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Antioxidative activity of phenolic acids on triacylglycerols and fatty acid methyl esters from olive oil

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The autoxidation of kinetically pure triacylglycerols and methyl esters of olive oil (TGOO and MEOO) in the presence of four different concentrations of *p*-coumaric, ferulic and caffeic acids at 100°C was studied. It was established that effectiveness and strength of the phenolic acids were greater in MEOO than in TGOO. In both lipid substates the molecules of phenolic acids participate in one side-reaction (with hydroperoxides). The rate constants of this reaction in TGOO and in MEOO are practically the same. The phenolic acids take part in chain initiation. The rate of this reaction for ferulic and caffeic acids has equal values in TGOO and in MEOO, whereas for *p*-coumaric acid it is twice as high in TGOO than in MEOO. The radicals of the phenolic acids participate in one reaction of chain propagation (with the lipid substrate) both in triacylglycerols and in methyl esters of olive oil. Comparison with the data published recently for sunflower oil triacylglycerols and methyl esters demonstrates the strong influence of the unsaturation type and degree of the lipid system on the kinetics and mechanism of the antioxidative action of the phenolic acids. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

The results of some investigations have shown that the binding of fatty acids to triacylglycerols affects both the trend to oxidation of the lipid substrate containing no antioxidants (Yanishlieva & Popov, 1973*b*; Katsuki *et al.*, 1987; Yanishlieva & Kortenska, 1989) and the inhibiting effect of the antioxidants (Yanishlieva & Popov, 1973*b*; Katsuki *et al.*, 1987).

Recently we clarified for the first time how the binding of the fatty acids to the natural triacylglycerols of sunflower oil (a lipid system with a high content of the easily oxidizable linoleic acid) influences the effectiveness, strength and mechanism of action of some phenolic acids (Yanishlieva & Marinova, 1995). It was established that the effectiveness and strength of the phenolic acids were higher in triacylglycerols of sunflower oil (TGSO) than in its methyl esters (MESO) because, during oxidation of TGSO, the contribution of the inhibitor molecules and radicals to chain initiation and propagation is smaller than is the case of MESO oxidation.

The purpose of the present study is to elucidate the same problem with olive oil—a lipid system with a high content of oleic acid which is a significantly less-oxidizable fatty acid than linoleic acid (Stirton *et al.*, 1945; Gunstone & Hilditch, 1945; Holman & Elmer, 1947; Silbert, 1962). We have also succeeded in clarifying the influence of the lipid system unsaturation on the mechanism of inhibited oxidation of natural triacylglycerols and their methyl esters.

The results are discussed taking into account that, in the case of triacylglycerols and methyl esters of olive oil (TGOO and MEOO, respectively), 40% of the hydroperoxides formed during the initial stage of the process are of a linoleate type and 60% are of an oleate type, whereas linoleate hydroperoxides alone are formed during TGSO and MESO autoxidation (Yanishlieva & Popov, 1973*a*). Moreover, oleate hydroperoxides are much more stable than the linoleate ones (Yanishlieva, 1973).

The investigations were performed using kinetically pure triacylglycerols of olive oil (TGOO) and its methyl esters (MEOO). The lipid substrates were inhibited with different concentrations (0.02–0.20%) of *p*-coumaric, ferulic and caffeic acids (Fig. 1).

MATERIALS AND METHODS

Materials

A commercially available olive oil was used. Its methyl esters were obtained according to Christie (1982). The

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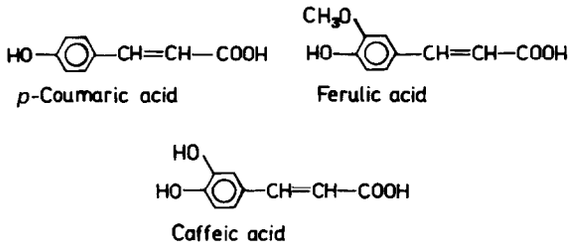

Fig. 1. Structures of the investigated phenolic acids.

lipid substrates were freed from pro- and antioxidants and trace metals by adsorption chromatography (Popov *et al.*, 1968; Yanishlieva & Marinova, 1995), and kinetically pure TGOO and MEOO were obtained. Control oxidation experiments at 80°C in the presence of 0.01 and 0.02 wt % citric acid, demonstrated that the chelating agent had no effect on the oxidation kinetics. The substrates were stored in an inert atmosphere at −20°C in the dark for no more than 10 days. The initial peroxide values (PV_o) at the start of each experiment were zero.

