Antioxidant Activity and Mechanism of Action of Some Synthesised Phenolic Acid Amides of Aromatic Amines

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Abstract

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The antioxidative activities and mechanism of action were studied of eight synthesised cinnamoyl- and hydroxycinnamoyl amides of biogenic amines (caffeoyldopamine, cinnamoyldopamine, *p*-coumaroyldopamine, feruloyldopamine, sinapoyldopamine, caffeoylphenylethylamine, caffeoyltyramine, and caffeoyltryptamine) in a wide concentration range ($2.5–20 \times 10^{-4}$M) during autoxidation of triacylglycerols of sunflower oil. It was established that all amides exhibited excellent antioxidant activity, higher than or comparable with that of caffeic acid. The best activity was shown by caffeoyldopamine followed by cinnamoyldopamine and caffeoyltyramine. The analysis of the kinetic data obtained showed that the presence of hydroxyl groups in the amino part (derivatives of dopamine and tyramine) led to direct oxidation of the molecules during the process and stabilisation of the resulting radicals. In contrast, the amides without hydroxyl groups in the amino part participated in the side reaction with peroxides and the resulting radicals took part in one side reaction of the chain propagation as did caffeic acid.

Keywords: cinnamic acid derivatives; phenylpropenoyl amides of aromatic amines; triacylglycerols of sunflower oil

Oxygen is critical for the life on the earth. Reactive oxygen species are continuously produced during normal physiological events. They may be required for the normal cell function at physiological concentrations, but are also capable to initiate destructive processes such as the peroxidation of lipids. Lipid peroxidation is one of the important primary events in the free radical-mediated oxidative damage of biological membranes and tissues. Antioxidants are organic molecules which can scavenge free radicals and thus avoid or delay the progress of lipid oxidation. Recently, natural antioxidants have received growing attention because they are known to function as chemoprotective agents against oxidative damage (Gordon 1996; Niki 2001). Antioxidant capacity is widely used as a parameter for medicinal bioactive components (Ko *et al*. 2009)

Phenolic compounds are widespread secondary plant metabolites with a broad spectrum of biological activities (Koshihara *et al.* 1984; Tanaka *et al.* 1993). They can bind to enzymes and other multisubunit proteins, modify their structural properties, and alter their biological activities (Suryaprakash *et al.* 2000; Sascha *et al.* 2002; Yen & Yen 2003). Hydroxycinnamic acid compounds have been described as chain-breaking antioxidants, probably acting through radical-scavenging, which is related to their hydrogen-donating capacity, and to their ability to stabilise the resulting phenoxyl radical (Siquet *et al*. 2006). On the other hand, some studies indicated that catecholamines and related compounds may possess antioxidant activity that seems to be correlated with the number of hydroxyl groups and

their position on the benzoic ring (Yen & Hsieh 1997; Sofic *et al.* 2001; Gulchin 2009). Cinnamic acid amides are a class of secondary metabolites. They have been identified as the main phenolic constituents in the reproductive organs of a range of flowering plants (Martin-Tanguy *et al.* 1978). They are also produced in plants in response to various stimuli – UV irradiation, injury, pathogenic infections, heavy metals (Muroi *et al.* 2009). Some authors discussed the antioxidatve properties of the phenolic amides (including cinnamoyl amides) (Ley 2001; Son & Lewis 2002; Choi *et al.* 2010; Liu *et al.* 2010; Roleira *et al.* 2010).

Today, there is an increasing interest into hybrid molecules, which combine two molecules with biological activity into a single molecule. Such conjugates can be more effective (Hadjipavlou-Litina *et al*. 2010). Taking into account the good antoxidative properties of hydroxyl cinnamic acids and the probable activity of aromatic amines, the objectives of this work were to examine and compare the antioxidative activities of eight synthesised cinnamoyl- and hydroxycinnamoyl amides of biogenic amines – caffeoyldopamine, cinnamoyldopamine, *p*-coumaroyldopamine, feruloyldopamine, sinapoyldopamine, caffeoylphenylethylamine, caffeoyltyramine, and caffeoyltryptamine (Figure 1) in a wide concentration range (2.5–20 × 10^{-4}M), and to clarify the mechanisms of their action. As a lipid substrate, we used kinetically pure (depleted from pro- and antioxidative micro components) triacylglycerols of sunflower oil (TGSO), because of its polyunsaturation degree and well known oxidation mechanism.

