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Activity of Natural Antioxidants on Lipids

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ABSTRACT

The kinetic behaviour of derivatives of benzoic and cinnamic acids, α-tocopherol, ascorbyl palmitate, flavonoids, coumarins, carnosol, thymol, carvacrol, resveratrol and carotenoids in lipid oxidation were studied. Most of the experiments were carried out with kinetically pure triacylglycerols or methyl esters of fatty acids. Some of the investigations were performed with fats and oils without purification in view to get information for the practice concerning the possibility for stabilization of real lipid systems. A new general parameter, activity A, for complex estimation of the effect of the antioxidants in lipids is proposed. It unifies the effectiveness of an inhibitor in termination of the autoxidation chain, on the one hand, and its ability to change the oxidation rate during the induction period, on the other. The analysis of the kinetic data obtained allowed the participation of the antioxidants in the side reactions of inhibited oxidation to be discussed. The extracts of different Bulgarian plant materials with solvents of various polarity were studied: leaves from *Rosemary officinalis* L., bark from *Fraxinus ornus* L., selected spieces of the family Lamiaceae, used as spices in Bulgaria, e.g. *Melissa officinalis* L., *Mentha piperita* L., *Mentha spicata* L., *Ocimum basilicum* L., *Origanum vulgare* L., and *Saturejae hortensis* L. Propolis, algae *Scenedesmus acutus*, *Silibum marianum* seed oil, *Capsicum annum* L. were also examined. The participation of carotenoids in the oxidation process differs from that of phenolic antioxidants. Our study on sunflower oil oxidation showed that in an antioxidant-free lipid system, the presence of carotenoids did not show any antioxidative protection, whereas in the presence of tocopherols and under light a synergism occurred.

Keywords: antioxidants, lipid oxidation, activity, kinetic analysis

Abbreviations: A, activity; **AH**, antioxidant; **F**, factor of stabilization (effectiveness); **IP**, induction period; **MEL**, methyl esters of lard; **MEOO**, methyl esters of olive oil; **MESO**, methyl esters of sunflower oil; **ORR**, oxidation rate ratio; **PV**, peroxide value; **TGL**, triacylglycerols of lard; **TGOO**, triacylglycerols of olive oil; **TGSBO**, triacylglycerols of soybean oil; **TGSO**, triacylglycerols of sunflower oil

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INTRODUCTION

Lipid oxidation occurring in food products is one of the major concerns in food technology. It is responsible for the rancid odours and flavors of the products, with a consequent decrease in nutritional quality and safety caused by the formation of secondary, potentially toxic compounds (St. Angelo 1996). Lipid oxidation products might also initiate the oxidative chain process in human constitution. The latter is responsible for the progress of cancerogenesis, atherosclerosis, infaction, allergies, inflammatory bowel and other diseases (Rice-Evans and Burdon 1993; Gordon 1996). For these reasons the problem of increasing the oxidation stability of lipids by antioxidant addition is very important for human health; it is also economically important (Valenzuela and Nieto 1996; Pokorny 1999; Yanishlieva-Maslarova 2001).

PRINCIPLES OF LIPID AUTOXIDATION

Lipids occur in almost all foodstuffs, and most of them are in the form of triacylglycerols, which are esters of fatty acids and glycerol. The two major components involved in lipid oxidation are unsaturated fatty acids and oxygen. Molecular oxygen behaves as a biradical by having two unpaired electrons in the ground state and it is said to be in triplet state. The free radical chain mechanism of autoxidation can be described by the reactions of non-inhibited oxidation presented in **Scheme 1**, where LH is the oxidizing lipid substrate, $LOO^\bullet$ is the peroxyl radical.

The primary oxidation products, the hydroperoxides LOOH, are odorless and tasteless. They are initators of the

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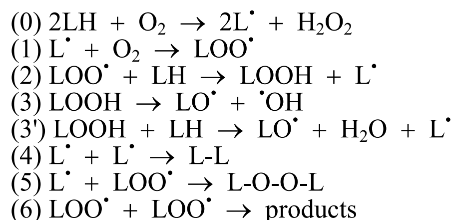
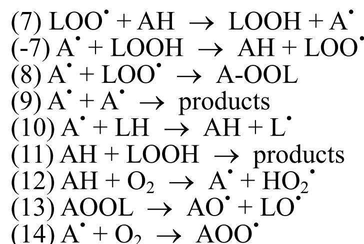
Invited Review



Scheme 1. Non-inhibited autoxidation.



Scheme 2. Inhibited autoxidation.

oxidative chains through decomposition to free radicals [reactions (3) and (3')]. As a result of further oxidation and cleavage of the hydroperoxide molecules, low molecular products of rancidity, e.g. aldehydes, ketones, acids, alcohols, esters, furans, lactones are formed. These products may further react in the organism with functional groups of proteins or DNA (Stahl 2000).

The only products formed during the initial stage of lipid oxidation in a kinetic regime (a sufficiently high oxygen concentration when the diffusion of oxygen does not influence the process rate) are the hydroperoxides (Popov and Yanishlieva 1976), and the kinetics of their accumulation is indicative of autoxidation kinetics. In a kinetic regime of oxidation reproducible results are achieved in view interpretation of the kinetic behaviour of the unsaturated lipids during autoxidation. The kinetic regime can be ensured by blowing oxygen or air through the samples, or by performing the process in thin layers (1 mm) (Yanishlieva *et al*. 1999).

Many years our research group has been working on the autoxidation and stabilization of lipids. The mechanism and kinetics of autoxidation of important lipid components such as fatty acids in form of methyl and glyceryl esters (Popov and Yanishlieva 1969, 1970; Yanishlieva and Popov 1971a, 1971b, 1971c, 1973b; Yanishlieva 1973a; Yanishlieva-Maslarova 1985), sitosterol (free and esterified) (Yanishlieva-Maslarova *et al*. 1982; Yanishlieva *et al*. 1985b) and various alkoxy lipids (alkylglycerols) (Yanishlieva-Maslarova 1983) were studied with a view to elucidating the possibilities for optimal stabilization of different lipid substrates.

STABILIZATION OF LIPIDS WITH NATURAL ANTIOXIDANTS

Some toxicological studies (Lindenschmidt *et al*. 1986; Shahidi and Wanasundara 1992; Kahl and Kappus 1993) have implicated the widely used synthetic inhibitors butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA) in promoting the development of cancerous cells in rats. These findings together with consumer interest to natural food additives have reinforced the interest in natural antioxidants (Pokorny 1991; Evans and Reynhout 1992; Angelo 1996), and in particular, in herbs and spices as harmless sources for obtaining natural antioxidants (Gordon and Weng 1992; Kikuzaki and Nakatani 1993; Kim *et al*. 1994; Cuvelier *et al*. 1994; Yanishlieva *et al*. 2006).

To obtain an objective information about the activity and mechanism of action of the antioxidants we have carried out the experiments in a kinetic regime of oxidation and with kinetically pure triacylglycerols or methyl esters of fatty acids, e.g. the lipid substrates were previously freed from pro- and antioxidative microcomponents by adsorption chromatography (Popov *et al*. 1968; Yanishlieva and Marinova 1995a) to avoid their participation in and contribution to the autoxidation process. The following kinetically pure lipid systems were used: triacylglycerols and methyl esters of lard (TGL and MEL), triacylglycerols and methyl esters of olive oil (TGOO and MEOO), triacylglycerols and methyl esters of sunflower oil (TGSO and MESO), and triacylglycerols of soybean oil (TGSBO). Some of the investigations were performed with fats and oils without purification (Yanishlieva and Marinova 1996a; Marinova and Yanishlieva 1997; Yanishlieva *et al*. 1997, 2001a, 2001b) to get information for the practice concerning the possibility for stabilization of the real lipid systems.