The fatty acid composition was as follows: palmitate 11.4%, palmitoleate 0.5%, stearate 0.4%, oleate 78.0%, linoleate 9.2%, linolenate 0.5%.

The phenolics, 4-hydroxycinnamic (*p*-coumaric) and 3-methoxy-4-hydroxycinnamic (ferulic) acids, were from Fluka, Switzerland, and 3,4-dihydroxycinnamic (caffeic) acid was from Merck, Germany.

Methods

Inhibition of the lipid substrate was achieved by adding aliquots of a solution of the antioxidant in purified acetone to a weighted lipid sample. Samples containing 0.02, 0.05, 0.10 and 0.20 wt % phenolic acids were prepared.

Oxidation was carried out at 100 ± 0.2°C by blowing air through the samples (5 g) in the dark at a rate of 100 ml min^{-1}. The process was followed by withdrawing samples (*ca* 0.1 g) at measured time intervals and subjecting them to iodometric determination of the peroxide concentration, i.e. the peroxide value, PV (Yanishlieva *et al.*, 1978).The effectiveness of the antioxidants was estimated on the basis of the induction period, IP, which was determined by the method of the tangents to the two parts of the kinetic curve (Yanishlieva & Popov, 1971; Le Tutour & Guedon, 1992). The rates of non-inhibited, W_o, and inhibited, W^{ie}_{inH}, oxidation was found from the tangents to the initial phase of the kinetic curves of peroxide accumulation and expressed as M s^{-1}. Recalculation of the rate from meq kg^{-1} h^{-1} into mol s^{-1} was performed according to the following formula (Marinova & Yanishlieva, 1992):

$$1\,\text{meq}\,\text{kg}^{-1}\,\text{h}^{-1} = 1.4\text{x}10^{-7}\,\text{M}\,\text{s}^{-1}$$

RESULTS AND DISCUSSION

The introduction of an inhibitor (antioxidant) into the oxidizing lipid system leads to a change in mechanism of the process and, as a result, in process kinetics. The effect of the inhibitor, InH, depends on the participation of its molecule and the radicals formed from the latter in a series of reactions (Denisov & Khudyakov, 1987):

(7) $LO_2^{\cdot} + InH \rightarrow LOOH + In^{\cdot}$

(−7) $In^{\cdot} + LOOH \rightarrow InH + LO_2^{\cdot}$

(8) $In^{\cdot} + LO_2^{\cdot} \rightarrow In\text{-}OOL$

(9) $In^{\cdot} + In^{\cdot} \rightarrow$ products

(10) $In^{\cdot} + LH \rightarrow InH + L^{\cdot}$

(11) $InH + LOOH \rightarrow$ products

(12) $InH + O_2 \rightarrow In^{\cdot} + HO_2^{\cdot}$

(13) $InOOL \rightarrow InO^{\cdot} + LO^{\cdot}$

(14) $In^{\cdot} + O_2 \rightarrow InOO^{\cdot}$

The peculiarities of the inhibitor action are described by two kinetic characteristics (Yanishlieva & Marinova, 1992): (i) effectiveness, representing the possibility of blocking the radical chain process by interaction with peroxide radicals [reaction (7)], which is responsible for the duration of the induction period, IP; and (ii) strength, expressing the possibility for the inhibitor moieties to participate in other reactions, e.g. (−7), (10), (11), (12), (14), which lead to a change in oxidation rate during the IP. A measure of the effectiveness is the stabilization factor *F*:

$$F = IP_{inH}/IP_o$$

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.