MATERIAL AND METHODS

Preparation of amides. The *N*-hydroxycinnamoyl amides were synthesised from the corresponding cinnamic acids and aromatic monoamines using coupling agents (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC) and 1-hydroxybenzotriazole (HOBT)), and triethylamine or *N*-methylmorpholine as a base (Lee *et al.* 2007). After purification, the yields were between 35% and 90%. Briefly, the phenylpropenoic acid (0.80mM), HOBt (0.80mM), and EDC (0.80mM) were dissolved in 3 ml DMF/CH_2Cl_2

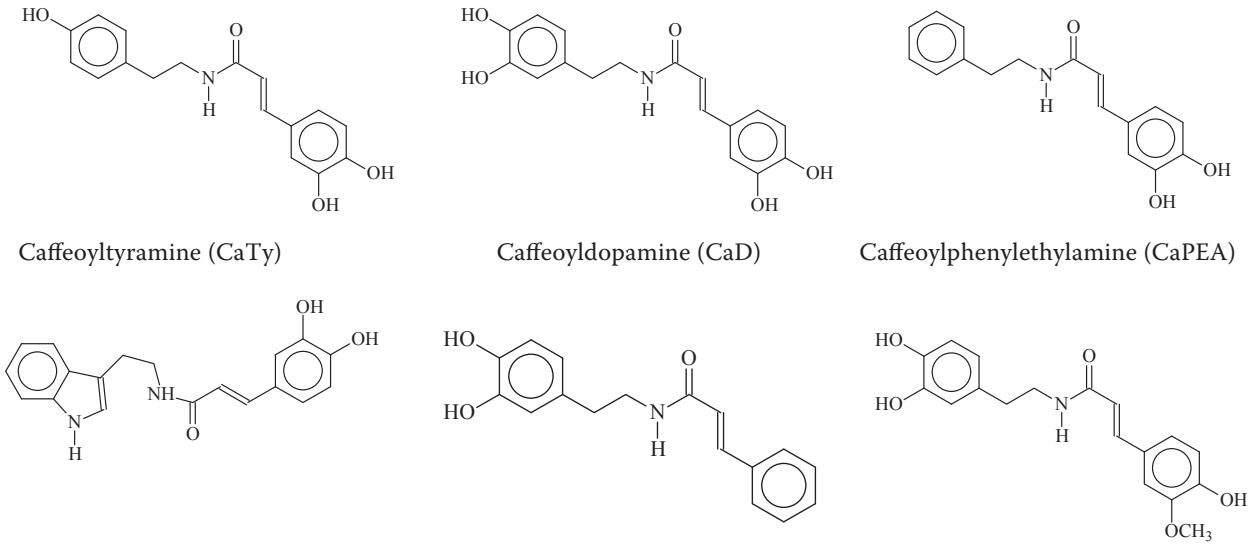

Figure 1. Chemical structure of the synthesised cinnamoyl and hydroxycinnamoyl amides of biogenic amines

and the solution was cooled to 0°C. After 15 min, the corresponding amine (0.80mM) and Et_3N dissolved in 3 ml DMF/CH_2Cl_2 were added. After 45 min, the mixture was removed from the ice bath and was stirred at room temperature for 4 h after which 50 ml of distilled water was added. The reaction was monitored by thin-layer chromatography on silica gel 60 F_{254} (Merck, Darmstadt, Germany). The product was extracted with ethyl acetate and washed consecutively with 5% $NaHCO_3$ and water. The amide was dried over anhydrouse Na_2SO_4 and purified by column chromatography or preparative TLC on silica gel with different proportions of the chromatographic system (CH_2Cl_2:CH_3OH). The structures and purity of all the amides were confirmed by spectral methods – UV, IR, ^1H and C^{13} NMR spectroscopy.

Preparation of triacylglycerols. Pure triacylglycerols of sunflower oil (TGSO) were obtained by cleaning the sunflower oil sample from pro- and antioxidants and trace metals by adsorption chromatography (Yanishlieva & Marinova 1995). Briefly, the lipid substrate (100 g in 1000 ml of distilled hexane) was 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 hours. The obtained triacylglycerols were collected in 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), iron, and copper (atom absorption spectroscopy, < 0.01 and 0.001 ppm, respectively). The 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. The initial peroxide value (PV) was 0.