Our investigations on antioxidants for lipid stabilization started 30 years ago. The antioxidative action of butylated hydroxyanisol BHA **1**, butylated hydroxytoluene BHT **2**, hydroquinone **3**, propyl gallate **4** and quercetin **5** in various lard samples (Yanishlieva and Popov 1974), of α-tocopherol **6** and α-tocotrienol **7** in MESO (Yanishlieva-Maslarova *et al*. 1977) was studied (**Fig. 1**).

Different extracts and individual compounds from natural sources, e.g. broad beans (Yanishlieva *et al*. 1983), propolis (Yanishlieva *et al*. 1984; Marinova *et al*. 1989), algae *Scenedesmus acutus* (Yanishlieva and Marinova 1985), *Silibum marianum* seed oil (Yanishlieva *et al*. 1985a), *Capsicum annum* L. (red pepper) (Yanishlieva and Marinova 1986) were also investigated. A thin layer chromatographic method for rapid determination of antioxidants in mixtures has been proposed (Marinova and Yanishlieva 1986).

Recently our research is directed to elucidate the dependence of antioxidant activity of different natural antioxidants on their structure (Yanishlieva and Marinova 1992; Marinova and Yanishlieva 1992a, 1992b; Marinova *et al*. 1994; Marinova and Yanishlieva 1994a, 1994b; Yanishlieva and Marinova 1996b; Marinova and Yanishlieva 1998; Yanishlieva *et al*. 1999; Marinova and Yanishlieva 2003; Yanishlieva and Marinova 2006), on the type of the lipid system being oxidized (Marinova and Yanishlieva 1992c, 1994a; Marinova *et al*. 1994, Marinova and Yanishlieva 1996; Marinova *et al*. 2002, 2004a, 2006), on temperature (Marinova and Yanishlieva 1992a, 1992c; Yanishlieva and Marinova 1996a; Marinova and Yanishlieva 1998), and on binding of the fatty acids to the natural triacylglycerols (Yanishlieva and Marinova 1995a; Marinova and Yanishlieva 1996).

The following antioxidants were studied at different concentration levels (**Fig. 1**): α-tocopherol (Marinova and Yanishlieva 1992a, 1998; Yanishlieva and Marinova 1996a; Yanishlieva *et al*. 1994, 2002), ascorbyl palmitate **8** (Marinova and Yanishlieva 1992c), *p*-coumaric **9**, ferulic **10**, caffeic **11**, and sinapic **12** acids (Marinova and Yanishlieva 1992a, 1992b, 1994a; Yanishlieva and Marinova 1995a; Marinova and Yanishlieva 1996; Yanishlieva and Marinova 1996a; Yanishlieva *et al*. 2005; Marinova *et al*. 2006), 3,4-dihydroxybenzoic **13**, vanillic **14** and syringic **15** acids (Marinova and Yanishlieva 1992b, 1994b; Yanishlieva and Marinova 1995a, 1996a), carnosol **16** (Marinova *et al*. 1991), esculetin **17** (Marinova *et al*. 1994; Yanishlieva and Marinova 1996a), esculin **18** (Marinova *et al*. 1994), fraxetin **19** (Marinova *et al*. 1994; Yanishlieva and Marinova 1996a), fraxin **20** (Marinova *et al*. 1994), quercetin and morin **21** (Yanishlieva and Marinova 1996b; Marinova and Yanishlieva 1998; Yanishlieva and Marinova 2006), 3,4-dihydroxyphenylacetic acid **22** (Yanishlieva *et al*. 1998), thymol **23** and carvacrol **24** (Yanishlieva *et al*. 1999; Yanishlieva and Marinova 2006), β-carotene **25** (Yanishlieva *et al*. 2001a), β-apo-8'-carotenoic acid **26** and its esters (Yanishlieva *et al*. 2001b), *trans*-resveratrol **27** (Marinova *et al*. 2002).

The synergism between resveratrol, caffeic acid, quercetin and α-tocopherol during lipid oxidation was studied (Marinova *et al*. 2004b). The influence of cholesterol on the antioxidative properties of α-tocopherol and quercetin in

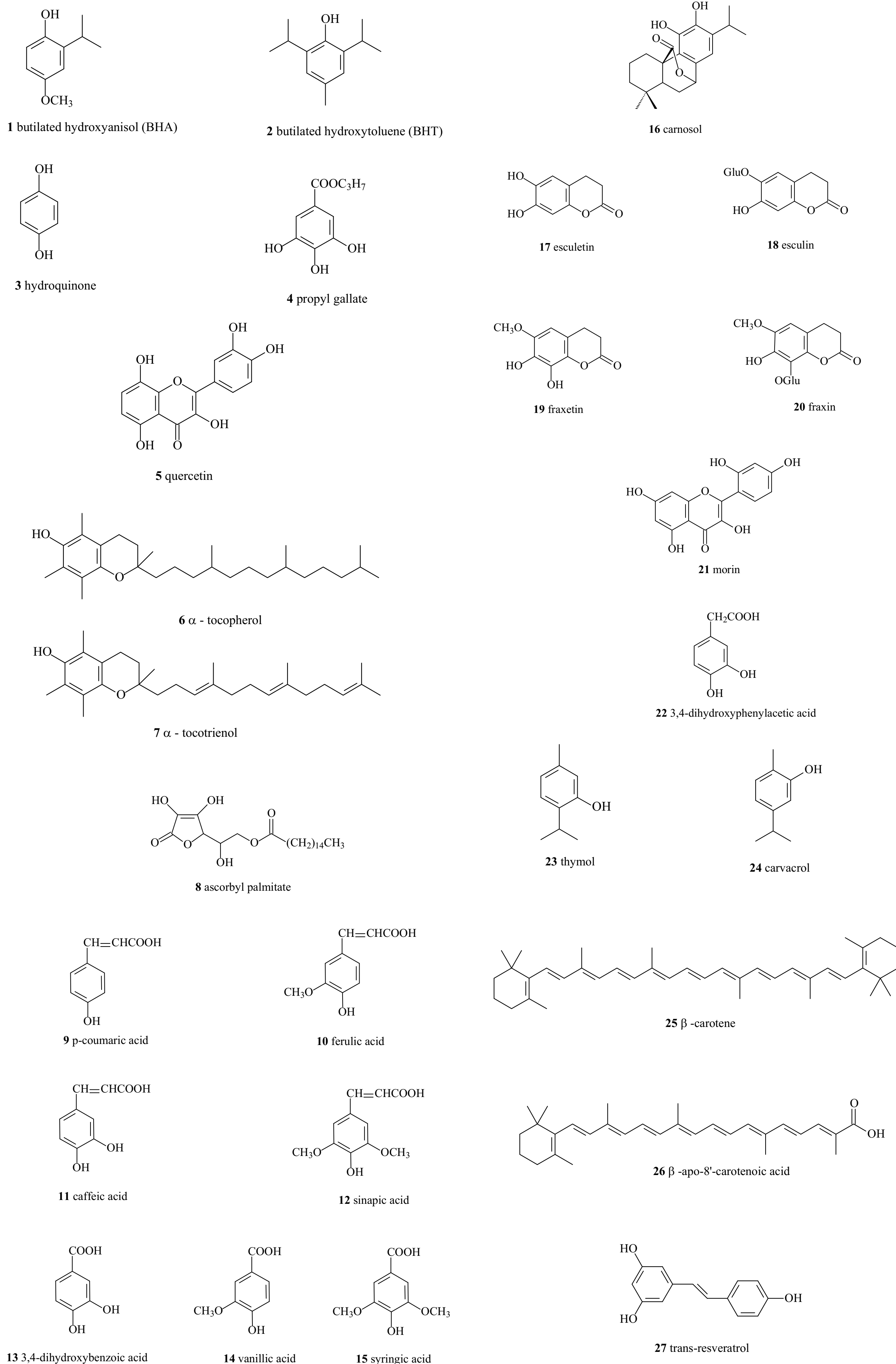


Fig. 1 Structures of the investigated antioxidants.

