

# Rate constants for the reaction of peroxy free radical with flavonoids and related compounds as determined by the kinetic chemiluminescence method

The late Vladimir A. Belyakov,<sup>a</sup> Vitalij A. Roginsky<sup>a</sup> and Wolf Bors<sup>\*,b</sup>

<sup>a</sup> N. N. Semenov Institute of Chemical Physics, Russian Academy of Sciences, Kosygin St. 4, 117977 Moscow, Russia

<sup>b</sup> Institute of Radiation Biology, GSF Research Center Neuherberg, 85758 Oberschleissheim, Germany

The kinetic chemiluminescence method was applied to determine rate constants ( $k_5$ ) for the reaction of the peroxy radical from diphenylmethane, ROO<sup>•</sup>, with a number of flavonoids, catechol derivatives as well as two 'standard' phenolic antioxidants at 50 °C in chlorobenzene. It was found that the kinetics of the interaction of ROO<sup>•</sup> with the majority of flavonoids and catechols is complicated by a slow process which could be optimally simulated by a mono-molecular transformation of the respective semiquinone radicals. The rate constants for this transformation ( $k_{17}$ ), indirectly derived from the kinetics of chemiluminescence are listed in parentheses. The following rate constants  $k_5$  (in dm<sup>-3</sup> mol<sup>-1</sup> s<sup>-1</sup>) and  $k_{16}$  (in s<sup>-1</sup>) have been obtained: quercetin,  $2.1 \times 10^7$  (2.0); dihydroquercetin,  $1.9 \times 10^7$  (0.92); luteolin,  $2.2 \times 10^7$  (0.8); catechin,  $6.6 \times 10^6$  (7.5); fisetin,  $1.2 \times 10^7$  (1.1); naringenin,  $3.4 \times 10^3$  (0.2); kaempferol,  $1.0 \times 10^6$  (0.8); caffeic acid,  $1.5 \times 10^7$  (1.9); 3,5-di-*tert*-butylcatechol,  $1.9 \times 10^7$  (0.65); nordihydroguaiaretic acid,  $1.0 \times 10^7$  (~0);  $\alpha$ -tocopherol,  $8.5 \times 10^6$  (0); 2,6-di-*tert*-butyl-4-methylphenol (BHT),  $3.6 \times 10^4$  (0).

Flavonoids, natural polyphenol compounds of plant origin, are known to be effective inhibitors of lipid peroxidation which is commonly associated with their ability to scavenge peroxy free radicals.<sup>1-8</sup> In addition, many flavonoids display a pronounced pharmacological and vitamin-like activity which is explained, at least partly, by their chain-breaking antioxidant ability.<sup>9-14</sup>

Detailed information on flavonoid reactivity towards a number of active free radicals, *e.g.* HO<sup>•</sup>, Bu<sup>•</sup>O<sup>•</sup>, N<sub>3</sub><sup>•</sup>, is available.<sup>7,15-17</sup> At the same time, rate constants for the reaction of flavonoids with peroxy radicals are limited to those with linoleic acid<sup>18</sup> or isopropyl peroxy radicals.<sup>19</sup> Surprisingly high values for quercetin [ $(3.4-4.2) \times 10^7$  dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>] and kaempferol [ $(1.5-1.8) \times 10^7$  dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>] were measured by the pulse radiolysis method in aqueous dispersions of linoleic acid at pH 11.5,<sup>18</sup> with values of  $2.1 \times 10^8$  dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup> and  $1.95 \times 10^9$  dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>, respectively, measured for the reaction with isopropyl peroxy radicals at pH 8.5.<sup>19</sup>

In this work a more detailed study of flavonoid reactivity with the peroxy free radical has been undertaken. The structures of the flavonoids tested (I-VII) are presented in Scheme 1. As an elevated reactivity of flavonoids towards free radicals is usually associated with the catechol moiety in the B-ring (ref. 16, see Scheme 1), catechol derivatives VIII-X were studied as well. Measurements were carried out by the kinetic chemiluminescence method that is known to be one of the most sensitive and informative methods with which to explore the reactivity of chain-breaking anti-oxidants.<sup>20-22</sup> In this method one evaluates the restitution of chemiluminescence (CL) after depletion of the inhibitors rather than the accumulation of the autoxidation products.

## Experimental

Quercetin, dihydroquercetin, naringenin (Serva), kaempferol, catechin (Fluka), luteolin, fisetin (Roth), caffeic acid, nordihydroguaiaretic acid (Aldrich),  $\alpha$ -tocopherol (Sigma), 2,6-di-*tert*-butyl-4-methylphenol (BHT, Sumimoto) and 3,5-di-*tert*-butylcatechol (a gift by G. A. Nikiforov) were used as received.

The substrate of oxidation (diphenylmethane, Aldrich), the solvent chlorobenzene (Aldrich), the initiator azoisobutyronitrile (Merck) and the chemiluminescence activator 9,10-dibromoanthracene (Merck) were purified by standard methods.

The kinetics of CL were studied by using a slightly modified arrangement for monitoring the autoxidation of DPM in chlorobenzene at 50 °C under air atmosphere<sup>20</sup> unless otherwise specified. The rate of free radical generation (rate of initiation,  $W_{in}$ ) was determined from the induction period,  $t_{ind}$ , caused by adding the standard inhibitor, 6-hydroxy-2,2,5,7,8-pentamethylchromane at known concentrations,  $[PhOH]_0$ , with the help of the simple relationship given in eqn. (1).<sup>23</sup> The