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

$$ORR = W_{inH}/W_o$$

where W_{inH} is the oxidation rate in the presence of an inhibitor and W_o is the initial oxidation rate of the non-inhibited system. ORR is an inverse measure of the strength.

When ORR is larger than 1, then the oxidation proceeds faster in the presence of an inhibitor than in the case of its absence, which, for example, is observed at high tocopherol concentrations (Marinova & Yanishlieva, 1992). The lower the ORR, the stronger the inhibitor.

Figure 2 illustrates, by way of example, the kinetic curves of peroxide accumulation during oxidation of TGOO and MEOO in the presence of 0.02 wt % of phenolic acids (Fig. 1). The kinetic curves of the oxidation of non-inhibited lipid substrates are also presented. All kinetic curves are the mean result of three independent experiments. It can be seen that the effectiveness and strength of *p*-coumaric, ferulic and caffeic acids depend not only on their structure, but also on the binding of the fatty acid moieties to triacylglycerol molecules. Moreover, every one of the phenolics shows a specific kinetic behaviour. This specificity is illustrated by the kinetic parameters, obtained after processing the kinetic curves for all investigated concentrations and is shown in Table 1 and Table 2. The mean rate of inhibitor consumption $\overline{W}_{InH}$ also is presented, as determined according to the formula:

$$\overline{W}_{InH} = [InH]_o/IP_{inH}(M\,s^{-1}) \quad (I)$$

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

Figures 3 and 4 illustrate the dependence of the stabilization factor F on the concentration of the phenolic acids. Obviously, for all acids in TGOO and MEOO, respectively, these dependences are not linear. In addition, the effectiveness of the antioxidants in both systems increases in the sequence *p*-coumaric < ferulic < caffeic acid. Besides, the phenolic acids are more effective in the methyl esters than in the triacylglycerols of the olive oil. The opposite is true when triacylglycerols and methyl esters of sunflower oil are oxidized in the presence of phenolic acids (Yanishlieva & Marinova, 1995).

The absence of linearity of the dependences in Figs 3 and 4 is due to the participation of the inhibitor molecules in reactions other than the main reaction of chain termination (7), namely reaction (11) and/or (12). In this case there is a relationship between the mean rate

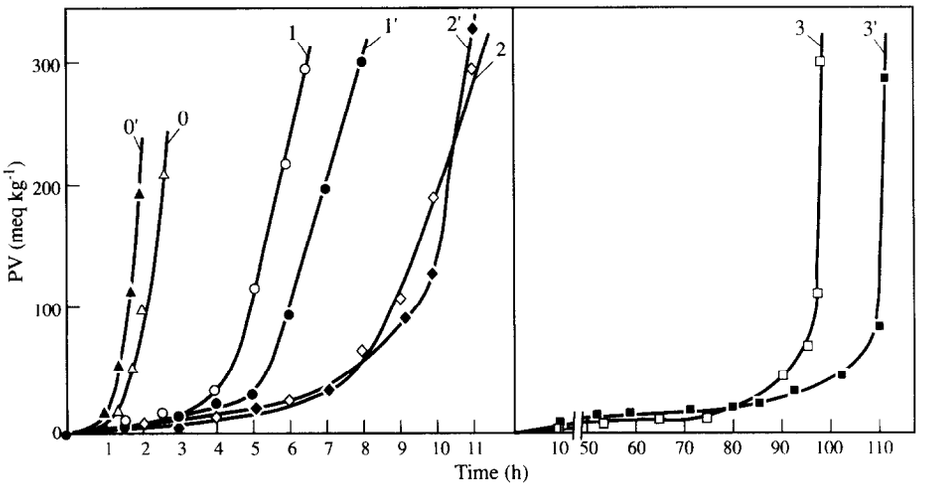

Fig. 2. Kinetic curves of peroxide accumulation (PV) during the oxidation of olive oil triacylglycerols TGOO (1, 2, 3, 4) and methyl esters MEOO (1′, 2′,3′,4′) at 100°C in the presence of 0.02% phenolic acids: 0 and 0′—without additive, 1 and 1′—*p*-coumaric acid, 2 and 2′—ferulic acid, 3 and 3′—caffeic acid.