TGSO autoxidation was discussed (Marinova *et al*. 2005). The effect of a fatty alcohol (1-octadecanol) on the oxidation stability of TGL in the presence of cinnamic acid derivatives (ferulic, sinapic and caffeic acids) was investigated (Kortenska-Kancheva *et al*. 2005)

Our efforts were concentrated also on searching of plant sources from Bulgarian origin for obtaining harmless antioxidants for lipids: leaves from *Rosemary officinalis* L. (rosemary) (Marinova *et al*. 1991), barks from *Fraxinus ornus* L.(ash) (Marinova *et al*. 1994), selected species of the family Lamiaceae grown in Bulgaria and used as spices, e.g. *Melissa officinalis* L. (common balm), *Mentha piperita* L. (peppermint), *Mentha spicata* L. (spearmint), *Ocimum basilicum* L. (common basil), *Origanum vulgare* L. (oregano), and *Saturejeae hortensis* L. (summer savory) (Yanishlieva and Marinova 1995b; Marinova and Yanishlieva 1997; Yanishlieva *et al*. 1997). The antioxidant activity of *Smilax excelsa* (sarsaparilla) rhizomes in TGL and TGSO was also studied (Ivanova *et al*. 2006).

Oxidation experiments were performed at different temperatures. Oxidation at 90 and 100°C was carried out by blowing air through the samples (2 g) in the dark at a rate of 100 ml/min. Oxidation at 25, 50 and 75°C was performed in the dark using a 1 mm layer in Petri dishes. Under the above 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 the primary oxidation products (peroxide) concentration, i.e. the peroxide value (PV). During the initial stage of the process, the rate of peroxide accumulation was equal to the oxidation rate (Popov and Yanishlieva 1976). Kinetic curves of peroxide accumulation were plotted. The effectiveness of the antioxidants was estimated on the basis of the induction period (IP), determined by the method of tangents to the two parts of the kinetic curve. The rates of non-inhibited Wo (control sample) and inhibited Winh oxidation were derived from the tangents applied to those parts of the kinetic curves of peroxide accumulation which represent the initial phase.

GENERAL PRINCIPLES OF INHIBITED OXIDATION

The free radical chain process of autoxidation can be retarded by two categories of inhibitors: chain-breaking inhibitors (or antioxidants AH) and preventive inhibitors (Yanishlieva-Maslarova 2001).

The introduction of an antioxidant AH into the oxidizing system leads to a change in the mechanism and kinetics of the process (Denisov and Khudyakov 1987) (compare **Scheme 1** for non-inhibited oxidation with **Scheme 2** for inhibited oxidation).

With a kinetic regime of oxidation, the system being oxidized contains no short-lived radicals $L^\bullet$, and the termination proceeds according to reaction (6) (**Scheme 1**) and/ or reactions (7) and (8) (**Scheme 2**). It has been found that the effect of the antioxidant depends on the participation of its molecules and radicals formed from the latter in a series of reactions presented in **Scheme 2**. (Denisov and Khudyakov 1987; Roginski 1990) The probability of reactions (7) - (14) taking place depends not only on the inhibitor structure but also on the type and degree of lipid unsaturation, on antioxidant concentration, on temperature, on binding the fatty acids to triacylglycerols, on the participation of different microcomponents (present or added to the lipid systems) in the oxidation process (Kortenska *et al*. 1991; Kortenska and Yanishlieva 1995).

The main type of lipid unsaturation is presented by monoenic fatty acid moieties, e.g. oleate, and fatty acids with two or more methylene interrupted double bonds, e.g. linoleate (two double bonds). By interpretation of the kinetic results one should take into consideration that the oxidation of linoleate is 10 times easier than that of oleate (Gunstone and Hilditch 1945; Stirton *et al*. 1945), and that the linoleate peroxyl radicals react several times faster than the oleate peroxyl radicals (Yanishlieva *et al*. 1970). Besides, the oleate hydroperoxides are much more stable than the linoleate ones (Yanishlieva 1973b). It is also established that both the linoleate and oleate moieties in triacylglycerols and methyl esters of lard and olive oil are oxidized during the initial stage of autoxidation, whereas in the case of triacylglycerols and methyl esters of sunflower oil the oxygen and the peroxyl radicals attack the linoleate units alone (Yanishlieva and Popov 1973a). That is why LH, $LOO^\bullet$ and LOOH in the different lipid systems have different compositions and reactivities, that may strongly influence the kinetic behaviour of the antioxidants in the various lipid substrates.

Irrespective of the fact that the reactions where the inhibitor moieties participate can be many in number, the mechanism of the process is determined only by some of them. Depending on the structure of the antioxidant, on the oxidizing substrate, and on the oxidation conditions, different side reactions can play the major role in the process. The most widely used antioxidants in foods are able to compete with the substrate for the chain-carrying species normally present in highest concentration in the system, the peroxyl radical $LOO^\bullet$, reaction (7) in **Scheme 2**. The efficient inhibitors are well known to terminate free-radical chain oxidation by trapping two peroxyl radicals according to reactions (7) and (8). The stoichiometric inhibition factor f (the number of kinetic chains broken per molecule of antioxidant) is normally two (Scott 1985).

KINETIC PARAMETERS OF INHIBITED OXIDATION

The antioxidative (inhibiting) action can be described by two kinetic characteristics (Yanishlieva and Marinova 1992):

Effectiveness, representing the possibility of blocking the radical chain process by interaction with the peroxyl radicals [reaction (7)], which is responsible for the duration of the induction period IP, and

Strength, expressing the possibility for the inhibitor moieties to participate in other reactions, e.g. (-7), (8), (9), (10), (11), (12), (13), (14), which leads to a change of the oxidation rate during the IP.

A measure of the effectiveness is the stabilization factor F:

$$F = IP_{inh}/IP_o \quad \text{(I)}$$

where IP_{inh} is the induction period in the presence of an inhibitor, and IP_o is the induction period of the non-inhibited system. Usually, IP_{inh} continues until the antioxidant has been destroyed.

The oxidation rate ratio ORR is a measure of the strength:

$$ORR = W_{inh}/W_o \quad \text{(II)}$$

where W_{inh} is the oxidation rate in the presence of an inhibitor, and W_o is the oxidation rate of the non-inhibited system.

The IP was determined by the methods of the tangents to the two parts of the kinetic curves (Yanishlieva and Popov 1971c; le Tutour and Guedon 1992). The rates of non-inhibited (W_o) and inhibited (W_{inh}) oxidation were found from the tangents to the initial phase of the kinetic curves and expressed as $M\ s^{-1}$.

When ORR is larger than 1, the oxidation proceeds faster in the presence of an inhibitor than in its absence; this is observed at high tocopherol concentrations (Marinova and Yanishlieva 1992a).