$$W_{in} = 2[PhOH]_0/t_{ind} \quad (1)$$

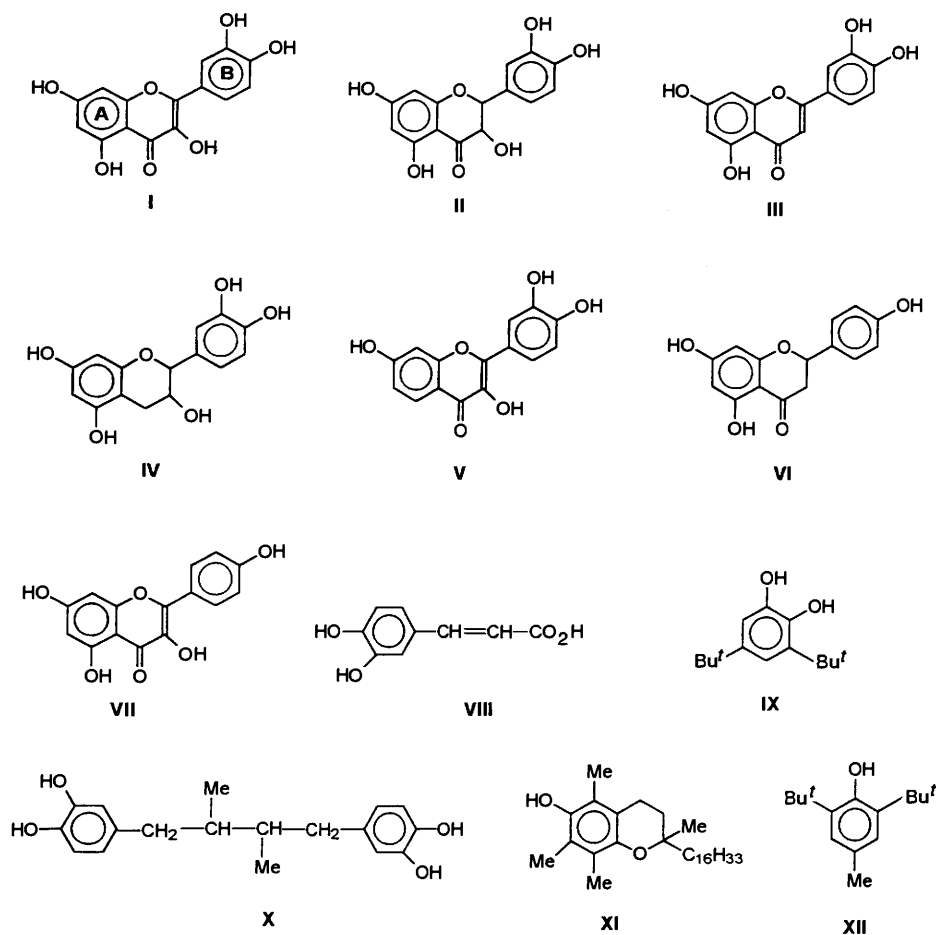
kinetic parameters of DPM autoxidation, *i.e.* rate constants for chain propagation ( $10$  dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>) and chain termination ( $1.5 \times 10^8$  dm<sup>3</sup> mol<sup>-1</sup> s<sup>-1</sup>) were determined elsewhere.<sup>24</sup> Computer simulations were performed with the help of the program 'Kinetics-2'.<sup>25</sup>

## Results and discussion

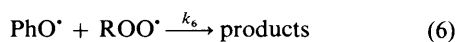
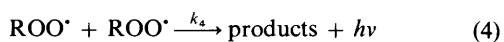
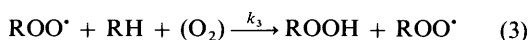
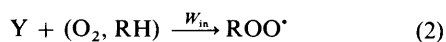
### 1. Determination of rate constants for the reaction of peroxy free radical with a chain-breaking antioxidant using the CL method for the case of a 'classic' phenol inhibitor †

The chain autoxidation of organic compounds, RH (hydrocarbons, lipids, *etc.*), initiated with a free radical initiator, Y, in the presence of a phenol antioxidant, PhOH, may be described by the following basic kinetic scheme (Q is either a quinone or a quinone methide, depending on the number and positions of

† The theory of CL accompanying the autoxidation and the use of the kinetic CL method to study chain-breaking antioxidants has been given in detail in refs. 21 and 22. Here only the most important features of this approach are outlined. The emphasis is on relations of practical importance and the deductions of equations are therefore omitted.



**Scheme 1** The structures of flavonoids (I–VII) and other phenolic antioxidants studied in this work: I, quercetin; II, dihydroquercetin; III, luteolin; IV, catechin; V, fisetin; VI, naringenin; VII, kaempferol; VIII, caffeic acid; IX, 3,5-di-*tert*-butylcatechol; X, nordihydroguaiaretic acid; XI,  $\alpha$ -tocopherol; XII, 2,6-di-*tert*-butyl-4-methylphenol (BHT)



the hydroxyl groups in the aromatic ring). Any behaviour which follows this simple kinetic scheme will be designated a 'classic' one. This scheme can be further simplified since under realistic conditions of the CL kinetic experiments  $PhO^*$  decay mainly *via* reaction (6) and reaction (7) can be neglected.<sup>21,22</sup> It is only this case which will be used in the subsequent consideration.

The concept of applying the CL method for the determination of  $k_5$  is as follows: resulting from the decay of  $ROO^*$  in reaction (4), excited states of carbonyl compounds are generated with a certain probability. Their deactivation is accompanied by

luminescence whose intensity,  $I$ , is proportional to the rate of reaction (4) [eqn. (8)] where  $\phi$  is an effective quantum yield

$$I = \phi k_4 [ROO^*]^2 \quad (8)$$

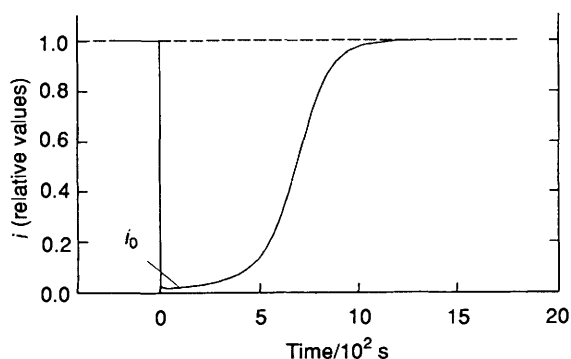
of CL for one elementary act of reaction (4). In the absence of phenols, all  $ROO^*$  molecules not taking part in the chain propagation [reaction (3)] can be accounted for by CL [eqn. (9)]. Adding a phenol to the system under consideration results