Table 1. Kinetic parameters characterizing inhibited oxidation of triacylglycerols of olive oil (TGOO) at 100°C, $PV_n = 0$ meq k^{-1}, $IP_o = 1.3$ h, $W_o = 2.14 \times 10^{-6}$ M s^{-1} (acids as labelled in Fig. 1)

No.	Antioxidant (phenolic acid)	Inhibitor concentration		F	ORR	W_{inh} ($\times 10^7$) (M s^{-1})	W_{InH} ($\times 10^8$) (M s^{-1})
		[InH] (%)	[InH] ($\times 10^3$) (M)				
1	*p*-Coumaric	0.02	1.22	3.3	0.30	6.46	11.3
		0.05	3.05	5.1	0.22	4.63	15.7
		0.10	6.10	6.9	0.16	3.39	22.0
		0.20	12.20	8.0	0.13	2.78	36.4
2	Ferulic	0.02	1.03	6.2	0.31	6.78	4.27
		0.05	2.53	11.5	0.20	4.28	5.23
		0.10	5.15	20.8	0.14	3.08	5.56
		0.20	10.30	29.6	0.10	2.19	7.69
3	Caffeic	0.02	1.11	73	0.015	0.33	0.33
		0.05	2.78	112	0.011	0.23	0.54
		0.10	5.56	175	0.006	0.14	0.68
		0.20	11.10	227	0.006	0.14	1.05

Table 2. Kinetic parameters characterizing inhibited oxidation of methyl esters of olive oil (MEOO) at 100°C, $PV_0 = 0$ meq kg^{-1}, $IP_0 = 1.0$ h, $W_0 = 2.78 \times 10^{-6}$ M s^{-1} (acids as labelled in Fig. 1)

No.	Antioxidant (phenolic acid)	Inhibitor concentration		F	ORR	W_{inh} ($\times 10^7$) (M s^{-1})	W_{inH} ($\times 10^8$) (M s^{-1})
		[InH] (%)	[InH] ($\times 10^3$) (M)				
1	*p*-Coumaric	0.02	1.22	5.4	0.23	6.46	7.7
		0.05	3.05	6.9	0.17	4.79	14.4
		0.10	6.10	8.0	0.15	4.08	24.2
		0.20	12.20	9.0	0.13	3.70	42.4
2	Ferulic	0.02	1.03	9.2	0.22	6.17	3.48
		0.05	2.53	19.5	0.13	3.47	3.87
		0.10	5.15	29.0	0.09	2.48	5.11
		0.20	10.30	40.8	0.06	1.74	7.20
3	Caffeic	0.02	1.11	109	0.014	0.40	0.28
		0.05	2.78	187	0.011	0.33	0.42
		0.10	5.56	256	0.010	0.28	0.61
		0.20	11.10	300	0.010	0.28	1.03

of inhibitor consumption $\overline{W}_{InH}$ and the inhibitor concentration [InH] (Emanuel *et al.*, 1965):

$$\overline{W}_{InH} = \overline{W}_i/f + K_{eff}[InH]^n \quad (II)$$

where $\overline{W}_i$ is the mean rate of initiation during the induction period of the inhibited oxidation (M s^{-1}) and f is the stoichiometric coefficient of inhibition determining how many radicals perish in an inhibitor molecule.

The presentation of the results as a dependence of the mean rate of consumption of phenolic acids $\overline{W}_{InH}$ on their concentration [InH] at different n showed that for all phenolic acids in both lipid systems this dependence was linear at $n = 1$ (Figs 5 and 6), which indicated their participation in one side-reaction. From the slopes of the dependences (Figs 5 and 6) the rate constants K_{eff} of this reaction were determined (Table 3). For the oxidation of TGOO and MEOO in the presence of ferulic and caffeic acids K_{eff} has equal values, whereas in the presence of *p*-coumaric acid K_{eff} has a somewhat higher value in MEOO than in TGOO.