Fig. 2 illustrates, by way of an example, the kinetic curves of peroxide accumulation during inhibited oxidation of TGL in presence of equal molar concentrations of α-tocopherol and ferulic acid at 100°C and 25°C (Marinova and Yanishlieva 1992a). The results presented in **Fig. 2**

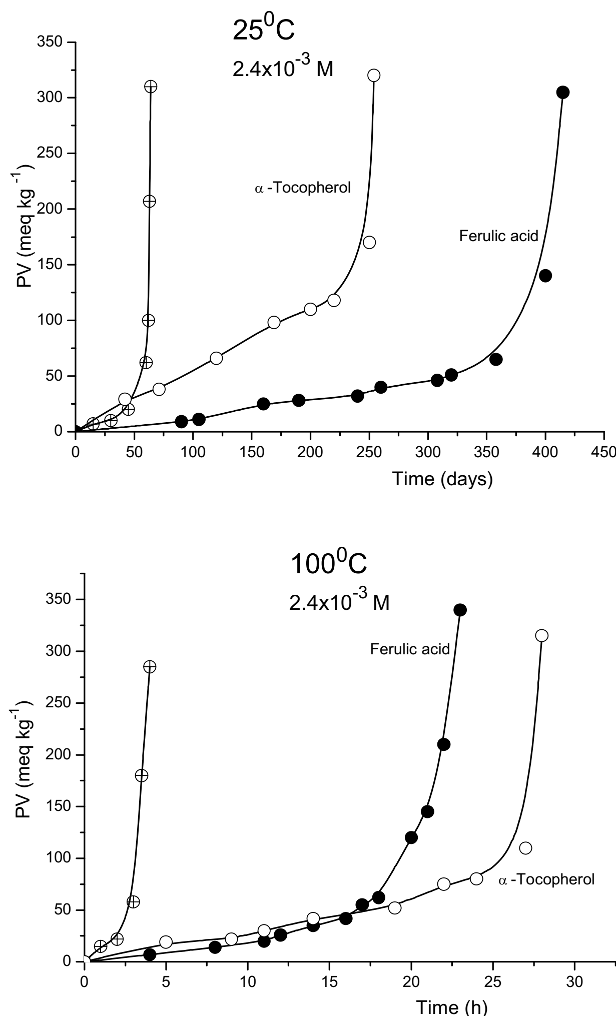


Fig. 2 Kinetic curves of peroxide accumulation during inhibited oxidation of TGL in the presence of 2.4 x 10^{-3} M α-tocopherol and ferulic acid at 100°C and 25°C. The curves without number present non-inhibited oxidation of TGL at the same temperatures (Marinova and Yanishlieva 1992a).

show that the variation of temperature changes the order of the antioxidant effectiveness – at 25°C ferulic acid is more effective, and at 100°C α-tocopherol exhibits a higher effectiveness. Moreover, in the presence of α-tocopherol at 25°C the oxidation rate during the IP is higher than is the case of non-inhibited system, which is not observed at 100°C.

Taking into account the complicated changes in the kinetic parameters of inhibited oxidation and the fact that the estimation of the antioxidative effect on the basis of IP or of the process rate may lead in many cases to different results we proposed a general kinetic parameter antioxidant activity A (Yanishlieva and Marinova 1992). This parameter unifies the effectiveness of an inhibitor in termination of the autoxidation chain, on one hand, and its ability to decrease the oxidation rate during the IP, on the other:

$$A = F/ORR \qquad (III)$$

Table 1 presents some data obtained for the general kinetic parameter antioxidant activity, A, during oxidation of various lipid (triacylglycerol) systems in the presence of different antioxidants.

PARTICIPATION IN SIDE REACTIONS

If the antioxidant participates in chain termination only, the stabilization factor F increases linearly with concentration (**Fig. 3A**), and the mean rate of inhibitor consumption W_{InH} is given by the formula $W_{InH} = W_i/f$ (Emanuel *et al.* 1965). With some of the antioxidants studied we have observed a nonlinear dependence of F on the antioxidant concentration (**Fig. 3B**).

The absence of linearity of the dependences is due to the participation of the inhibitor molecules in reactions other than the main reaction (7) of chain termination, namely reaction (11) or/and (12). In this case there is a relationship between the mean rate of inhibitor consumption W_{InH} and the inhibitor concentration [AH] (Emanuel *et al.* 1965):

$$W_{InH} = W_i/f + k_{eff}[AH]^n \qquad (IV)$$

where W_i is the mean rate of initiation during the IP (M s^{-1}), f is the stoichiometric coefficient of inhibition, and n is the number of side reactions, in which the antioxidant participates.

After processing of the kinetic curves the mean rates of inhibitor consumption W_{InH} were determined according to the formula (V):

$$W_{InH} = [AH]_o / IP, \; M\, s^{-1} \qquad (V)$$

Table 1 Antioxidative activity **A** of various antioxidants at concentration levels 0.02, 0.05 and 0.10% during oxidation of different lipid systems at 100°C.

Antioxidant	Lipid system	A			Reference
		0.02%	0.05%	0.10%	
α-Tocopherol	TGL	50.0	43.5	21.6	Yanishlieva and Marinova 1992
α-Tocopherol	TGSO	222	222	220	Yanishlieva *et al*. 2002
3,4-Dihydroxybenzoic acid	TGL	191	705	1477	Marinova and Yanishlieva 1992b
3,4-Dihydroxybenzoic acid	TGSO	6.0	11.6	17.6	Yanishlieva and Marinova 1995a
p-Coumaric acid	TGL	3.9	8.8	17.9	Marinova and Yanishlieva 1992b
p-Coumaric acid	TGOO	11.0	23.2	43.1	Marinova and Yanishlieva 1996
Ferulic acid	TGL	5.2	17.6	52	Marinova and Yanishlieva 1992b
Ferulic acid	TGOO	20.0	57.5	148	Marinova and Yanishlieva 1996
Ferulic acid	TGSO	4.3	5.3	7.8	Yanishlieva and Marinova 1995a
Sinapic acid	TGL	95	333	1015	Marinova and Yanishlieva 1992b
Sinapic acid	TGSO	28.1	34.8	48.1	Yanishlieva and Marinova 1995a
Caffeic acid	TGL	10350	20350	28917	Marinova and Yanishlieva 1992b
Caffeic acid	TGOO	4867	10182	29167	Marinova and Yanishlieva 1996
Caffeic acid	TGSO	448	900	1364	Yanishlieva and Marinova 1995a
Esculetin	TGL	712	1290	1462	Marinova *et al*. 1994
Esculetin	TGSO	231	627	824	Marinova *et al*. 1994
Fraxetin	TGL	2877	10400	34000	Marinova *et al*. 1994
Fraxetin	TGSO	302	1800	6250	Marinova *et al*. 1994
Resveratrol	TGL	3750	6675	10675	Marinova *et al*. 2002
Resveratrol	TGSO	79	119	178	Marinova *et al*. 2002