$$I_0 = \phi k_4 [ROO^*]_0^2 = \phi W_{in} \quad (9)$$

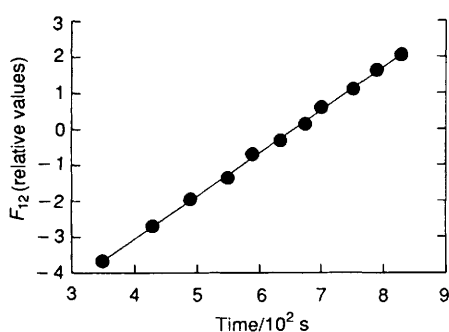
in the decrease in  $[ROO^*]$  because of reaction (5) and hence in the reduction of CL intensity. The determination of  $k_5$  actually involves the evaluation of the competition between reactions (5) and (4). In practice, the use of the relative value of CL intensity [eqn. (10)] seems to be more convenient.

$$i = \frac{I}{I_0} = \frac{k_4 [ROO^*]^2}{W_{in}} \quad (10)$$

A representative plot of a kinetic CL run is given in Fig. 1. After adding the phenol to oxidizing RH,  $i$  drops quickly reaching a minimum value equal to  $i_0$ ; the system arrives at the start of the induction period. As the phenol is progressively consumed in reaction (5),  $[ROO^*]$  increases with time and the CL intensity is restored, a so-called 'CL restitution' is observed. The relationship between  $i_0$  and the initial concentration of phenols is given by eqn. (11). The change in  $i$  during the



**Fig. 1** CL trace accompanying the oxidation of  $0.5 \text{ mol dm}^{-3}$  DPM at  $50^\circ\text{C}$  in the presence of  $0.1 \text{ }\mu\text{mol dm}^{-3}$  XI. The antioxidant was added at  $t = 0$ ;  $i$  relative intensity of CL. Conditions are: air-saturated solution, rate of initiation,  $3.1 \times 10^{-10} \text{ mol dm}^{-3} \text{ s}^{-1}$ .



**Fig. 2** Linear depiction of CL restitution in the plot of eqn. (12)

$$F_{11} = \frac{1 - i_0}{2\sqrt{i_0}} = \frac{k_5}{\sqrt{k_4 W_{in}}} [\text{PhOH}]_0 \quad (11)$$

restoration of the full CL intensity is described by eqn. (12). In the kinetic traces of  $i$  the point of maximum slope

$$F_{12} = \ln \frac{1 + \sqrt{i}}{1 - \sqrt{i}} - \frac{1}{\sqrt{i}} = \frac{k_5}{\sqrt{k_4}} \sqrt{W_{in} t} + \text{const} \quad (12)$$

lies at  $i_{\text{max}} = 0.535$  and the maximum slope is given by eqn. (13). The stoichiometric inhibition coefficient,  $f$ , that is equal to

$$\left(\frac{di}{dt}\right)_{\text{max}} = 0.237 \frac{k_5}{\sqrt{k_4}} \sqrt{W_{in}} \quad (13)$$

the number of  $\text{ROO}^{\bullet}$  scavenged by one molecule of  $\text{PhOH}$  may be determined through the integration of kinetic traces of  $i$  according to eqn. (14). Reactions (5) and (6) of the kinetic scheme predict a value of two for  $f$ .

$$f = \frac{W_{in} \int_0^{\infty} (1 - i) dt}{[\text{PhOH}]_0} \quad (14)$$

The eqns. (11)–(13) offer three independent ways to determine  $k_5$  from CL kinetics. Eqn. (11) allows  $k_5$  to be calculated from the value of  $i_0$  if  $[\text{PhOH}]_0$  is known. A value of  $k_5$  may be calculated as well from the slope of the linear depiction of CL restitution in the plot of eqn. (12) (Fig. 2). Finally, eqn. (13) makes it possible to determine  $k_5$  from the maximum value of  $(di/dt)$  at the inflection point of the trace of  $i$  vs. time,  $t$ . It should

be pointed out that the last two ways to determine  $k_5$  do not require the knowledge of the absolute concentration of the respective phenol. In the case of  $\alpha$ -tocopherol (XI in Scheme 1), values of  $k_5$  were found to be  $8.4 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  from  $i_0$  by using eqn. (11),  $8.7 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  from the kinetic traces of  $i$  in the plot of eqn. (12) (Fig. 2) and  $8.3 \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  from  $(di/dt)_{\text{max}}$  using eqn. (13). The results are only marginally higher than those compiled by Niki *et al.*<sup>26</sup> For the value of  $f$  calculated from the integral  $\int_0^{\infty} (1 - i) dt$  by eqn. (14), we obtained 2.06, *i.e.* very close to the theoretical value of 2.00. Similar values of  $k_5$  and  $f$  were obtained from different runs with variable  $[\text{PhOH}]_0$  and  $W_{in}$  (data not shown). These observations demonstrate the validity of the kinetic scheme and the applicability of the approach under consideration to determine  $k_5$ . Previously this was demonstrated for a number of phenolic antioxidants.<sup>21,22</sup>

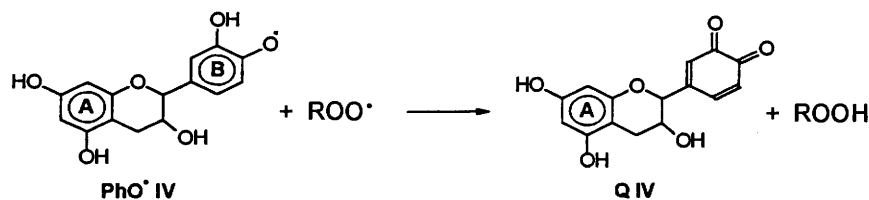
## 2. The peculiarities of CL kinetics during the oxidation inhibited by flavonoids

