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ScienceDirect
Food Chemistry 106 (2008) 628–633
Food Chemistry
www.elsevier.com/locate/foodchem

Synergistic antioxidant effect of α-tocopherol and myricetin on the autoxidation of triacylglycerols of sunflower oil

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Received 19 January 2007; received in revised form 2 May 2007; accepted 19 June 2007

Abstract

The synergistic antioxidant effect of different concentrations (50–250 ppm) of α-tocopherol and myricetin during autoxidation of triacylglycerols of sunflower oil (TGSO) at 100 °C was studied. The process was followed by monitoring the peroxide values and the formation of conjugated dienes. It was established that myricetin is a more effective and stronger antioxidant than α-tocopherol. All mixtures investigated exhibited a synergistic effect. The best synergistic effect was achieved with an equal molar ratio of α-tocopherol and myricetin, and at total concentration of the mixtures lower than 10×10^{-4} M. The kinetic analysis of the results demonstrated that α-tocopherol regenerates myticetin during autoxidation of TGSO at 100 °C.

Keywords: Synergism; Myricetin; α-tocopherol; Triacylglycerols; Sunflower oil; Autoxidation

1. Introduction

Lipid oxidation has been recognized as the major source of deteriorative changes in the chemical, sensory and nutritional properties of fats and oils. In addition, lipid oxidation has been shown to be associated with the disturbance of the structure and the function loss of the cell membranes, and the oxidative modification of low-density lipoproteins and is an important initial event for the pathogenesis of atherosclerosis (Niki, 2000; Stahl, 2000). The inhibition of deleterious lipid oxidation has received much attention and the role and action of antioxidants have been the subjects of extensive studies (Gordon, 1996; Niki, 2001). Since, the oxidation process follows a complicated set of mechanisms, no single antioxidant is effective for all stages and circumstances under which it might occur. That is why, it may be successfull to use antioxidant combinations in which the antioxidants produce a synergistic effect (Jaswir, Kitts, Che Man, & Hassan, 2004; Judde, Villeneuve, Rossignol-Castera, & Le Guillou, 2003; Rossetto et al., 2002).

Flavonoids are polyphenolic compounds present in substantial amounts in plants. They exert multiple biological effects including antioxidative activity (Jovanovich, Steenken, Tosic, Marjanovic, & Simic, 1994). Flavonoids have been widely investigated in recent years, because of their potential antioxidant activity, which improves the stability of lipid containing foods and their possible beneficial effects on human health (Hirano et al., 2001; Hopia & Heinonen, 1999; Roedig-Penman & Gordon, 1998). Myricetin is an important dietary flavonoid and occurs mainly in blackcurrants, black grapes, cranberries, bilberries, broad beans, red wine and grape juice (Hertog, Hollman, & Katan, 1992).

Tocopherols are the most widely used natural antioxidants (Kamal-Eldin & Appelqvist, 1996). It has been reported that they are much more effective when used in combination with other antioxidants (Bandarra, Campos, Batista, Nunes, & Empis, 1999; Judde et al., 2003; Pedrielli & Skibsted, 2002).

To achieve a complete understanding of the antioxidant mechanism of flavonoids in food and living organisms,

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doi:10.1016/j.foodchem.2007.06.022

their interaction with other antioxidants must be explored. In multi-component systems, some antioxidants can reinforce each other by cooperative effects known as synergism (Frankel, 1998). The synergistic effect of antioxidant activity is very important to protect lipids from degradation. There has been some evidence of a higher antioxidant effect when combinations of flavonoids and α-tocopherol were used (Pedrielli & Skibsted, 2002; Zhu, Huang, Tsang, & Chen, 1999). The combination of 25 μM myricetin and 25 μM α-tocopherol were synergistic compared to 50 μM myricetin or 50 μM α-tocopherol alone during autoxidation of methyl linoleate at 40 °C, while the combination of quercetin with α-tocopherol showed no synergistic effect (Pekkarinen, Heinonen, & Hopia, 1999). However, the mechanism of such interaction is not well understood. It has been suggested that the regeneration of α-tocopherol from its radical by some flavonoids proceeded in analogy to the well-known synergism between α-tocopherol and ascorbate (Jia, Zhou, Yang, Wu, & Liu, 1998; Pedrielli & Skibsted, 2002). On the other hand, Jorgensen, Madsen, Thomsen, Dragsted, and Skibsted (1999) showed that flavonoids were regenerated by tocopherol under certain conditions. Hiramoto, Miura, Ohnuki, Kato, and Kikugava (2002) established that α-tocopherol inhibited decomposition of some water-soluble antioxidants in the radical initiator-induced methyl linoleate oxidation system.