Which is the side-reaction in which the phenolic acid molecules participate during the oxidation of the triacylglycerols and methyl esters: (11) or (12)?

The consumption of the phenolic acids according to the reaction of chain initiation (12) presupposes that K_{eff} should not depend on the character of the lipid medium. Comparison of the K_{eff} values published recently for inhibited oxidation of sunflower oil triacylglycerols and methyl esters in the presence of phenolic acids (Marinova & Yanishlieva, 1994; Yanishlieva & Marinova,

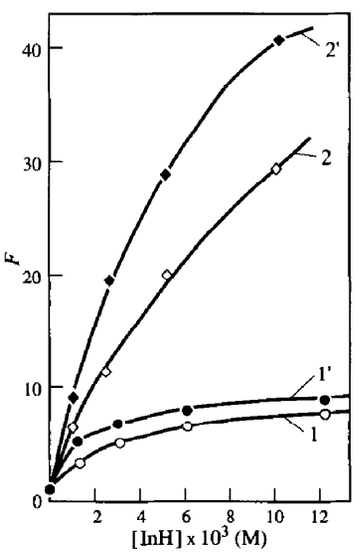

Fig. 3. Dependence of the stabilization factor F on the concentration of *p*-coumaric (1, 1′) and ferulic (2, 2′) acids during the oxidation of TGOO (1, 2) and MEOO (1′,2′) at 100°C.

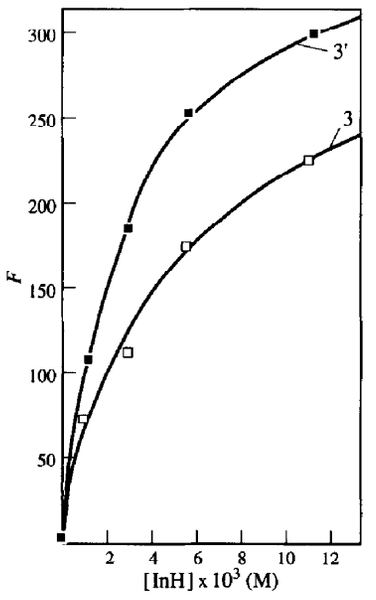

Fig. 4. Dependence of the stabilization factor F on the concentration of caffeic acid during the oxidation of TGOO (3) and MEOO (3′) at 100°C.

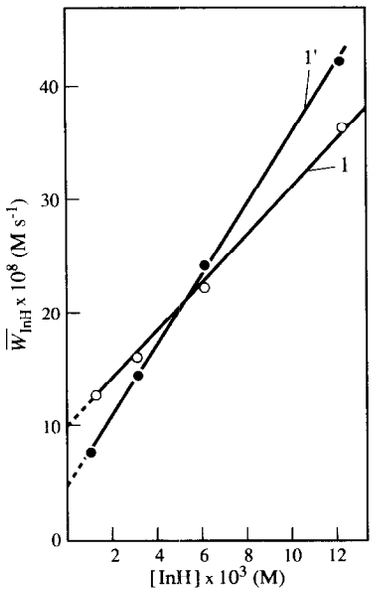

Fig. 5. Dependence of the mean rate of consumption of *p*-coumaric acid $\overline{W}_{InH}$ on its concentration [InH] during oxidation of TGOO (1) and MEOO (1') at 100°C.