Preparation of samples for oxidation. Lipid samples containing different concentrations of the amides were prepared by adding aliquots of their solutions in purified acetone to a weighed amount of TGSO followed by the removal of the solvent with nitrogen.

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

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 the samples at measured time intervals, estimating the degree of oxidation by iodometric determination of the PV (Yanishlieva *et al.* 1978).

Kinetic curves of PV accumulation were plotted. All of them represent the mean result of three independent experiments. The effectiveness of the antioxidants 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 (Marinova & Yanishlieva 1992).

Determination of the kinetic parameters. The results obtained are interpreted on the basis of the main regularities of the inhibited lipid oxidation. The introduction of an antioxidant (inhibitor – InH) into the oxidising lipid system leads to a change in the mechanism of the process and, as a result, in the process kinetics. The effect of the inhibitor depends on the participation of its molecule and the radicals formed from the latter in a series of reactions (Denisov & Khudyakov 1987).

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, which is responsible for the duration of the induction period, and (*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. The measure of the effectiveness is the stabilisation factor *F*:

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

where:

IP_{inh} – induction period in the presence of an inhibitor

IP_o – induction period of the non-inhibited system

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

$$ORR = W_{inh}/W_{noninh}$$

where:

W_{inh} – oxidation rate in the presence of an inhibitor

W_{noninh} – initial oxidation rate of the non-inhibited system

ORR – inverse measure of the strength

As a combined parameter to evaluate the antioxidant properties, the general kinetic parameter, antioxidant activity (A), was used. This parameter combines the effectiveness of an inhibitor in terminating the oxidation chains with its ability to influence the oxidation rate during the IP, i.e.

$$A = F/ORR$$

The mean rate of the inhibitor consumption, W_{InH}, is determined according to the formula, taking into

Table 1. Kinetic parameters characterising inhibited oxidation of triacylglycerols of sunflower oil at 100°C (IP_o = 0.4 h, $W_o = 9.26 \times 10^{-6}$ M/s)

Compound	Concentration ($\times 10^4$) (M)	*F*	W_{inh} ($\times 10^6$) (M/s)	ORR	W_{inh} ($\times 10^8$) (M/s)	A
Caffeoyltyramine	2.5	9.0	1.39	0.15	2.17	60
	5.0	17.2	0.87	0.094	2.14	183
	10	27.5	0.48	0.052	2.62	529
	20	37.5	0.35	0.038	3.81	987
Caffeoyldopamine	2.5	13.5	0.79	0.085	1.39	159
	5.0	22.5	0.51	0.055	1.61	409
	10	45.0	0.34	0.038	1.58	1184
	20	54.5	0.25	0.027	2.60	2019
Caffeoylphenylethylamine	2.5	11.2	1.54	0.17	1.69	66
	5.0	16.8	1.11	0.12	2.20	140
	10	31.0	0.92	0.10	2.31	310
	20	41.2	0.62	0.067	3.45	615
Caffeoyltryptamine	2.5	8.3	1.98	0.21	2.39	40
	5.0	13.5	1.16	0.12	2.78	112
	10	25.0	0.69	0.074	2.89	337
	20	31.3	0.52	0.056	4.59	559
Cinnamoyldopamine	2.5	15.0	0.99	0.11	1.24	136
	5	22.5	0.69	0.074	1.61	321
	10	45.0	0.40	0.043	1.57	1047
	20	45.0	0.37	0.036	3.16	1250
Feruloyldopamine	2.5	7.5	2.31	0.25	2.67	30
	5.0	13.0	1.32	0.14	2.89	93
	10	22.5	0.69	0.074	3.23	304
	20	25.0	0.62	0.067	5.78	373
p-Coumaryldopamine	2.5	9.0	2.14	0.23	2.10	39
	5.0	16.5	1.26	0.14	2.24	118
	10	28.8	0.69	0.074	2.50	389
	20	30.5	0.50	0.054	4.71	564
Sinapoyldopamine	2.5	4.5	2.78	0.30	4.96	15
	5.0	7.5	1.74	0.19	5.34	39
	10	13.0	1.11	0.12	5.78	108
	20	15.5	0.79	0.085	9.58	182
Caffeic acid (Marinova *et al.* 2009)	2.8	7.0		0.23		30
	5.6	13.2		0.14		94
	11.1	24.0		0.077		312
	27.8	48.0		0.063		762

F – stabilisation factor; ORR – inverse measure of the strength; A – antioxidant activity

account that the concentration of an antioxidant is proportional to the length of the induction period (Niki & Noguchi 2000; Shahidi & Zhong 2005):