Hence, the information on antioxidant interactions of different kind of natural antioxidants would, therefore, provide a better understanding of the antioxidant effects of phytochemicals in complex mixtures.

The objective of the present work was to investigate the potential synergistic effect of myricetin with α-tocopherol (Fig. 1), in order to obtain knowledge about the probable mechanism responsible for this synergistic effect and to select the most efficient combinations for inhibiting the oxidation of triacylglycerols of sunflower oil (TGSO). TGSO is a nonpolar lipid model, free from antioxidants and was widely used in antioxidant activity testing. When used without added initiator, the main inhibiting reactions take place between antioxidants and lipid peroxyl radicals (Hopia & Heinonen, 1999). The oxidation mechanism of TGSO is well-known to give quantitatively conjugated diene hydroperoxides (Frankel, 1998).

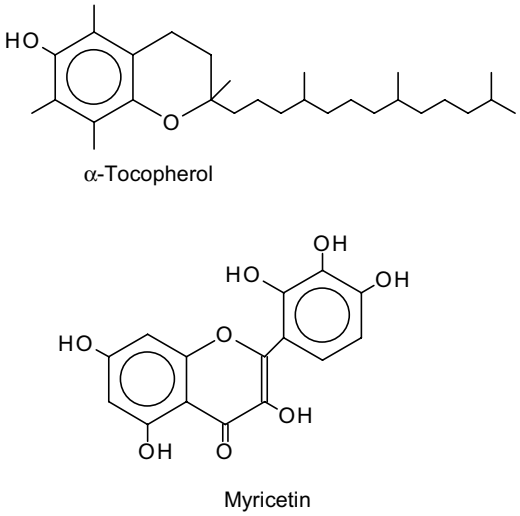

Fig. 1. Formulae of the investigated antioxidants.

2. Materials and methods

2.1. Materials

TGSO were obtained by cleaning commercially available sunflower oil sample from pro- and antioxidants and trace metals by adsorption chromatography (Yanishlieva & Marinova, 1995). Briefly, lipid substrate (100 g in 1000 ml distilled hexane) were passed through a 2 cm i.d. column filled with 70 g alumina (type 507C, neutral, activity stage II, Fluka, Buchs, Switzerland) activated at 180 °C for 4 h. The obtained triacylglycerols were collected under nitrogen in the dark and were stored under nitrogen at −20 °C for no more than 10 days. The TGSO were found to contain undetectable amounts of tocopherols (HPLC, <0.5 ppm) and iron and copper (atom absorption spectroscopy, < 0.01 and 0.001 ppm, respectively). Control oxidation experiments at 80 °C in the presence of 0.01% and 0.02% citric acid demonstrated that the chelating agent had no effect on the oxidation kinetics. α-Tocopherol was obtained from E. Merck (Darmstadt, Germany) and myricetin was obtained from Fluka (Buchs, Switzerland).

2.2. Methods

2.2.1. Sample preparation

Lipid samples containing different concentrations of the antioxidants were prepared by adding aliquots of their solutions in purified hexane (for α-tocopherol) or in purified acetone (for myricetin) to a weighted amount of TGSO followed by removal of the solvent with nitrogen.