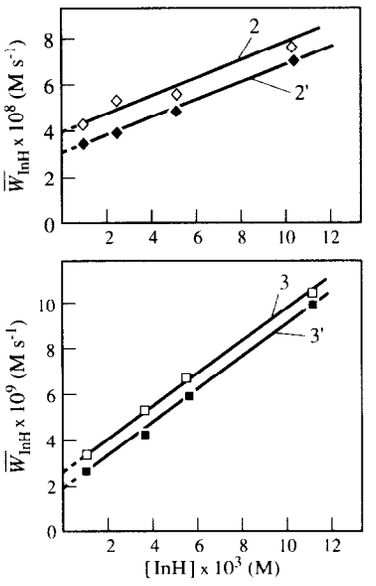

Fig. 6. Dependence of the mean rate of consumption of ferulic (2, 2') and caffeic (3,3') acids $\overline{W}_{InH}$ on their concentration [InH] during oxidation of TGOO (2, 3) and MEOO (2',3').

1995) with K_{eff} obtained for TGOO and MEOO (Table 3), shows that its values are much higher in sunflower oil systems than in olive oil systems; e.g. K_{eff} depends on the character of the lipid system. Therefore the phenolic acid molecules took part in the side-reaction with the hydroperoxides, reaction (11). This fact was confirmed by the different composition and, hence, different stabilities of sunflower and olive oil hydroperoxides, previously discussed, which signified that the rate constants of consumption of phenolic acids [reaction (11)] would be higher in TGSO and MESO, than in TGOO and MEOO. This is confirmed by the K_{eff} values obtained; compare the data in Table 3 with those for TGSO and MESO (Marinova & Yanishlieva, 1994; Yanishlieva & Marinova, 1995: the K_{eff} values for ferulic and caffeic acids in TGSO are 10.60×10^{-5} and 0.82×10^{-5} s^{-1}, respectively, and in MESO are 10.0×10^{-5} and 0.88×10^{-5} s^{-1}, respectively.

The results obtained show that the binding of the fatty acids to the triacylglycerol structure does not change the mechanism of phenolic acid consumption in side-reactions.

Figure 7 presents the dependence of the ORR on the concentration of the phenolic acids in TGOO and MEOO. Comparison of these dependences with those concerning the stabilization factor, F (Figs 3 and 4), shows that the sequence of change in strength of the phenolic acids is the same as the sequence of their effectiveness. Besides, the phenolic acids act as stronger inhibitors in methyl esters than in triacylglycerols of olive oil. The opposite is true for the sunflower oil systems (Yanishlieva & Marinova, 1995).

As already discussed, ORR is a measure of the oxidation rate during the IP. This rate is in a direct ratio to the rate of chain initiation and propagation. The weaker the participation of the inhibitor moieties in chain initiation and propagation, the lower the oxidation rate during the IP and the stronger the inhibitor.

How do the phenolic acids participate in chain initiation and propagation?

The parameter $\overline{W}_i/f$ takes into account the participation of the antioxidant in the initiation reactions. $\overline{W}_i/f$ was determined from Figs 5 and 6 by extrapolation to zero concentration of the phenolic acids and is presented in Table 3. Comparison of the data for $\overline{W}_i/f$ in TGOO and in MEOO shows that they increase in the sequence:

Table 3. Kinetic parameters characterizing the mean rate of initiation, $\overline{W}_i/f$, and the effective rate constant of inhibitor consumption, K_{eff}, during oxidation of olive oil triacylglycerols (TGOO) and methyl esters (MEOO) at 100°C in the presence of different phenolic acids

Antioxidant (phenolic acid)	W_i/f ($\times 10^7$) M s^{-1}		K_{eff} ($\times 10^5$) (s^{-1})	
	TGOO	MEOO	TGOO	MEOO
p-Coumaric	0.98	0.50	2.14	3.12
Ferulic	0.40	0.32	0.38	0.38
Caffeic	0.027	0.02	0.07	0.07

caffeic < ferulic < *p*-coumaric acids. In addition, $\overline{W}_i/f$ has similar values during the oxidation of TGOO and MEOO in the presence of ferulic and caffeic acids. In the presence of *p*-coumaric acid $\overline{W}_i/f$ has half the values in MEOO than in TGOO. Quite different are the data with triacylgycerols and methyl esters of sunflower oil: during the oxidation of MESO the phenolic acids cause a 3–6 times higher initiation rate than during the oxidation of TGSO (Yanishlieva & Marinova, 1995).