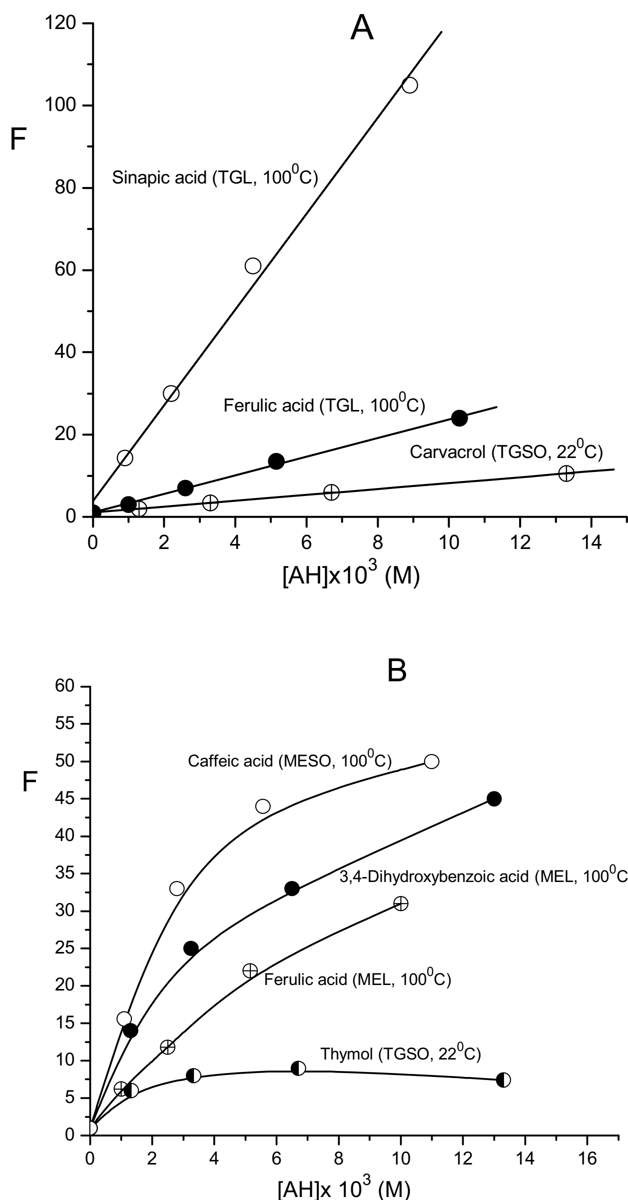


Fig. 3 Dependence of the stabilization factor F on the concentration of various antioxidants at different oxidation conditions. Adapted from Marinova and Yanishlieva (1992b, 1994b) and Yanishlieva *et al.* (1999).

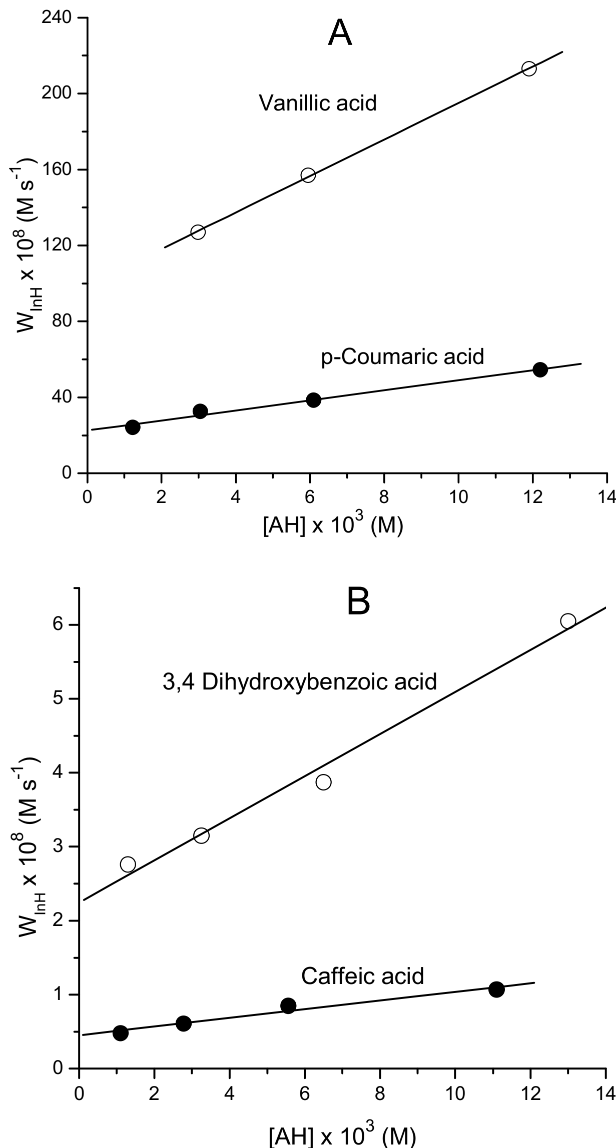


Fig. 4 Dependence of the rate of consumption, W_{InH}, of different phenolic acids on their concentration [AH]. Oxidation of TGL at 100°C (Marinova and Yanishlieva 1992b).

where $[AH]_o$ is the initial concentration of the antioxidant (M), and IP is the duration of the induction period(s).

The W_{InH} obtained for different initial concentration of the antioxidants were presented as dependence (IV) for different n. As an illustration, **Fig. 4** shows the dependences of W_{InH} of the concentration of vanillic, *p*-coumaric, 3,4-dihydroxybenzoic and caffeic acids (n=1).

The kinetic results showed that for most of the investigated antioxidants n=1 or n=0, e.g. their molecules participate in one side reaction, (11) or (12), or do not participate in such reactions. From the dependence (IV) the kinetic parameters W_i/f and K_{eff} were also found and discussed. W_i/f was determined by extrapolation to zero concentration of the antioxidant, and K_{eff} was obtained from the slope of the dependence (IV). In **Table 2** some of the obtained data for W_i/f and K_{eff} are given.

The consumption of the inhibitors according to the reaction of chain initiation (12) presupposes that K_{eff} should not depend on the character of the lipid medium, which is not the case (**Table 2**).

Therefore, the antioxidant molecules take part in side reactions with the hydroperoxides, reaction (11). This statement is confirmed by the different composition, and hence, different stability of the hydroperoxides formed during oxidation of various types of lipid substrates (Yanishlieva 1973b; Yanishlieva and Popov 1973a), previously discussed. This means that the rate constants of consumption of the inhibitors should be higher in TGSO than in TGL, which is demonstrated by the Keff values obtained (**Table 2**).

From **Table 2** it can be seen that ferulic and sinapic acids, and fraxetin in TGL oxidation at 100°C, as well as fraxetin in TGSO oxidation at 100°C, do not change the rate of their consumption with rising concentration (n=0), and K_{eff} = 0, respectively. The molecules of these antioxidants do not participate in side reactions at these oxidation conditions.

Previous research (Denisov and Khudyakov 1987) showed that if the antioxidant radical ($A^•$) participates in one reaction of chain propagation [reaction (-7), or (10), or (14)], the dependence (VI) is valid:

$$W_{InH} \approx [AH]^{-0.5} \quad \text{(VI)}$$

When $A^•$ does not participate in chain propagation, dependence (VII) is valid:

$$W_{InH} \approx [AH]^{-1} \quad \text{(VII)}$$

Table 2 Kinetic parameters K_{eff} and W_i/f determined for various antioxidants during oxidation of different lipid substrates at 100°C (Marinova and Yanishlieva 1992b; Marinova *et al.* 1994; Yanishlieva and Marinova 1995a; Marinova and Yanishlieva 1996; Marinova *et al.* 2002; Yanishlieva *et al.* 2002).