The antioxidants were added to TGSO in the following quantities: α-tocopherol – 50 ppm (1.2×10^{-4} M), 125 ppm (3.0×10^{-4} M), 250 ppm (6.0×10^{-4} M) and 500 ppm (12.0×10^{-4} M); myricetin – 50 ppm (1.6×10^{-4} M), 100 ppm (3.2×10^{-4} M), 200 ppm (6.4×10^{-4} M), and 400 ppm (12.8×10^{-4} M). The mixtures were prepared in the following quantities: MIX-1 – 50 ppm α-tocopherol + 50 ppm myricetin, MIX-2 – 125 ppm α-tocopherol + 50 ppm myricetin, MIX-3 – 50 ppm α-tocopherol + 100 ppm myricetin, MIX-4 – 125 ppm α-tocopherol + 100 ppm myricetin, MIX-5 – 250 ppm α-tocopherol + 100 ppm myricetin, MIX-6 – 125 ppm α-tocopherol + 200 ppm myricetin, MIX-7 – 250 ppm α-tocopherol + 200 ppm myricetin. For control, the sample without added antioxidant was used.

2.2.2. Gas chromatography

The fatty acid composition of the starting oil was determined by gas chromatography of its methyl esters using a Pye Unicam instrument, model 304, equipped with a dual

flame-ionization detector and a glass capillary column (30 m × 0.2 mm id) coated with SILAR 10C (Supelco Inc., Bellefonte, PA). The carrier gas was nitrogen at a flow rate of 14 ml/min. The temperature was maintained at 165 °C for 5 min, then increased to 200 °C by 2 °C/min.

2.2.3. Oxidation procedure

Oxidation at 100 °C (±0.2 °C) was carried out by blowing air through the samples (2 g) in the dark at a rate of 50 ml/min. Under these conditions the process took place in a kinetic regime, i.e. at a sufficiently high oxygen concentration at which the diffusion rate does not influence the oxidation rate. The process was followed by withdrawing samples at measured time intervals, estimating the degree of oxidation by iodometric determination of peroxide value, PV (Yanishlieva, Popov, & Marinova, 1978), and measuring the content of conjugated dienes, CD, by UV spectroscopy. CD values were determined by dissolving weighted samples in iso-octane and reading the sample absorbance at 232 nm (A_{232}), using a Cecil Series 8000 UV/VIS double-beam scanning spectrophotometer (Cecil Instruments Ltd., Cambrige, United Kingdom). The A_{232} (0.1%) was calculated from the absorbance reading.

Kinetic curves of PV accumulation were plotted. All of them represent a mean result of three independent experiments. The effectiveness of the antioxidants and the mixtures was estimated on the basis of the induction period, IP, determined by the method of the tangents to the two parts of the kinetic curves (Le Tutour & Guedon, 1992). The rates of non-inhibited W_{noninh} (control sample) and inhibited W_{inh} oxidation were found from the tangents to the initial phase of the kinetic curves of peroxide accumulation and expressed as M s^{-1} (Marinova & Yanishlieva, 1992).

2.2.4. Statistical analysis

The coefficient of variation for the PV determination was 7–8% irrespective of the measured value. The reported values for the IP were a mean result from three independent experiments. The coefficient of variation ranged from 6% to 13% and was inversely related to the induction period. The W_{noninh} and W_{inh} were varying by not more than 5%. Linear relationship between PV and CD was obtained using the Linear fit tool of Origin 6.1 software (OriginLab Corporation, One Roundhouse Plaza, Northampton MA, USA).

3. Results and discussion

The fatty acid composition of TGSO was as follows: palmitate 6%, stearate 5%, oleate 27%, linoleate 62%.

The peculiarities of the inhibiting action of the antioxidants can be described by two kinetic characteristics (Yanishlieva & Marinova, 1992):

(i) Effectiveness, representing the possibility of blocking the chain radical process by interaction with the peroxyl radicals, which is responsible for the duration of the IP.