$W_{inh} = [InH]_o/\tau$ (M/s)

where:

$[InH]_o$ – initial molar concentration of the antioxidant

$\tau = IP_{inh} - IP_o$

Statistical analysis. The coefficient of variation for the PV determination was 7–8% irrespective of the value measured. The reported values for the IP were the mean result from three independent experiments. The coefficient of variation ranged from 6–13% and was inversely related to the induction period. The W_{noninh} and W_{inh} varied by no more than 5%. Linear relationships between the parameters investigated were obtained using the Linear fit tool of Origin 6.1 software (OriginLab Corporation, Northampton, USA).

RESULTS AND DISCUSSION

The fatty acid composition of sunflower oil, as determined by gas chromatographic analysis of its methyl esters, was: saturated acids – 13%, monounsaturated acids – 26%, polyunsaturated acids – 61%.

For example, Figure 2 illustrates the kinetic curves of peroxide accumulation during the oxidation of TGSO in the presence of different concentrations of cinnamoyldopamine (CiD). The kinetic parameters, obtained after processing all the kinetic curves of peroxides accumulation during

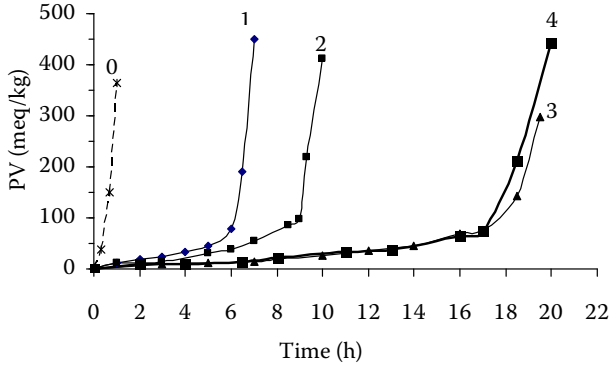

Figure 2. Kinetic curves of peroxides accumulation during autoxidation of triacylglycerols of sunflower oil at 100°C in the absence (0) and in the presence of cinnamoyldopamine 1 – 2.5×10^{-4}M; 2 – 5×10^{-4}M; 3 – 10×10^{-4}M; 4 – 20×10^{-4}M

the TGSO oxidation in the presence of 2.5, 5, 10, and 20×10^{-4}M of caffeoyldopamine (CaD), cinnamoyldopamine (CiD), *p*-coumaroyldopamine (*p*-CD), feruloyldopamine (FD), sinapoyldopamine (SD), caffeoylphenylethylamine (CaPEA), caffeoyltyramine (CaTy), caffeoyltryptamine (CaTr), are given in Table 1.

Figure 3 presents the dependences of the stabilisation factors (*F*) on the concentrations of the investigated phenolic amides. For comparison, the stabilisation factor of caffeic acid under the same oxidation conditions is given (Marinova *et al.* 2009). In Figure 3 can be seen that all compounds possessed excellent antioxidant effectiveness, higher than or comparable with that of caffeic acid except sinapoyldopamine. Among caffeoyl amides (Figure 3A), the antioxidant effectiveness decreases in the following order: caffeoyldopamine > caffeoylphenylethylamine ≅ caffeoyltyramine > caffeoyltryptamine. Among cinnamoyl and hydroxycinnamoyl dopamines (Figure 3B), the antioxidant activity decreases in the following order: caffeoyldopamine ≅ cinnamoyldopamine > *p*-coumaroyldopamine > feruloyldopamine > sinapoyldopamine.

Figure 4 illustrates the dependence of the combined parameter, antioxidant activity A, on the concentrations of the synthesised cinnamoyl- and hydroxycinnamoyl amides. It can be seen that caffeoyldopamine possessed the best activity followed by cinnamoyldopamine and caffeoyltyramine. *p*-Coumaroyldopamine, caffeoylphenylethylamine, caffeoyltryptamine, and feruloyldopamine showed activities comparable with that of caffeic acid (CA). Sinapoyldopamine exhibited the weakest activity.

It should be noted that in these oxidation conditions, pure cinnamic acid acted as a prooxidant, pure *p*-coumaric acid had no effect, and pure ferulic and sinapic acids were less active antioxidants than the investigated compounds (Yanishlieva-Maslarova & Marinova 2007).

