seen that a 5-fold higher concentration of AscPH does not result in a better oxidation stability and inhibiting activity of TOH. The oxidative

Table II - Induction periods and effects between components obtained in different concentrations of the synergist (AscAH, AscPH) and ratios.

[TOH] mM	[Synergist] mM	IP _{Asc} , h	IP _{TOH} , h	Ratio	Σ IP _{TOH+IP_{Asc}} h	IP _{ΣTOH+Asc} h	Effect, %	Ref.
0.1	0.1	1.3 ± 0.2	10.5 ± 0.8	1:1 (Mix1)	11.8 ± 0.8	16.3 ± 0.9	Synergism, 38.1%	tw
1.0	1.0	1.3 ± 0.2	27.5 ± 2.0	1:1 (Mix2)	28.8 ± 2.0	41.0 ± 3.0	Synergism 42.4%	[52]
0.1	0.5	1.3 ± 0.2	10.5 ± 0.8	1:5 (Mix3)	11.8 ± 0.8	10.5 ± 0.8	No effect	tw
1.0	5.0	1.3 ± 0.2	27.5 ± 2.0	1:5 (Mix4)	28.8 ± 2.0	42.5 ± 2.7	Synergism 47.6%	tw
0.1	1.0	1.3 ± 0.2	10.5 ± 0.8	1:10 (Mix5)	11.8 ± 0.8	11.3 ± 0.8	Additive/No effect	tw
1.0	10	2.3 ± 0.2	27.5 ± 2.0	1:10 (Mix6)	29.8 ± 2.0	46.3 ± 3.1	Synergism 55.4%	tw

stability of lipid substrate is the same as with the presence of TOH alone.

- ✓ 1.0 mM TOH and 5.0 mM AscPH (Mix 4)

In this case a stronger synergism (47.6%) is observed in presence of 5.0 mM AscPH instead of 1.0 mM AscPH. The lipid oxidative stability is 1.5-fold higher than with TOH as an individual component.

- Ratio TOH/AscPH = 1:10

- ✓ 0.1 mM TOH and 1.0 mM concentration of AscPH (Mix 5)

There is no synergism obtained, only summary effect between the individual components. It is seen that the oxidative stability of lipid substrate is the same as in presence of TOH as individual component. This means that TOH and AscPH "prefer" to act separately, not in tandem in this concentrations at ratio 1:5.

- ✓ 1.0 mM TOH and 10 mM AscPH (Mix 6)

Strongest synergism is obtained for this binary mixture (55.4%). The oxidative stability of lipid substrate is much higher in presence of the mixture 6 instead of only TOH.

- Comparative analysis

Both equimolar binary mixtures demonstrated strong synergism and are able to ensure the high oxidative stability of lipid substrate, being oxidized. All binary mixtures with 1.0 mM TOH manifest strong synergism. However, binary mixture TOH₁ + AscPH₁₀ (Mix 6) manifests the highest activity. Antioxidant efficiency and reactivity of the mixture expressed with the main kinetic parameters *PF* (protection factor) and *ID* (inhibition degree) and the antioxidant capacity *R_m* are between 1.5- and 2-fold higher in comparison with the same parameters obtained for the individual TOH in 1.0 mM. If we compare the values for *PF* and *R_m* for all binary mixtures, in which TOH is in 1.0 mM, we noticed that there is no significant difference between them. Antioxidant reactivity of these three mixtures is quite different. *ID* values of the equimolar mixture TOH₁ + AscPH₁ (Mix 2) and individual TOH don't differ, i.e. they are equal (29.3). This value remains constant for TOH in the concentration diapason 0.1 + 1.0 mM. Adding AscPH to TOH in 5- and 10-fold higher doses, when concentration of TOH in the mixture is 1.0 mM, leads to a considerable increase of antioxidant reactivity. Conversely – when TOH is in the lower concentration (0.1 mM), 5- and 10-fold higher concentrations of AscPH in binary mixture (Mix 3 and Mix 5) decrease the inhibition degree. Only if both components are in equimolar ratio (TOH_{0,1} + AscPH_{0,1}) we observe *ID* higher than those for individual TOH in 0.1 mM. Therefore, TOH in low concentration 0.1 mM

is effective enough and the level of side reactions responsible for its pro-oxidant activity is neglectable. Addition of AscAH/AscPH in 5- and 10-fold higher doses respectively in this case doesn't result in a more effective inhibition. From the new orders of the main kinetic parameters obtained, we see that the antioxidant reactivity of these mixtures is even lower than that of TOH in 0.1 mM.