Antioxidant	K_{eff} (s^{-1})			W_i/f ($M\ s^{-1}$)		
	TGL	TGOO	TGSO	TGL	TGOO	TGSO
Caffeic acid	7.0×10^{-7}	7.0×10^{-7}	8.2×10^{-6}	0.04×10^{-7}	0.27×10^{-8}	0.1×10^{-7}
Ferulic acid	0	3.8×10^{-6}	10.6×10^{-5}	1.2×10^{-7}	0.40×10^{-7}	3.0×10^{-7}
p-Coumaric acid	2.6×10^{-5}	2.1×10^{-5}	-	2.5×10^{-7}	0.98×10^{-7}	-
Sinapic acid	0	-	3.2×10^{-5}	0.2×10^{-7}	-	0.6×10^{-7}
Esculetin	2.5×10^{-6}	-	2.0×10^{-5}	0.05×10^{-7}	-	0.35×10^{-7}
3,4-Dihydroxy-benzoic acid	2.8×10^{-6}	-	7.3×10^{-5}	0.20×10^{-7}	-	1.5×10^{-7}
Fraxetin	0	-	0	0.03×10^{-7}	-	0.41×10^{-7}
α-Tocopherol	0.98×10^{-5}	-	1.5×10^{-5}	0.35×10^{-8}	-	0.15×10^{-7}
Resveratrol	2.3×10^{-6}	-	3.4×10^{-5}	0.14×10^{-7}	-	0.9×10^{-7}

Table 3 Antioxidative activity **A** of some phenolic acids during oxidation of different lipid substrates at 100°C (Marinova and Yanishlieva 1992b, 1994a, 1994b, 1996; Yanishlieva and Marinova 1996).

Antioxidant	Concentration	A					
		TGL	MEL	TGSO	MESO	TGOO	MEOO
3,4-Dihydroxy-benzoic acid	1.30×10^{-3} M (0.02%)	191	55	6.0	2.0	-	-
	3.25×10^{-3} M (0.05%)	705	210	11.6	3.7	-	-
	6.49×10^{-3} M (0.10%)	1477	412	17.6	6.8	-	-
Ferulic acid	1.03×10^{-3} M (0.02%)	5.2	11.1	4.3	1.0	20.0	42.0
	2.53×10^{-3} M (0.05%)	17.6	35.7	5.3	1.7	58	150
	5.15×10^{-3} M (0.10%)	52	100	7.8	2.2	149	322
Sinapic acid	0.89×10^{-3} M (0.02%)	95	103	28.1	3.4	-	-
	2.23×10^{-3} M (0.05%)	333	315	34.8	7.3	-	-
	4.46×10^{-3} M (0.10%)	1015	588	48.1	14.2	-	-
Caffeic acid	11.10×10^{-3} M (0.02%)	10350	2652	448	74	4867	7786
	2.78×10^{-3} M (0.05%)	20350	6444	900	237	10182	17000
	5.56×10^{-3} M (0.10%)	28917	9500	1364	400	29167	25600

The W_{InH} values for vanillic acid during oxidation (100°C) of MEL and for ferulic acid during oxidation (100°C) of MESO showed no linear dependence on either $[AH]^{-0.5}$ or $[AH]^{-1}$ (Marinova and Yanishlieva 1994b), which indicated that the radicals of these phenolic acids were involved in more than one reaction of chain propagation. The same was true for α-tocopherol in TGL oxidation (Marinova and Yanishlieva 1992a), as well as for α- and γ-tocopherols at higher concentrations in TGSO and TGSBO oxidation (Yanishlieva *et al.* 2002; Marinova *et al.* 2004a).

It has been established that the radical of esculetin did not participate in chain propagation during oxidation of TGL and TGSO, and the radical of fraxetin did not participate in chain propagation during TGL oxidation (Marinova *et al.* 1994). On the other hand, the radical of fraxetin took part in one reaction of chain propagation in TGSO oxidation (Marinova *et al.* 1994). The same was true for 3,4-dihydroxybenzoic and caffeic acids in TGSO (Yanishlieva and Marinova 1995a), for *p*-coumaric, ferulic and caffeic acids in TGOO and MEOO (Marinova and Yanishlieva 1996), and for vanillic, *p*-coumaric, ferulic, syringic and 3,4-dihydroxybenzoic acid in TGL (Marinova and Yanishlieva 1992b). The interpretation of the kinetic results obtained for the oxidation of different lipid substrates in presence of the antioxidants studied allowed the assumption that this reaction should be reaction (10) (Marinova and Yanishlieva 1994b; Marinova *et al.* 1994; Yanishlieva and Marinova 1995a).

INFLUENCE OF THE LIPID SYSTEM ON THE ANTIOXIDATIVE ACTION

As can be seen from **Table 1**, all the antioxidants studied, with the exception of α-tocopherol, show lower activity in TGSO than in the more saturated lipid system TGL. Moreover, *p*-coumaric and ferulic acids are more active antioxidants in TGOO than in TGL.

It has been found that in the concentration range 0.02-0.10% thymol possessed higher activity in TGSO than in TGL at 22°C, whereas the opposite was true for carvacrol (Yanishlieva *et al.* 1999). We have also established that α- and γ-tocopherols at levels 0.005-0.20% were more active antioxidants in TGSBO than in TGSO at 100°C (Yanishlieva *et al.* 2002).

The antioxidative action of some phenolic acids in triacylglycerols and methyl esters of sunflower and olive oils and lard at 100°C is compared in **Table 3**. These results illustrate that the activity of the antioxidants is in most cases higher in TGL and TGSO than in MEL and MESO, respectively, indicating that the binding of the fatty acids to the triacylglycerol structure offers a greater stabilizing effect by the antioxidants. The opposite is true for TGOO and MEOO. This result allow the assumption that the oleate moiety plays a specific role with respect to the antioxidative stability of lipids (Marinova and Yanishlieva 1994a), which should be examined in connection with the triacylglycerol structure of the olive oil.

The influence of the lipid substrate on the kinetic parameters W_i/f and K_{eff} is presented in **Table 2**. It can be seen that in TGL W_i/f and K_{eff} have lower values than in TGSO. **Table 4** summarizes the data for the antioxidant activity and mechanism of action of some of the investigated antioxidants in different lipid substrates at 100°C.

It has been also established that the effectiveness of the antioxidants differed depending on whether the process took part in a bulk phase or in a liposome bilayer (Yanishlieva *et al.* 1994). It was found that the effectiveness of α-tocopherol and caffeic acid was considerably lower in the case of liposome oxidation. In addition, the sequence of effectiveness of α-tocopherol and caffeic acid was reversed when passing from bulk phase oxidation to liposome oxidation. In the first case, caffeic acid was twice as effective as α-tocopherol, whereas in the second case, α-tocopherol was 2.5 times more effective than caffeic acid.

The inhibiting effect of the added antioxidants depends also on the presence of other antioxidants or prooxidants in the lipid systems being stabilized. A comparison of the data

Table 4 Antioxidative activity **A** and mechanism of action of some of the investigated antioxidants at 100°C (Marinova *et al.* 1991, 1994; Marinova and Yanishlieva 1992b, 1996; Yanishlieva and Marinova 1995a).