For various reasons, the kinetics of CL accompanying the autoxidation inhibited by flavonoids do not follow the 'classic' theory given in section 1. The first reason seems to be the fact that the theory was developed for phenols with only one active OH group, while flavonoids and catechol derivatives presented in Scheme 1 have several OH groups. At first glance, the existence of two neighbouring OH groups in the catechol moiety of flavonoids (B-ring, Scheme 1) should create no serious complications (one should remember that the enhanced reactivity of flavonoids towards free radicals is commonly associated with this moiety). We assume that  $\text{PhO}^{\bullet}$  formed by reaction (5) most likely reacts with  $\text{ROO}^{\bullet}$  in the manner depicted in Scheme 2 (to be specific, the reaction is given for IV). This pathway of the reaction may be thermodynamically favoured, owing to the very low strength of the O–H bonds in the B-ring of  $\text{PhO}^{\bullet}$ .<sup>22,27</sup>

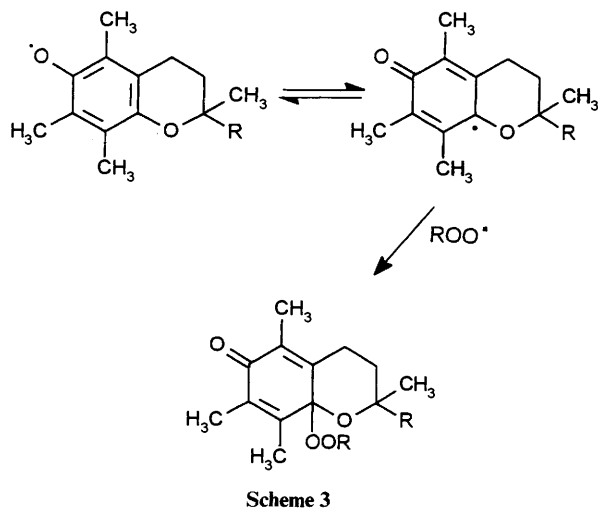
Incidentally, for  $\alpha$ -tocopherol (XI in Scheme 1) a similar mechanism for reaction (6) was proposed,<sup>28</sup> yet in this case formation of non-radical products cannot involve oxidation to quinones as in Scheme 2, but rather recombination of the radicals *via* a tautomeric structure of the phenoxy radical (Scheme 3). While in a careful study on the products of  $\alpha$ -tocopherol and the model compound 6-hydroxy-2,2,5,7,8-pentamethylchromane in a peroxy radical-generating system such adducts were not found,<sup>29</sup> a more recent study reported the isolation and identification of analogous products.<sup>30</sup> Whether semiquinone radicals, *e.g.* from flavonoids, are similarly capable of forming peroxy adducts will remain speculative, as such compounds are probably too unstable,<sup>31</sup> yet they may form quinoid tautomers.<sup>32</sup> Both pathways of reactions between  $\text{PhO}^{\bullet}$  and  $\text{ROO}^{\bullet}$  outlined in Schemes 2 and 3 correspond kinetically to reaction (6).

The reactivities of OH-groups in the A-ring and the heterocyclic ring are expected to be much lower than that of *o*-di-OH-groups in the B-ring. As the parent flavonoid is consumed in the course of the process and products of the type Q IV accumulate, the contribution of hydroxy groups of the A-ring of the products for the interaction with  $\text{ROO}^{\bullet}$ , being neglected at the outset, may increase with time. This will contribute to the overall process at relatively high starting concentrations of phenols, especially in the region of CL restitution, in agreement with experiments. For instance, with II at  $[\text{PhOH}]_0 = 1.2 \text{ }\mu\text{mol dm}^{-3}$  and  $W_{in} = 2.7 \times 10^{-10} \text{ mol dm}^{-3} \text{ s}^{-1}$ , the value of  $k_5$  estimated from kinetics of CL restitution was found to be  $5.6 \times 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  (data not shown). This is much lower than that typical of OH groups associated with B-ring catechols (see below).

As our goal is the determination of  $k_5$  for parent flavonoids rather than for products of their transformations, conditions



Scheme 2



Scheme 3

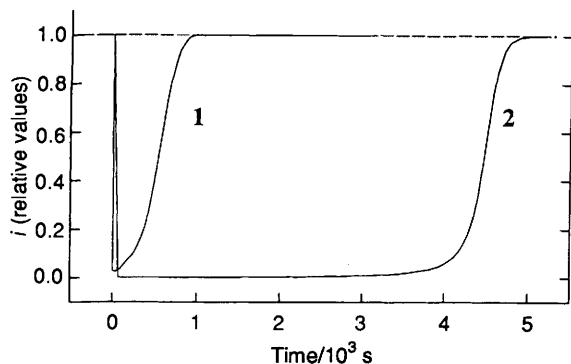


Fig. 3 Comparison of CL traces accompanying the autoxidation of  $0.5 \text{ mol dm}^{-3}$  DPM at  $50^\circ\text{C}$  in the presence of  $0.75 \text{ }\mu\text{mol dm}^{-3}$  IV (trace 1) and  $0.75 \text{ }\mu\text{mol dm}^{-3}$  XI (trace 2).  $i$ , relative intensity of CL. Antioxidants were added at  $t = 0$ . Conditions are: air-saturated solution, rate of initiation,  $3.4 \times 10^{-10} \text{ mol dm}^{-3} \text{ s}^{-1}$ .