The results showed that, in contrast to the hydroxy derivatives of cinnamic acid (Yanishlieva-Maslarova & Marinova 2007), the presence of methoxy groups in the molecules of the compounds studied (feruloyldopamine and sinapoyldopamine) decreases the antioxidant activity. Moreover, cinnamoyldopamine exhibited an activity comparable with that of caffeoyldopamine. These findings indicated that the antioxidant potential of the synthesised amides does not directly depend on the phenolic part of the molecules.

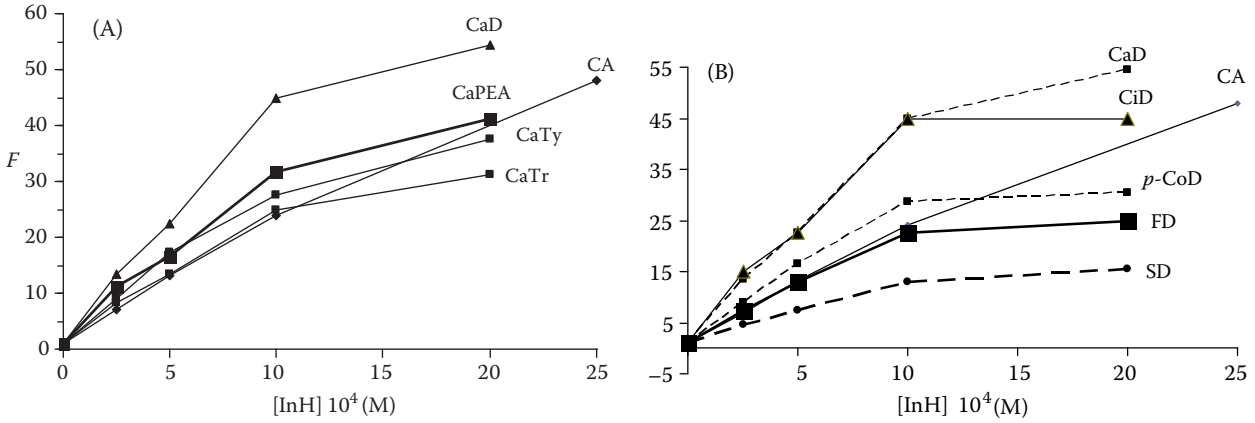

Figure 3. Dependence of the stabilization factor (F) of the investigated amides on their concentration, [InH], during autoxidation of TGSO at 100°C

The absence of linearity of the dependences of F on the concentrations is due to the participation of the inhibitor molecules in reactions other than the main reaction of the chain termination – with hydroperoxides ($LO_2^\bullet$ + InH → LOOH + $In^\bullet$), namely the reactions with lipid substrate ($In^\bullet$ + LH → InH + $L^\bullet$) or/and with oxygen (InH + O_2 → $In^\bullet$ + HO_2). In this case, a relationship exists between the mean rate of the inhibitor consumption W_{InH} and the inhibitor concentration [InH] (Emanuel *et al.* 1965):

$$W_{InH} = W_i/f + K_{eff}\,[InH]^n \quad \text{(I)}$$

where:

W_i – mean rate of initiation during the induction period of the inhibited oxidation

f – stoichiometric coefficient of inhibition determining how many radicals perish in the inhibitor molecule

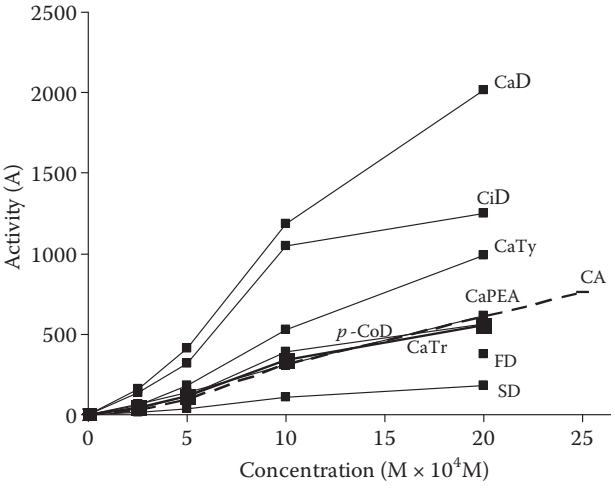

Figure 4. Dependence of the activity (A) of the investigated amides and caffeic acid (CA) on their concentration, [InH], during autoxidation of triacylglycerols of sunflower oil at 100°C