Antioxidant	Concentration interval	Conditions	A (range)	Participation in side reactions of autoxidation
Caffeic acid	$1.1\text{-}11.1 \times 10^{-3}$ M (0.02-0.20%)	TGL	10350-45900	Molecules are consumed in one side reaction (11) $K_{eff} = 7.0 \times 10^{-7}\ s^{-1}$ $W_i/f = 0.04 \times 10^{-7}\ Ms^{-1}$
Fraxetin	$0.5\text{-}4.8 \times 10^{-3}$ M (0.01-0.10%)	TGL	764-34000	Molecules are not consumed in side reactions $W_i/f = 0.03 \times 10^{-7}\ Ms^{-1}$ Radicals do not participate in chain propagation
3,4-Dihydroxy-benzoic acid	$1.3\text{-}13.0 \times 10^{-3}$ M (0.02-0.20%)	TGL	191-2890	Molecules are consumed in one side reaction (11) $K_{eff} = 2.8 \times 10^{-6}\ s^{-1}$ $W_i/f = 0.2 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)
Carnosol	$0.3\text{-}6.1 \times 10^{-3}$ M (0.01-0.20%)	TGL	590-1643	Molecules are consumed in one side reaction (11) $K_{eff} = 3.6 \times 10^{-6}\ s^{-1}$ $W_i/f = 0.03 \times 10^{-7}\ Ms^{-1}$
Sinapic acid	$0.9\text{-}8.9 \times 10^{-3}$ M (0.02-0.20%)	TGL	95-2617	Molecules are not consumed in side reactions $W_i/f = 0.2 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)
Esculetin	$0.6\text{-}5.6 \times 10^{-3}$ M (0.01-0.10%)	TGL	324-1462	Molecules are consumed in one side reaction (11) $K_{eff} = 2.5 \times 10^{-6}\ s^{-1}$ $W_i/f = 0.05 \times 10^{-7}\ Ms^{-1}$ Radicals do not participate in chain propagation
p-Coumaric acid	$1.2\text{-}12.2 \times 10^{-3}$ M (0.02-0.20%)	TGOO	11.0-62	Molecules are consumed in one side reaction (11) $K_{eff} = 2.14 \times 10^{-5}\ s^{-1}$ $W_i/f = 0.98 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)
Ferulic acid	$1.0\text{-}10.3 \times 10^{-3}$ M (0.02-0.20%)	TGOO	20.0-296	Molecules are consumed in one side reaction (11) $K_{eff} = 0.38 \times 10^{-5}\ s^{-1}$ $W_i/f = 0.40 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)
Caffeic acid	$1.1\text{-}11.1 \times 10^{-3}$ M (0.02-0.20%)	TGOO	4867-37833	Molecules are consumed in one side reaction (11) $K_{eff} = 0.07 \times 10^{-5}\ s^{-1}$ $W_i/f = 0.027 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)
3,4-Dihydroxy-benzoic acid	$1.3\text{-}13.0 \times 10^{-3}$ M (0.02-0.20%)	TGSO	6.0-17.6	Molecules are consumed in one side reaction (11) $K_{eff} = 7.30 \times 10^{-5}\ s^{-1}$ $W_i/f = 1.5 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)
Ferulic acid	$1.0\text{-}10.3 \times 10^{-3}$ M (0.02-0.20%)	TGSO	4.3-9.0	Molecules are consumed in one side reaction (11) $K_{eff} = 10.6 \times 10^{-5}\ s^{-1}$ $W_i/f = 3.0 \times 10^{-7}\ M\ s^{-1}$ The radicals are involved in more than one reaction of chain propagation
Sinapic acid	$0.89\text{-}8.93 \times 10^{-3}$ M (0.02-0.20%)	TGSO	28.1-448	Molecules are consumed in one side reaction (11) $K_{eff} = 3.2 \times 10^{-5}\ s^{-1}$ $W_i/f = 0.6 \times 10^{-7}\ Ms^{-1}$ The radicals are involved in more than one reaction of chain propagation
Caffeic acid	$1.1\text{-}11.1 \times 10^{-3}$ M (0.02-0.20%)	TGSO	448-1463	Molecules are consumed in one side reaction (11) $K_{eff} = 0.82 \times 10^{-5}\ s^{-1}$ $W_i/f = 0.1 \times 10^{-7}\ Ms^{-1}$ Radicals participate in one side reaction of chain propagation (10)

for F of different extracts from some species of the familiy Lamiaceae during oxidation of sunflower oil at 100°C (Marinova and Yanishlieva 1997) with F of the extracts in TGSO oxidation (Yanishlieva and Marinova 1995b) shows that the natural sunflower oil is much more difficult to stabilize than are its pure triacylglycerols. The tocopherol concentration in sunflower oil is close to the optimal concentration required for its stabilization, which explains the effect observed. The same effect was established with other antioxidants studied in TGSO and sunflower oil oxidation, e.g. caffeic acid, esculetin and fraxetin (Yanishlieva and Marinova 1996a).

It has been found that the effectiveness, strength and activity of α-tocopherol were greater in cholesterol containing TGSO than in pure TGSO, whereas these parameters for quercetin were practically the same in both lipid systems (Marinova *et al*. 2005).

The kinetic behaviour of β-carotene (Yanishlieva *et al*. 2001a) and β-apo-8'-carotenoic acid and its esters (Yanishlieva *et al*. 2001b) in lipid oxidation differs from that of phenolic antioxidants. The carotenoids at concentrations 0.001-0.02% did not show any antioxidative effect during oxidation of TGSO at room temperature, whereas they increased the stability of tocopherol-containing sunflower oil in day light. The synergism between the carotenoids and tocopherols was also discussed (Yanishlieva *et al*. 2001a, 2001b).

INFLUENCE OF TEMPERATURE ON THE ANTIOXIDATIVE ACTION

Very often the oxidation stability of fats and oils, as well as the antioxidative action of the inhibitors in different lipid systems were estimated by accelerated methods performed at high temperatures. The values for the oxidation stability thus obtained cannot always be used for quantitative and

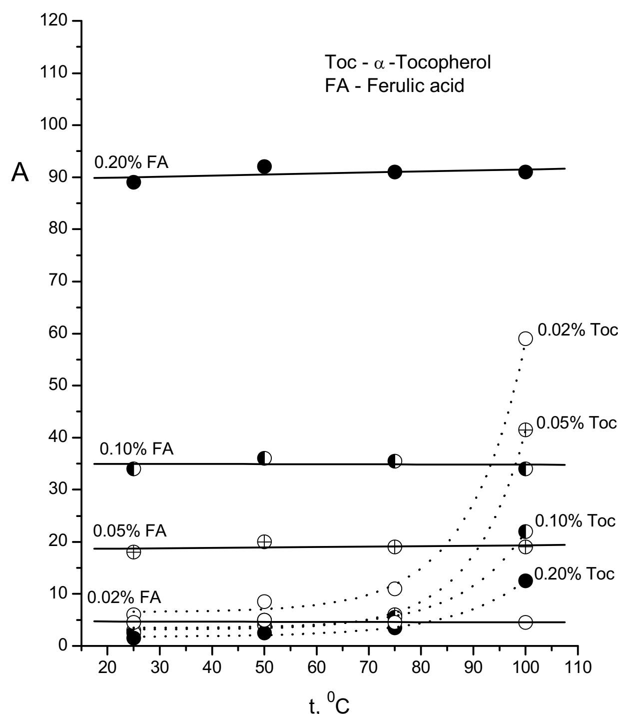


Fig. 5 Activity A for various concentrations of α-tocopherol and ferulic acid during oxidation of TGL at different temperatures. Adapted from Marinova and Yanishlieva (1992a).

even for semi-quantitative estimation of this important storage characteristic of the lipids at ambient temperature.

We have examined the effect of temperature (25°C, 50°C, 75°C, and 100°C) on the antioxidative action of the wide-spread typical lipid antioxidant α-tocopherol, and ferulic acid, which is widely distributed in the plant kingdom, during oxidation of TGL (Marinova and Yanishlieva 1992a). After processing the obtained kinetic results the data for F and ORR were determined. It was established that F and ORR for ferulic acid did not depend on temperature, whereas F increased, and ORR decreased with increasing temperature for α-tocopherol. These results show that the change in temperature does not affect the activity of ferulic acid, and with rising temperature the activity of α-tocopherol increases (**Fig. 5**).