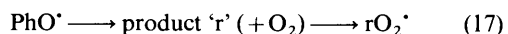
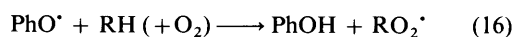
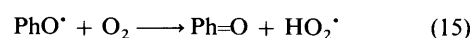
have to be adopted where the contribution of products is minimal. This may be achieved by the use of diphenylmethane (DPM) as a substrate of oxidation and a very low  $W_{\text{in}}$  of the order of  $1 \times 10^{-10} \text{ mol dm}^{-3} \text{ s}^{-1}$ . As the self-decay of  $\text{ROO}^\bullet$  from DPM is very rapid ( $k_4 = 1.5 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ; ref. 24), it is possible to observe reduction of CL intensity at  $[\text{PhOH}]$  of the order of  $0.1 \text{ }\mu\text{mol dm}^{-3}$  and even lower provided that  $k_5 \geq 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ . Under these conditions products with  $k_5 < 10^4 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  do not affect CL intensity.

However, even under these very mild conditions, the behaviour of I–V, VII–X differs from the ‘classic’ one. Fig. 3 shows CL traces for the DPM oxidation inhibited by  $0.75 \text{ }\mu\text{mol dm}^{-3}$  IV (1) and, for comparison, that of XI (2) at the same concentration. Another observation that engages our attention is that the value of  $f$  calculated from  $\int_0^\infty (1 - i) dt$  for IV is lower than the expected one. Furthermore,  $f$  shows a tendency

Table 1 The values of the stoichiometric coefficient,  $f$ , and the rate constant  $k_5$  obtained from simulated kinetic traces of  $i$  during the autoxidation of RH in the presence of PhOH with  $k_5$  fixed at  $1 \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  at various  $[\text{PhOH}]_0$  and  $k_{17}$ . The details of the computer simulations and the other kinetic parameters used in the simulations are given in the legend of Fig. 4. The protocols of  $k_5$  calculations from these data are explained in the text

$[\text{PhOH}]_0 / \mu\text{mol dm}^{-3}$	$k_{17} / \text{s}^{-1}$	$f$	$k_5 / \text{dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	
			from $i_0$	from $(di/dt)_{\text{max}}$
0.02	1	0.76	3.2	8.4
0.05	1	0.68	2.7	8.4
0.1	1	0.60	2.3	8.4
0.2	0.1	1.38	5.3	9.6
0.2	0.2	0.97	4.3	9.4
0.2	0.5	0.68	2.9	8.8
0.2	1	0.50	1.9	8.4
0.2	2	0.21	1.5	8.3
0.5	1	0.37	1.8	8.4
1.0	1	0.28	1.7	8.4
2.0	1	0.21	1.5	8.4

to decrease with  $[\text{PhOH}]_0$  (see column 3 in Table 1). A similar ‘non-classical’ behaviour was typical for most flavonoids and catechol derivatives studied with the exception of VI and X. ‘Non-classical’ behaviour of phenolic antioxidants is not a unique phenomenon and is commonly associated with increased contribution of side reactions,<sup>21</sup> of which reactions (–5), (15)–(17) should be considered (‘r’ denotes a radical with



undefined structure, derived from the original aroxyl radical). Under conditions of a normal CL run, reaction (–5) seems to be of little importance because of the very low concentration of ROOH accumulated. According to our preliminary experiments with I, IV and IX, the alteration of  $[\text{O}_2]$  from  $1.5$  to  $0.02 \text{ mmol dm}^{-3}$  and  $[\text{RH}]$  from  $0.5$  to  $1.5 \text{ mol dm}^{-3}$  exerted little if any effect on the kinetics of CL and the values of  $k_5$  and  $f$  calculated from CL traces (data not shown). This means that the side reactions (15) and (16) are of little importance either.

In the light of the foregoing observations, reaction (17), a monomolecular transformation of  $\text{PhO}^\bullet$  (dissociation or isomerization), seems to be the most probable reason for ‘non-classical’ behaviour of the majority of the antioxidants studied. Reaction (17) is conceived to result in the transformation of  $\text{PhO}^\bullet$  of little activity into (a) more active radical(s) ‘r’ (or ‘ $\text{rO}_2^\bullet$ ’) that facilitate the propagation of the peroxidation chain reaction. In the case of  $\alpha$ -tocopherol, such addition of oxygen

**Table 2** Kinetic parameters characterizing the chain-breaking activity of flavonoids and other antioxidants during the autoxidation of DPM calculated from the kinetics of CL. The notations of antioxidants are the same as in Fig. 1. The letters in parentheses denote the method of  $k_5$  calculations from the experimental data: *A*, from  $(di/dt)_{\max}$  by using eqn. (13), *B*, from  $i_0$  through the optimization of the parameters of eqn. (11a), *C*, from the linear depiction of the kinetic traces of  $i$  trace in the plot of eqn. (12)

Antioxidant	$k_5/10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	$k_{17}/\text{s}^{-1}$
I	$21 \pm 1$ (A)	$2.0 \pm 0.3$
II	$19 \pm 1$ (A)	$0.92 \pm 0.05$
III	$22 \pm 2$ (A)	$0.8 \pm 0.2$
IV	$6.6 \pm 0.5$ (A)	$7.5 \pm 0.7$
V	$12 \pm 1$ (A)	$1.1 \pm 0.2$
VI	$0.0034 \pm 0.0004$ (B)	$\sim 0.2$
VII	$1.0 \pm 0.1$ (B)	$0.8 \pm 0.2$
VIII	$15 \pm 3$ (A)	$0.65 \pm 0.07$
IX	$19 \pm 4$ (A)	$\sim 0$
XI	$8.5 \pm 0.5$ (A, B, C)	0
XII	$0.036 \pm 0.002$ (A, B, C)	0