The presentation of the results as the dependence (I) at different n showed that for CaPEA and CaTr these dependences were linear at n = 1 (Figure 5A), and for CaD, CiD, *p*-CoD, FD, SD, and CaTy – at n = 2 (Figure 5B). This indicated that CaPEA and CaTr participate in one side reaction, while CaD, CiD, *p*-CoD, FD, SD, and CaTy in two side reactions. The main side reaction in which the antioxidants should participate in inhibited lipid oxidation is the reaction with hydroperoxides (Yanishlieva-Maslarova & Marinova 2007). From the slopes of the dependences, the rate constants K_{eff} of this reaction were determined. The value of K_{eff} was for CaPEA – 9×10^{-6}/s, and for CaTr – 12×10^{-6}/s. For comparison, caffeic acid participates in one side reaction and its K_{eff} was 5×10^{-6}/s (Marinova *et al.* 2009) i.e., caffeoylphenylethylamine and caffeoyltryptamine took part in a greater extent in the side reactions with hydroperoxides than did caffeic acid.

On the other hand, CaD, CiD, *p*-CoD, FD, SD, and CaTy participate in both side reactions of the inhibited oxidation (11) and (12). K_{eff} were as follow: for CaD – 3×10^{-3}/s; CiD – 5×10^{-3}/s; *p*-CoD – 7×10^{-3}/s; FD – 8×10^{-3}/s; SD – 12×10^{-3}/s, and CaTy – 4×10^{-3}/s, respectively. These findings indicated that these compounds, in parallel with the reaction with hydroperoxides, were also destroyed by direct oxidation.

Denisov and Khudyakov (1987) have proved that, if the inhibitor radical $In^\bullet$ participates in one reaction of the chain propagation, reaction (In + LOOH → InH + $LO_2^\bullet$), or ($In^\bullet$ + LH → InH + $L^\bullet$), or ($In^\bullet$ + O_2 → $InOO^\bullet$), the dependence (II) is valid:

$$W_{inh} \sim [InH]^{-0.5} \quad \text{(II)}$$

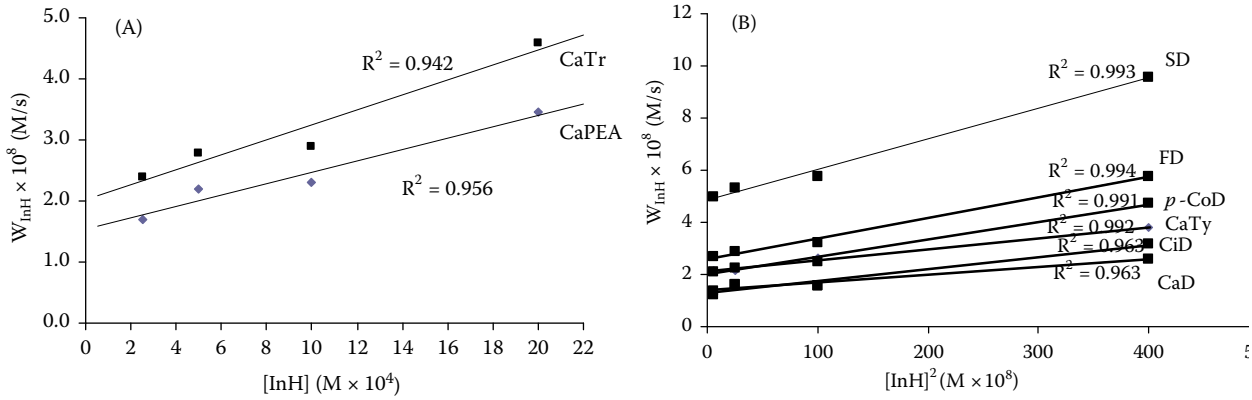

Figure 5. Dependence of the mean rate of consumption (W_{InH}) of the investigated amides on their concentration: [InH] (A) and [InH]^2 (B), during autoxidation of TGSO at 100°C

If it does not participate in the chain propagation, the dependence (III) is valid:

$$W_{inh} \sim [InH]^{-1} \quad \text{(III)}$$

The processing of the results obtained (Table 1) on the basis of these dependences showed that for CaPEA and CTr dependence (II) (Figure 6A) was valid, and for CaD, CiD, *p*-CoD, FD, SD, and CaTy dependence (III) was valid (Figure 6B). This means that the radicals derived from CaD, CiD, *p*-CoD, FD, SD, and CaTy are stable and did not participate in the side reaction of the chain propagation. CaPEA and CaTr resulting radicals participated in one reaction of the chain propagation as did caffeic acid. As discussed previously (Yanishlieva-Maslarova & Marinova 2007), this reaction should be the reaction with the lipid substrate.