(ii) Strength, expressing the possibility of the inhibitor moieties to participate in other (side) reactions which may lead to a change in the oxidation rate during the IP.

A measure for the effectiveness is the stabilization factor (F):

$$F = \text{IP}_{\text{inh}}/\text{IP}_0,$$

where IP_{inh} is the induction period in the presence of an inhibitor, and IP_0 is the induction period of the uninhibited oxidation.

The strength of the antioxidant can be estimated by the oxidation rate ratio (ORR):

$$\text{ORR} = W_{\text{inh}}/W_0,$$

where W_{inh} is the oxidation rate during the induction period in the presence of an inhibitor, and W_0 is the initial oxidation rate of the uninhibited oxidation. ORR is an inverse measure of the strength.

The mean rate of inhibitor consumption, W_{InH}, is determined by the formula

$$W_{\text{InH}} = [\text{InH}]_0/\text{IP}_{\text{inh}}\ (\text{M s}^{-1}),$$

where $[\text{InH}]_0$ is the initial molar concentration of the antioxidant, and IP_{inh} is the duration of the induction period in the presence of the inhibitor, in seconds.

The percent synergism is calculated on the basis of the induction periods (IP) observed as follows (Frankel, 1998):

$$\%\text{Synergism(SN \%)} = \frac{[(\text{IP}_{\text{mix}} - \text{IP}_0) - [(\text{IP}_{\text{InH1}} - \text{IP}_0) + (\text{IP}_{\text{InH2}} - \text{IP}_0)]}{[(\text{IP}_{\text{InH1}} - \text{IP}_0) + (\text{IP}_{\text{InH2}} - \text{IP}_0)]} \times 100$$

where IP_{mix}, IP_0, IP_{InH1} and IP_{InH2} are the induction periods of the samples containing the mixture of additives, of the control sample, and of the samples containing the individual antioxidants, InH1 and InH2, respectively. A positive value defines a synergistic effect between the implicated antioxidants, while a negative value corresponds to an antagonism between them.

Fig. 2 illustrates, by way of example, the kinetic curves of peroxide accumulation during inhibited oxidation of TGSO in the presence of equal molar concentrations (~3×10^{-4} M) of α-tocopherol, myricetin and their mixture – MIX-4 at 100 °C. The results presented in Fig. 2 clearly shows that an extended induction period, IP, and a reduced oxidation rate, W_{inh} are observed in the presence of MIX-4. The effects of the different concentrations of myricetin, α-tocopherol and their mixtures on the main kinetic parameters (IP, W_{inh}, W_{InH}, and ORR), are presented in Table 1. As can be seen from Table 1, myricetin is a more effective and stronger antioxidant than α-tocopherol during autoxidation of TGSO at 100 °C. All mixtures of myricetin and α-tocopherol cause a longer IP compared to the sum of the IP determined in the presence of each antioxidant alone. This observation indicates a synergistic effect at all concentrations investigated. The calculated

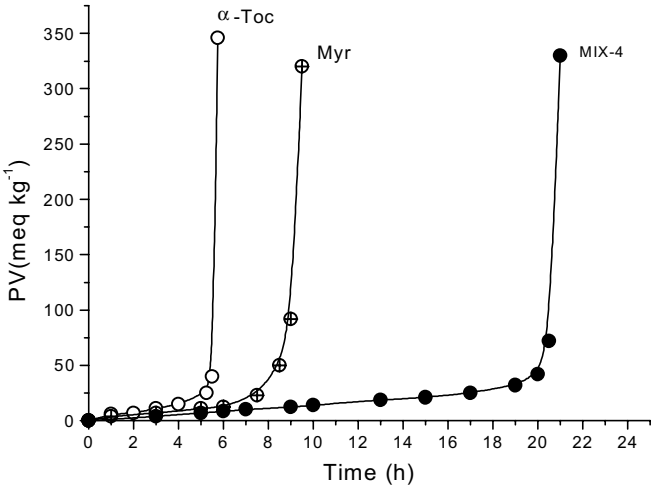

Fig. 2. Kinetic curves of peroxide accumulation during autoxidation of TGSO at 100 °C in the presence of 125 ppm (3.0×10^{-4} M) α-tocopherol, (α-Toc), 100 ppm (3.2×10^{-4} M) myricetin, (Myr), and MIX-4 (125 ppm α-tocopherol + 100 ppm myricetin).