The results obtained allowed the following conclusion to be made (Marinova and Yanishlieva 1992a): a change of temperature does not affect the mechanism of action of ferulic acid; therefore its effectiveness and strength, i.e. activity, remain the same at different temperatures. With rising temperature both the effectiveness and the strength, i.e. activity, of α-tocopherol increase, which is due to the change in mechanism of its participation in the different reactions of inhibited oxidation. As it was established (Marinova and Yanishlieva 1992a), in the case of α-tocopherol the increase of temperature leads to a decrease in the contribution of reactions (-7), (10), (13), and (14). Thus, the results for the oxidative stability of lipids, obtained at high temperature, can be used for quantitative estimation of the stability at room temperature only when no change occurs in the mechanism of participation of the antioxidant and its radical in the reactions of inhibited oxidation.

We investigated the oxidation kinetics of TGL and TGSO, containing 0.05% α-tocopherol, in the presence of different concentrations of ascorbyl palmitate (AP) at 25°C and 100°C (Marinova and Yanishlieva 1992c). It has been established that the rise of temperature was associated with an increase in A of AP, which was more pronounced with the lipid system of lower oxidizability, e.g. TGL.

The influence of temperature on the antioxidative action of quercetin and morin in TGL (Yanishlieva and Marinova 1996b) and in TGSO (Marinova and Yanishlieva 1998) was also studied. At 22°C and in the concentration interval (2.2-8.9) × 10^{-4} M (0.0075-0.03%) the values of A for morin and quercetin in TGL did not differ significantly, whereas they differed at 90°C for both inhibitors by one order of magnitude (in morin's favour) (Yanishlieva and Marinova 1996b). Quercetin was a more active antioxidant than morin in TGSO at both temperatures. In addition, with rising temperature the activity of both antioxidants increased significantly (Yanishlieva and Marinova 1996b; Marinova and Yanishlieva 1998).

It was also established that the antioxidative activity of syringic and 3,4-dihydroxybenzoic acids in TGSO were practically the same at 22 and 90°C, whereas sinapic and caffeic acids showed a greater activity at 90°C than at ambient temperature (Marinova and Yanishlieva 2003).

The investigation of the antioxidative effect of the ethanol extract from *Saturejae hortensis* L. (summer savory) in lipids has shown that the effect of the additive was stronger at room temperature than at 100°C (Yanishlieva and Marinova 1998).

EXTRACTS FROM PLANT SOURCES FOR INCREASING THE OXIDATIVE STABILITY OF LIPIDS

The results obtained on the antioxidative effectiveness of different concentrations of hexane, ethylacetate and ethanol extracts from *Melissa officinalis* L. (common balm), *Mentha piperita* L. (peppermint), *Mentha spicata* L. (spearmint), *Ocimum basilicum* L. (common basil), *Origanum vulgare* L. (oregano), and *S. hortensis* at 100°C have shown that the ethanol extracts were the most active in retarding the autoxidation of TGSO (Yanishlieva and Marinova 1995b). The most effective were also the extracts from *S. hortensis*.

In TGL, 0.1, 0.3 and 0.5% ethanol extract from *S. hortensis* L. at 100°C had a stabilization factor F equal to 10.1, 22.0 and 31.3, respectively. It was also established that the stabilizing effect of the ethanol extract from *S. hortensis* in

Table 5 Stabilization factor **F** of different extracts from Bulgarian plant sources, determined during oxidation of TGL and TGSO at 100°C.

Plant source	Extract	Concentration (%)	F (Lipid system)	Reference
Leaves from	Hexane	0.05	35.0 (TGL)	Marinova *et al.* 1991
Rosemary officinalis L.	Ethanol	0.05	20.0 (TGL)	Marinova *et al.* 1991
Bark from	Ethanol	0.05	4.8 (TGL)	Marinova *et al.* 1994
Fraxinus ornus L.	Ethanol	0.10	6.1 (TGL)	Marinova *et al.* 1994
	Ethanol	0.05	3.6 (TGSO)	Marinova *et al.* 1994
	Ethanol	0.10	4.0 (TGSO)	Marinova *et al.* 1994
Leaves from				
Saturejae hortensis L.	Ethanol	0.10	9.6 (TGSO)	Yanishlieva and Marinova 1995b
Mentha piperita L.	Ethanol	0.10	4.5 (TGSO)	Yanishlieva and Marinova 1995b
Melissa officinalis L.	Ethanol	0.10	4.2 (TGSO)	Yanishlieva and Marinova 1995b
Mentha spicata L.	Ethanol	0.10	3.4 (TGSO)	Yanishlieva and Marinova 1995b
Origanum vulgare L.	Ethanol	0.10	7.0 (TGSO)	Yanishlieva and Marinova 1995b
Ocimum basilicum L.	Ethanol	0.10	2.7 (TGSO)	Yanishlieva and Marinova 1995b

the more saturated lipid system TGL was close to its effect in the more unsaturated system TGSO (F for TGSO being 9.6, 17.7 and 24.0, respectively) (Yanishlieva and Marinova 1995b). Hence, the ethanol extract from *S. hortensis* is suitable for inhibition of the autoxidation of highly unsaturated lipids.

Table 5 summarizes some of the results obtained on the effectiveness F of the extracts from different Bulgarian plant sources. The data, presented in **Table 5**, confirm the reported strong antioxidative effect of the extracts from *Rosmarinus officinalis* L. (rosemary) (Dugan 1980). As already discussed, the ethanol extract from *S. hortensis* possesses also a high antioxidative effectiveness. This extract leads also to a decrease in the oxidative and thermal changes occurring in sunflower oil during its high temperature (180°C) treatment in air (Yanishlieva *et al*. 1997), and it also stabilizes lipids against autoxidation at room temperature (Yanishlieva and Marinova 1998). Under the conditions of simulated fat frying the ethanol extract from *S. hortensis* inhibits the oxidative processes more strongly than the pure thermal ones (Yanishlieva *et al*. 1997)

PRACTICAL APPLICATIONS

Herbs, spices and their extracts with antioxidant capacity could be used as stabilizers of fats, in order to improve quality and shelf-life of meat and fat-containing foods. Summer savory and rosemary significantly improved the oxidation stability of heat-treated meat balls (Madsen *et al*. 1996). Dried leaves of rosemary added to cooked meat balls retarded the development of warmed over flavor during cold storage (Huisman *et al*. 1994). The addition of rosemary extract to the minced meat balls delayed the oxidation of lipid fraction (Karpinska *et al*. 2000). The rosemary extract displayed potential for maintaining sensory eating quality in processed pork products (Nissen *et al*. 2004). The effectiveness of mint leaves, as a natural antioxidant for radiation-processed lamb meat was established (Kanatt *et al*. 2007). Water-soluble oregano extract has potential for maintaining sensory eating quality in processed pork products (Rojas and Brewer 2007). Dietary oregano essential oil increased the stability of both raw and cooked turkey meat to lipid oxidation (Botsoglou *et al*. 2003).

CONCLUDING REMARKS

The stabilizing effect of the natural antioxidants in lipid oxidation depends not only on their structure and concentration, but also it is influenced strongly by the type of the lipid system, being oxidized, and temperature. In this respect the participation of the antioxidants in the side reactions of inhibited auto-oxidation should be taken into consideration. The proposed general kinetic parameter antioxidant activity allows a complex estimation of the effect of the antioxidnats in lipid oxidation. It unifies the effectiveness of an inhibitor in the termination of the autoxidation chain (stabilization factor, F) and its ability to change the oxidation rate during the induction period (oxidation rate ratio, ORR). The three parameters (F, ORR and A) also enable the evaluation of the effect of the main factors, e.g. the type of the lipid substrate and temperature, of different micro-components present or added in the lipid system, on the efficacy of the antioxidants.

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