Our results showed that the synthesised amides can be divided into two groups with respect to their antioxidative properties:

Phenoyl amides like caffeic acid – caffeoylphenylethylamine and caffeoyltryptamine (amino part without hydroxyl group). The molecules of these compounds participated in one side reaction (with hydroperoxides) and the radicals derived from them took part in one reaction of the chain propagation as did caffeic acid. Given that they exhibited effectiveness and activity similar to those of caffeic acid, it can be concluded that their antioxidative properties were determined thoroughly by caffeoyl moiety.

The compounds whose molecules were destroyed by direct oxidation during the process and whose resulting radicals were stable, did not participate in the chain propagation. Caffeoyldopamine, caffeoyltyramine, *p*-coumaroyldopamine, cinnamoyldopamine, feruloyldopamine, and sinapoyldopamine belong to this group. Contrary to hydroxycinnamic acids, the presence of methoxy groups in the acyl part of these amides (feruloyldopamine and sinapoyldopamine) decreased

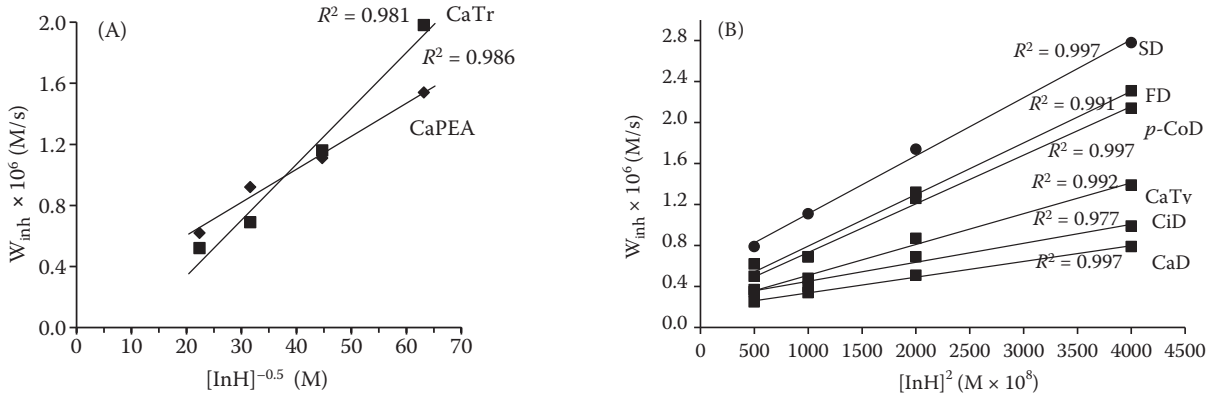

Figure 6. Dependence on the initial rate of inhibited oxidation of triacylglycerols of sunflower oil at 100°C (W_{inh}) on the concentration of the investigated amides: [InH]$^{-0.5}$ (A) and [InH]$^{-1}$ (B)

their antioxidant activity, and cinnamoyldopamine exhibited an activity comparable with that of the caffeoyldopamine. These findings indicated that the presence of hydroxyl groups in the amino parts promoted the direct oxidation of the molecules, stabilised radicals, and hence modified the antioxidative potentials of these amides.

CONCLUSIONS

(1) During the autoxidation of TGSO, the synthesised cinnamoyl- and hydroxycinnamoyl amides of biogenic amines exhibited an excellent antioxidant activity, higher than or comparable with that of caffeic acid. The best activity was shown by caffeoyldopamine followed by cinnamoyldopamine and caffeoyltyramine.

(2) From the analysis of the kinetic data obtained, it was found that the presence of hydroxyl groups in the amino part (derivatives of dopamine and tyramine) led to direct oxidation of the molecules during the process, and to stabilisation of the resulting radicals. In contrast, amides without hydroxyl groups in the amino part (caffeoylphenylethylamine and caffeoyltryptamine) participated in one side reaction with peroxides and the resulting radicals took part in one side reaction of the chain propagation as did caffeic acid.

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