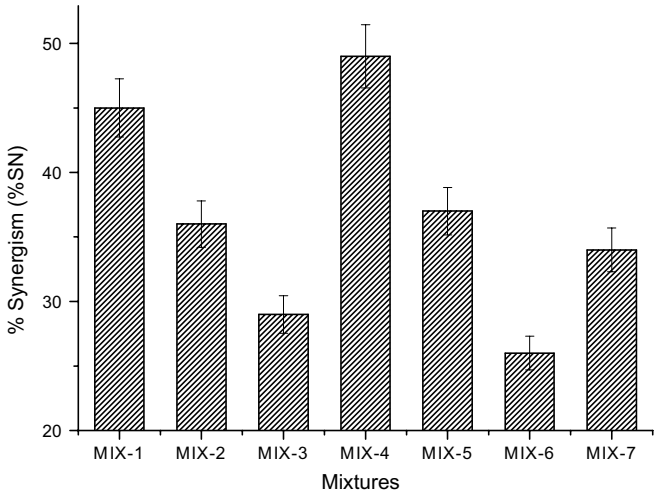

Fig. 3. The percent synergism (% SN) of the investigated mixtures of α-tocopherol and myricetin. The symbols are as in Table 1.

synergism of the mixtures of α-tocopherol and myricetin are presented in Fig. 3. The highest values of % synergism (*SN*%) are observed in the MIX-1 and MIX-4. This means that the best synergistic effect was achieved with equal molar ratio of α-tocopherol and myricetin, and total concentration of the mixtures lower than 10×10^{-4} M. From the results obtained, it can be deducted that the molar ratio was not the only parameter influencing the synergism between α-tocopherol and myricetin, but that the total concentration of the mixture was also of importance. The lower synergism in the MIX-7 (molar ratio 1:1) may be due to the fact that at higher concentration of α-tocopherol the participation of its molecules and radicals in chain initiation and propagation should be taken into account (Yanishlieva, Kamal-Eldin, Marinova, & Toneva, 2002).

The extended IP measured in the presence of the combinations of α-tocopherol and myricetin confirmed the existence of an antioxidant interaction, which produced a more effective inhibition of lipid peroxidation. The data in Table 1 shows that the oxidation rates during the IP (W_{inh}, ORR) in the presence of the mixtures were the same as that during the IP observed when myricetin was used alone at higher concentration. The W_{InH} values for α-tocopherol increase with increasing concentration, which indicates the participation of its molecules in side reaction with hydroperoxides (Yanishlieva et al., 2002). On the other hand, W_{InH} values for myricetin did not depend on its concentration. The results presented in Table 1 shows that W_{InH} for all mixtures tested were also concentration independent and are comparable to these for myricetin. All these observations provide evidence for the regeneration of myricetin by α-tocopherol. In addition, a larger

Table 1
Kinetic parameters characterizing inhibited oxidation of TGSO at 100 °C ($IP_0 = 0.5$ h, $W_0 = 6.94 \times 10^{-6}$ M s^{-1})