The results obtained demonstrate the strong influence of the unsaturation type and degree of the lipid system on the kinetics and mechanism of the antioxidative action of the phenolic acids.

Previous research (Denisov & Khudyakov, 1987) showed that if the inhibitor radical In˙ participates in one reaction of chain propagation [reaction (−7) or (10) or (14)], the dependence (III) is valid:

$$W_{inH} \sim [InH]^{-0.5} \quad (III)$$

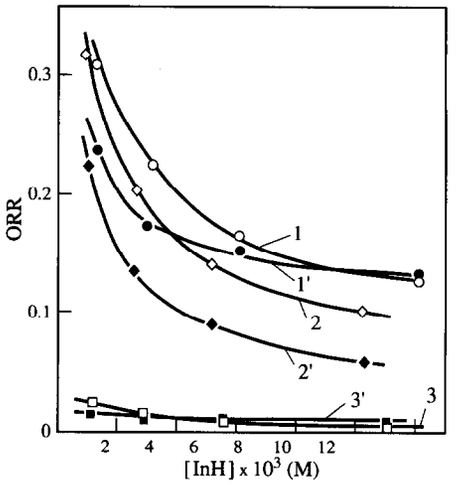

Fig. 7. Dependence of the oxidation rate ratio ORR on the concentration of *p*-coumaric (1, 1′), ferulic (2, 2′) and caffeic (3, 3′) acids [InH] during oxidation of TGOO (1, 2, 3) and MEOO (1′, 2′, 3′) at 100°C.

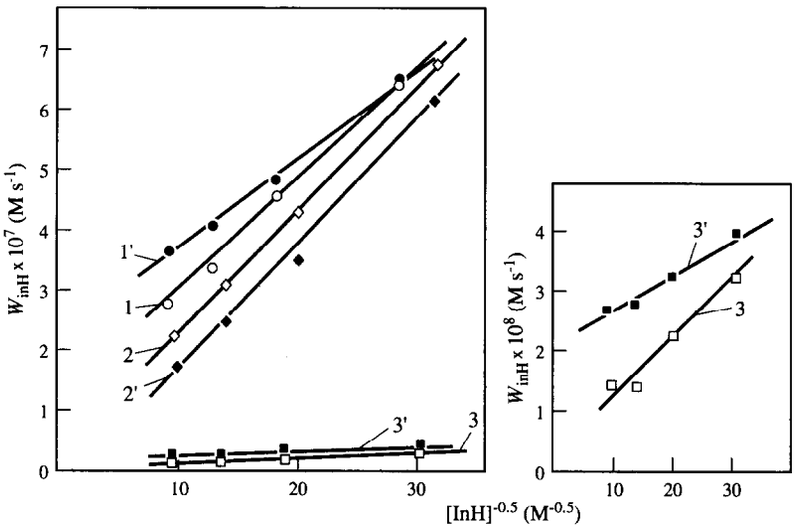

Fig. 8. Dependence of the rate of inhibited oxidation W_{inH} on the concentration of *p*-coumaric (1, 1′), ferulic (2, 2′) and caffeic (3, 3′),acids, $[InH]^{-0.5}$, during oxidation of TGOO (1, 2, 3) and MEOO (1′, 2′, 3′).

When In˙ does not participate in chain propagation, dependence (IV) is valid:

$$W_h \sim [InH]^{-1} \quad (IV)$$

Processing of the results obtained (Tables 1 and 2) on the basis of the dependences (III) and (IV) showed that, for all phenolics investigated during TGOO and MEOO oxidation dependence (III) is valid (Fig. 8). This means that the radicals of the phenolic acids participate in one reaction of chain propagation. As discussed previously (Yanishlieva & Marinova, 1992), this reaction should be reaction (10): $In^{\cdot} + LH \rightarrow InH + L^{\cdot}$.

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