Regarding the kinetic parameter *R_m* (antioxidant consumption mean rate during the induction period *IP_{TOH}*) that is a measure for the antioxidant capacity; the following conclusions can be drawn:

- ✓ When there are higher concentrations of TOH (1.0 mM) – in all cases the addition of AscPH leads to a decrease of the TOH rate of consumption in the course of the oxidation process.
- ✓ When there are lower concentrations of TOH, (0.1 mM) the addition of AscAH and/or AscPH in ratios 1:5 and 1:10 does not change *R_m* and its value is the same of those obtained for individual TOH (in 0.1 mM). In equimolar quantities (1:1) of both components we observe the lowest rate of TOH consumption.

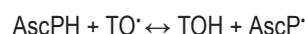
By varying concentration of the synergist (AscAH/AscPH) in the two-component system, at the lower concentration (0.1 mM) of TOH, at which the level of side reactions (Scheme 1 B) can be neglected, we observe synergistic effect only for the equimolar ratio 1:1. In the remaining cases, the addition of the synergist AscAH/AscPH in 5- and 10-fold higher concentration does not lead to synergism, but to additive effect or to the lack of it. (Tab. II).

When TOH is in 1.0 mM, at all studied ratios (1:1, 1:5 и 1:10) between both compounds, we observe a synergism. Furthermore, the synergistic effect increases with the increase of the concentration of the synergist (Tab. II). 1.0 mM is considered to be a "critical" concentration by different authors but it is the optimal concentration of TOH in this study.

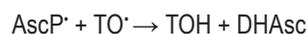
Synergism occurs mostly when more effective free radical scavenger is regenerated by a less effective free radical scavenger. This reaction of H-atom transfer is reversible. However, in case of synergism between AscPH and TOH, the reaction is shifted to the right with regeneration of TOH, which is the stronger antioxidant.

There are several reactions in which TOH can be regenerated:

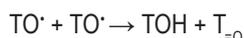
- 1) H-atom transfer (HAT) from molecules of AscPH to tocopheryl radicals TO* (a reversible reaction):



- 2) cross-disproportionation between AscP* and TO* with regeneration of TOH and dehydroascorbylpalmitate DHAsc formation:



- 3) homo-disproportionation of $\text{TO}\cdot$ leading to re-generation of TOH:



However, in binary mixtures the rate of cross-recombination reaction is much faster than that of homo-disproportionation reaction [42]. For that reason, the cross-recombination reactions are important for the synergism obtained in various binary mixtures, containing TOH.

4. CONCLUSIONS

Six binary mixtures of TOH and AscAH/AscPH: two equimolar (1:1), two with ratio 1:5 and two with ratio 1:10 between the individual components were studied for possible synergism. Four of these mixtures demonstrated strong synergism and the remaining two – without effect or additive effect. Both equimolar (1:1) binary mixtures of TOH and AscAH/AscPH in 0.1mM and 1.0mM concentrations demonstrated strong synergism (38.5 and 42.4%, resp.).

Binary mixtures containing TOH in a 1.0 mM concentration and ratios 1:5 and 1:10 with AscPH ensured maximal oxidative stability of the lipid substrate as a result of a stronger synergism between both components (47.6% and 55.4%, resp.).

Antioxidant mixtures with ratios between components 1:1; 1:5 and 1:10 when the concentration of TOH is 1.0 mM can be used for further preparations of multi-component antioxidant and bio-antioxidant mixtures.

The results obtained proved the significant effect of adding the synergist AscPH when the antioxidant TOH is at high concentrations because of a higher level of side reactions responsible for the additional generation of free radicals.

The “critical” concentration of TOH - 2.5 mM has been established in this study and thus a higher TOH concentration is not recommended to be used for TOH as an individual inhibitor.

The binary mixtures with ratios 1:5 and 1:10 of TOH and AscPH are not able to ensure a higher oxidative stability of the lipid substrate in comparison with the individual inhibiting effect of TOH in 0.1 mM and thus are not preferred to be applied in practice.

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