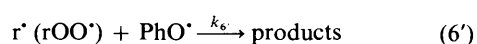
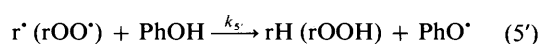
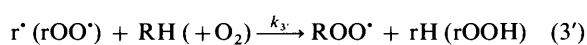
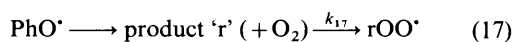
**Table 3** The kinetic parameters of IV calculated from CL traces accompanying the inhibited autoxidation of  $0.5 \text{ mol dm}^{-3}$  DPM at  $50^\circ\text{C}$  and  $W_{\text{in}} = 3.4 \times 10^{-10} \text{ mol dm}^{-3} \text{ s}^{-1}$ . Values of the stoichiometric coefficient,  $f$ , were estimated using eqn. (14);  $k_{17}$  was calculated by eqn. (11a) with  $k_5$  determined from the maximum slope of the kinetic trace of  $i$  [ $(di/dt)_{\max}$ ]. The details of the determination of  $k_5$  from CL kinetics are specified in the text

[PhOH] <sub>0</sub> / μmol dm <sup>-3</sup>	<i>f</i>	$k_{17}/\text{s}^{-1}$	$k_5/10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$		
			by eqn. (13)	by eqn. (11)	by eqn. (11a)
0.05	0.32	7.7	7.1	0.70	6.7
0.14	0.24	7.9	7.0	0.52	7.1
0.75	0.13	7.4	6.4	0.29	6.8

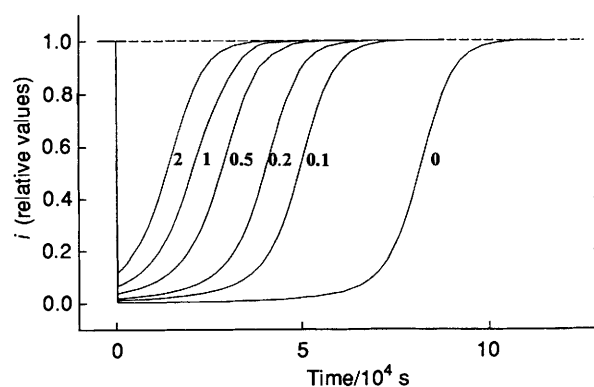
to form a peroxy radical adduct seems to be of minor importance,<sup>28</sup> in line with our assumption of  $k_{17}$  for XI as zero (see Table 2). Earlier pulse-radiolytic studies in aqueous solutions<sup>33</sup> have only rarely found such first-order decay on the timescale of these experiments (up to 1 s).

### 3. Kinetics of CL accompanying the inhibited peroxidation with regard to monomolecular transformation of PhO<sup>•</sup>

The goal of this consideration is to find a reasonable explanation for the unusual kinetics of CL accompanying the oxidation inhibited by flavonoids and related compounds and to develop a procedure for determining true values of  $k_5$ . In this case, the original scheme must be supplemented with the following reactions.



With the solution of corresponding arrays of differential equations a set of equations similar to that given in section 1



**Fig. 4** The simulated kinetic traces of  $i$  during the autoxidation of  $0.5 \text{ mol dm}^{-3}$  RH in the presence of  $0.2 \text{ μmol dm}^{-3}$  PhOH with  $k_5 = 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  at various rate constants for reaction (17) which are noted next to the individual traces. Simulations were performed on the basis of Scheme 1 [minus reaction (7)] with reactions (3'), (5'), (6') and (17') added. The other kinetic parameters used in the calculations were:  $W_{\text{in}} = 3 \times 10^{-10} \text{ mol dm}^{-3} \text{ s}^{-1}$ ;  $k_3 = k_{3'} = 10 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ;  $k_4 = 1.5 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ;  $k_5 = 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ ;  $k_6 = k_{6'} = 5 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ .

may be obtained. The main difference is the appearance of the parameter in eqn. (18) that reflects the contribution of reaction (17) as well as the parameter in eqn. (19).

$$C = \frac{k_{17}}{k_6} \sqrt{\frac{k_4}{W_{\text{in}}}} \quad (18)$$

$$D = \frac{k_3 [\text{RH}]}{k_3 [\text{RH}] + k_5 [\text{PhOH}]} \quad (19)$$

With  $k_5$  generally much slower than  $k_3$ , under these 'non-classical' conditions, eqn. (19) approaches unity and subsequent relations are given under the assumption that  $D = 1$ . The relationship between  $i_0$  and  $[\text{PhOH}]_0$  is now given by eqn. (11a). The kinetics of the CL restitution is alternatively

$$\frac{1 - i_0}{2\sqrt{i_0}} = \frac{k_5}{\sqrt{k_4 W_{\text{in}}}} \times \left( \frac{\sqrt{i_0}}{C + \sqrt{i_0}} \right) [\text{PhOH}]_0 \quad (11a)$$

described by relation (12a). It is notable that eqns. (11a) and

$$\ln \frac{1 + \sqrt{i}}{1 - \sqrt{i}} + \frac{1}{C} \ln \frac{C + \sqrt{i}}{\sqrt{i}} - \frac{1}{\sqrt{i}} = \frac{k_5}{\sqrt{k_4}} \sqrt{W_{\text{in}}} t + \text{const} \quad (12a)$$

(12a) can be reduced to the 'classic' eqns. (11a) and (12a) at  $C \rightarrow 0$ . The maximum slope of the plot of  $i$  vs. time is given by eqn. (13), but with a somewhat reduced coefficient (down to 0.186 instead of 0.237 in the limiting case when  $C \rightarrow \infty$ ). As for the stoichiometric coefficient of inhibition,  $f$  may be calculated using the 'classic' eqn. (14), but now  $f$  will not be equal to two.