Antioxidant	Concentration		Molar ratio ([α-TH]/[Myr])	IP (h)	W_{inh} ($\times 10^7$) (M s^{-1})	W_{InH} ($\times 10^8$) (M s^{-1})	F	ORR
	(ppm)	(M $\times 10^4$)						
α-Tocopherol	50	1.2		3.2	5.79	1.23	6.4	0.08
α-Tocopherol	125	3.0		5.5	5.05	1.61	11.0	0.07
α-Tocopherol	250	6.0		7.4	4.27	2.33	14.8	0.06
Myricetin	50	1.6		4.7	5.10	1.06	9.2	0.07
Myricetin	100	3.2		8.9	3.27	1.03	17.8	0.05
Myricetin	200	6.4		16.3	2.13	1.11	32.6	0.03
MIX-1	100	2.8	1:1	10.5	4.23	0.78	21.0	0.07
MIX-2	175	4.6	2:1	13.0	3.47	1.00	26.0	0.05
MIX-3	150	4.4	1:2	14.8	2.13	0.84	29.6	0.03
MIX-4	225	6.2	1:1	20.5	1.98	0.83	41.0	0.03
MIX-5	350	9.2	2:1	21.5	1.98	1.19	43.0	0.03
MIX-6	325	9.4	1:2	26.7	1.98	0.98	53.4	0.03
MIX-7	450	12.4	1:1	31.1	1.98	1.10	62.2	0.03

MIX-1 – 50 ppm α-tocopherol + 50 ppm myricetin, MIX-2 – 125 ppm α-tocopherol + 50 ppm myricetin, MIX-3 – 50 ppm α-tocopherol + 100 ppm myricetin, MIX-4 – 125 ppm α-tocopherol + 100 ppm myricetin, MIX-5 – 250 ppm α-tocopherol + 100 ppm myricetin, MIX-6 – 125 ppm α-tocopherol + 200 ppm myricetin, MIX-7 – 250 ppm α-tocopherol + 200 ppm myricetin.

ratio of α-tocopherol/myricetin in the mixtures with identical total concentrations (compare MIX-2 and MIX-3; MIX-5 and MIX-6) leads to a higher synergetic effect. This result is in agreement with the fact that, if α-tocopherol regenerates myricetin, then the length of the extended IP should be dependent on the tocopherol concentration (Pedrielli & Skibsted, 2002).