Fig. 4 depicts results of computer simulations which illustrate the kinetic peculiarities of the system under study. It demonstrates that the shape of the kinetic traces of  $i$  and the value of  $\int_0^\infty (1 - i) dt$  for  $f$  are very sensitive to  $k_{17}$ , whereas the assumption that  $k_{6'} = k_6$  was without effect. Table 1 shows how the values of  $f$  and  $k_5$  calculated from CL kinetics depend on  $k_{17}$  and  $[\text{PhOH}]_0$ . In accord with experimental data, the simulation predicts the decrease in  $f$  and  $k_5$  calculated from  $i_0$  by using eqn. (11) with fixed values for both  $k_{17}$  and  $[\text{PhOH}]_0$ . At the same time the value of  $k_5$  calculated from  $(di/dt)_{\max}$

**Table 4** The minimum values of the relative CL intensity,  $i_0$ , during the autoxidation of  $0.5 \text{ mol dm}^{-3}$  DPM at  $50^\circ\text{C}$  in the presence of VII and  $k_5$  values calculated from these data. The protocols of  $k_5$  calculations are given in detail in the text

[PhOH] <sub>0</sub> / μmol dm <sup>-3</sup>	$i_0$	$k_5/10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$	
		by eqn. (11)	by eqn. (11a) <sup>a</sup>
0.092	0.696	0.46	1.12
0.18	0.533	0.41	1.12
0.37	0.381	0.32	1.04
0.55	0.294	0.28	1.03
1.47	0.140	0.18	0.98
2.85	0.075	0.14	1.09
4.69	0.047	0.11	1.02

<sup>a</sup>  $(k_5)_{av} = (1.04 \pm 0.06) \times 10^6 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ .

appears to be fairly close to the true value of  $k_5$ , as it is almost insensitive to  $k_{17}$  and [PhOH]<sub>0</sub>.

The results of these theoretical considerations suggest the way to calculate  $k_5$  and  $k_{17}$  from the kinetics of CL for phenols with 'non-classical' behaviour. When a phenol is sufficiently active and it is possible to observe a CL restitution in the region of  $i < 0.5$ , the estimation of  $k_5$  from  $(di/dt)_{\max}$  using eqn. (13) seems to be the best way to determine this rate constant. When  $k_5$  has been determined, the parameter  $C$  and subsequently  $k_{17}$  may be estimated from  $i_0$  with the help of eqn. (11a). Table 4 illustrates the validity of this approach to the calculation of  $k_5$  and  $k_{17}$  for IV. It is seen in Table 3 that the values of  $k_5$  and  $k_{17}$  calculated from CL kinetics by eqns. (11a) and (13) are constant over a rather wide range of [PhOH]<sub>0</sub>.

With a less active phenol the complete CL restitution can be observed only at rather high concentrations of the phenol when the contribution of products is significant. In this case the values of  $k_5$  and  $k_{17}$  may be estimated from the relationship between  $i_0$  and [PhOH]<sub>0</sub> by the optimization of parameters of eqn. (11a). Table 4 gives an example of this procedure. One can see that with an optimized  $k_{17}$  value of  $0.83 \text{ s}^{-1}$ , the value of  $k_5$  calculated by eqn. (11a) is kept practically constant within a 50-fold change in [PhOH]<sub>0</sub>, in contrast eqn. (11), by neglecting reaction (17), results in a decrease in  $k_5$  with [PhOH].

#### 4. The relationship of the reactivity towards ROO' with the structure of flavonoid and catechol derivatives

The kinetic parameters of flavonoids and the other antioxidants studied are listed in Table 2. Flavonoids I–V as well as compounds VIII–X, which have catechol moieties in their structure, display very high  $k_5$  rate constants, of the order of  $(1-2) \times 10^7 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ .<sup>‡</sup> These are comparable or even higher than that of  $\alpha$ -tocopherol (XI), one of the most active natural chain-breaking antioxidants that prevents lipids from peroxidation in biological membranes.<sup>35</sup> Note that the reactivity of XII, a very popular synthetic antioxidant that is in common use for stabilization of foodstuffs and other lipid products, is smaller by a factor of ca. 500 compared with that of the most active flavonoids.

In contrast, VII and especially VI, which have only one OH group in the B ring, show much lower reactivity. This observation confirms the concept that the existence of two adjacent OH groups in the B-ring is a key factor in the high reactivity of flavonoids towards ROO'. As for the effect of other structural details on the reactivity of flavonoids, the currently available data are insufficient to consider this problem.

<sup>‡</sup> As has been found with a number of phenols,<sup>24</sup> the reactivity of the peroxy radical derived from DPM is nearly twice as high compared with that of ROO' formed from other hydrocarbons used in kinetic studies such as styrene, cumene, ethylbenzene, etc.<sup>21,34</sup>

At this time the existence of reaction (17) is only kinetically verifiable and any mechanistic implications remain speculative. To solve this problem, a detailed study of products of oxidative transformations of flavonoids would be necessary, yet owing to the inherent instability of such compounds,<sup>31</sup> may be futile. In any case, the fact that flavonoid aroxyl radicals may be converted in monomolecular reactions into more highly reactive derivatives is expected to reduce the true value of these natural polyphenols as chain-breaking antioxidants under the realistic conditions of their use. Recently this was confirmed in our laboratory,<sup>36</sup> as experiments revealed only moderate antioxidant capability of flavonoids during the autoxidation of a model lipid, methyl linoleate.

## Appendix

### I. A classical phenol antioxidant

The kinetic scheme includes reactions (2)–(6). The following consideration aims to show that reaction (7) can be neglected under realistic conditions of a kinetic CL experiment. The criterion of inessentiality of reaction (7) for the total rate of chain termination may be deduced from the stationary state relation eqn. (1A). Assuming  $k_7[\text{PhO}']^2 = \gamma W_{in}$ , where  $\gamma$  is

$$W_{in} = k_4[\text{ROO}']^2 + 2k_6[\text{ROO}'][\text{PhO}'] + k_7[\text{PhO}']^2 \quad (1A)$$

the contribution of reaction (7) to the chain termination and with regard to eqn. (2A) then eqn. (3A) may be obtained. For

$$i = \frac{k_4[\text{ROO}']^2}{W_{in}} \quad (2A)$$

$$k_7 \leq \frac{k_6^2}{k_4} \times \frac{4\gamma i}{(1 - \gamma - i)^2} \quad (3A)$$

the case of DPM oxidation ( $k_4 = 1.5 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ , ref. 22) and assuming  $k_6 = 5 \times 10^8 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$  (ref. 20) the contribution of reaction (7) will be less than 10% for  $i$  near 0.5 provided that  $k_7 \leq 2.1 \times 10^9 \text{ dm}^3 \text{ mol}^{-1} \text{ s}^{-1}$ . In fact all semiquinones and phenoxy radicals meet this requirement.<sup>20,25</sup>