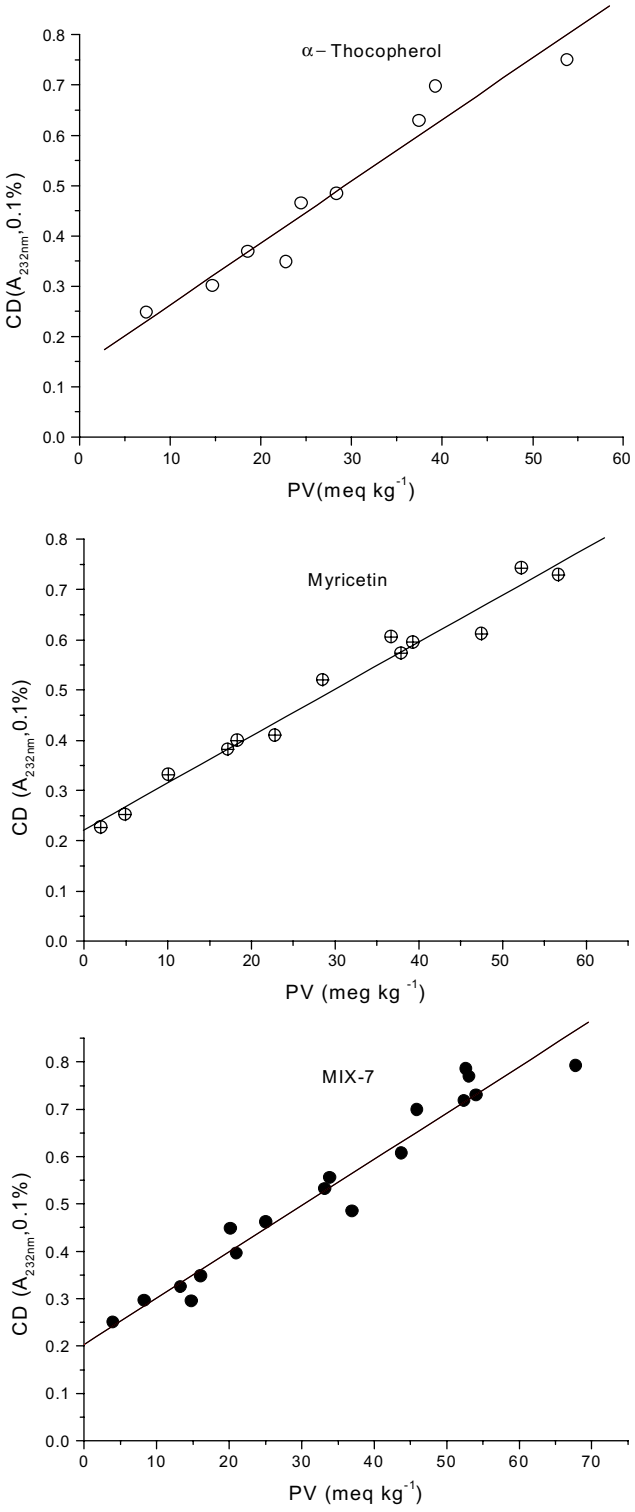

Fig. 4. Correlation between the absorbance of conjugated diene structures, A_{232} (0.1%), and the concentration of peroxides, PV (meq/kg), during autoxidation of TGSO at 100 °C in the presence of 12.0×10^{-4} M α-tocopherol, 12.8×10^{-4} M myricetin and MIX-7 (6.0×10^{-4} M α-tocopherol + 6.4×10^{-4} M myricetin).

Table 2
Regression coefficients (±standard error) and coefficients of determination (r^2) from the linear regression of PV and CD

Antioxidant	Molar concentration ($M \times 10^{-4}$)	Slope	r^2
α-Tocopherol	12.0	$0.0123 \pm 1.2\ 10^{-4}$	0.940
Myricetin	12.8	$0.0093 \pm 5.0\ 10^{-4}$	0.975
MIX-7	12.4	$0.0098 \pm 5.4\ 10^{-4}$	0.954

Since, the lipid oxidation is a complex multistep process it is important to study the ability of antioxidants to inhibit the various steps of this process. The formation of hydroperoxides and their decomposition are discrete steps, which are affected quite differently by various antioxidants (Hopia, Huang, & Frankel, 1996). As far as PV is a direct measure of peroxide concentration, it appears likely that oxidation products of other origins with conjugated diene structures, e.g. hydroxy compounds, contribute to the CD value (Kulas & Ackman, 2001).

The PV and CD values measured during the IP of the samples containing *ca* 12×10^{-4} M myricetin, α-tocopherol and MIX-7 were strongly correlated (Fig. 4.), and linear relationships between the two data are found (Table 2). The data in Table 2 shows that the value of the regression line slope for α-tocopherol is higher than for myricetin. From these results it appears that α-tocopherol results in the formation of a higher proportion of oxidation products, other than hydroperoxides, with a conjugated diene structure. It is known that α-tocopherol reacts not only with peroxyl radicals (ROO•), but also with alkoxyl radical intermediates (RO•) to form hydroxy compounds (Hopia, Huang, Schwarz, German, & Frankel, 1996). The linoleate hydroxy compounds were the only oxidation products, besides the hydroperoxides, that appeared to absorb at 232 nm, but not contribute to the PV (Kulas & Ackman, 2001). From the results in Table 2 it can be seen that the value of the regression line slope for the MIX-7 is the same as that for myricetin. Obviously, the mixture (MIX-7) exhibited properties observed when myricetin was used alone, which also confirmed the assumption for the regenerating of myricetin by α-tocopherol.

4. Conclusion

The presented results support a synergistic effect of myricetin in combination with α-tocopherol. The analysis of the kinetic data obtained demonstrates that α-tocopherol regenerates the highly efficient and strong antioxidant myricetin, during autoxidation of TGSO. Therefore, the simultaneous presence of these antioxidants produces an increase of their protective effect.

Acknowledgement

The authors are grateful to the National Council for Scientific Research in Bulgaria for the partial financial support under Contract TK-X-1610.

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