The original system of the differential equations is given by eqns. (4A)–(6A). Further resolution will be carried out assuming

$$\begin{aligned} d[\text{ROO}']/dt = & W_{in} - k_4[\text{ROO}']^2 - k_5[\text{ROO}'][\text{PhOH}] - \\ & k_6[\text{ROO}'][\text{PhO}'] \quad (4A) \end{aligned}$$

$$d[\text{PhO}']/dt = k_5[\text{ROO}'][\text{PhOH}] - k_6[\text{ROO}'][\text{PhO}'] \quad (5A)$$

$$-d[\text{PhOH}]/dt = k_5[\text{ROO}'][\text{PhOH}] \quad (6A)$$

a quasi-stationary state for all free radicals. Combining eqns. (5A) and (6A) with eqn. (2A) results in a function correlating certain values of  $i$  with [PhOH] [eqn. (7A)]. This relation may

$$\frac{1 - i}{\sqrt{i}} = \frac{2k_5}{\sqrt{k_4} W_{in}} \times [\text{PhOH}] \quad (7A)$$

be applied for the determination of  $k_5$  provided that the concentration of the phenol is known, e.g. at the time of addition [eqn. (7Aa)]. To obtain the relation between  $di/dt$  and

$$\frac{1 - i_0}{\sqrt{i_0}} = \frac{2k_5}{\sqrt{k_4} W_{in}} \times [\text{PhOH}]_0 \quad (7Aa)$$

$d[\text{PhOH}]/dt$ , eqn. (7A) must be differentiated. The substitution of the relation thus obtained into eqn. (6A) results in eqn. (8A).

$$di/dt = \frac{k_5\sqrt{W_{in}}}{\sqrt{k_4}} \times \frac{2i\sqrt{i}(1-i)}{(1+i)} \quad (8A)$$

The integration of eqn. (8A) yields eqn. (9A). To find  $i_b$ , the

$$\ln \frac{1+\sqrt{i}}{1-\sqrt{i}} - \frac{1}{\sqrt{i}} = \frac{k_5\sqrt{W_{in}}}{\sqrt{k_4}} t + \text{const} \quad (9A)$$

point of inflection for the kinetic trace of  $i$ , it is necessary to integrate eqn. (8A) one more time with the second derivative obtained equalling zero. The solution of eqn. (10A) yields  $i_b =$

$$3/2i_b^2 + 2i_b - 3/2 = 0 \quad (10A)$$

0.535 18. The substitution of this value of  $i_b$  into eqn. (8A) gives the relation (11A), which allows  $k_5$  to be calculated from the maximum slope of the kinetic trace of  $i$ .

$$(di/dt)_{\max} = 0.237 09 \frac{k_5\sqrt{W_{in}}}{\sqrt{k_4}} \quad (11A)$$

## II. 'Non-classical' phenol antioxidants

The kinetic scheme includes reactions (2)–(6), (17), (3') and (5'). The following system of equations is an intermediate solution of the original system obtained for the quasi-stationary assumption for all free radicals.

$$-d[\text{PhOH}]/dt = k_5[\text{ROO}^*][\text{PhOH}] - \frac{k_5[\text{PhOH}]}{k_3[\text{RH}] + k_5[\text{PhOH}]} \times k_{17} \frac{W_{in} - k_4[\text{ROO}^*]^2}{2k_6[\text{ROO}^*]} \quad (12A)$$

$$-k_5[\text{ROO}^*][\text{PhOH}] - \frac{W_{in} - k_4[\text{ROO}^*]^2}{2} - \frac{k_3[\text{RH}]}{k_3[\text{RH}] + k_5[\text{PhOH}]} \times k_{17} \frac{W_{in} - k_4[\text{ROO}^*]^2}{2k_6[\text{ROO}^*]} = 0 \quad (13A)$$

Combining eqns. (12A) and (13A) with eqn. (2A) gives eqn. (14A).

$$-d[\text{PhOH}]/dt = \frac{W_{in}}{2} (1-i) \left( 1 + \frac{k_{17}\sqrt{k_4}}{k_6\sqrt{W_{in}}\sqrt{i}} \right) \quad (14A)$$

The contribution of reaction (17) is determined by the following parameter [eqn. (15A)]. The integration of eqn. (14A)

$$C = \frac{k_{17}\sqrt{k_4}}{k_6\sqrt{W_{in}}} \quad (15A)$$

under starting conditions of  $[\text{PhOH}] = 0$  at  $t = 0$  yields eqn. (16A). As in the 'classical' case the stoichiometric coefficient of

$$[\text{PhOH}]_0 = \frac{W_{in}}{2} \int_0^\infty (1-i)dt + \frac{W_{in}}{2} \int_0^\infty \frac{1-i}{\sqrt{i}} dt \quad (16A)$$

inhibitor,  $f$ , is determined from the kinetic trace of  $i$  by using the relation (17A). Substitution of eqn. (17A) into eqn. (16A) and

$$f = \frac{W_{in} \int_0^\infty (1-i)dt}{[\text{PhOH}]_0} \quad (17A)$$

**Table 1A** The dependence on the parameter  $C$  of the point of inflection,  $i_b$ , and the coefficient  $B$  in eqn. (23A) for the maximum slope of the kinetic trace of  $i$ , calculated for a non-classical phenolic antioxidant

$C$	0	1	2	5	$\infty$
$i_b$	0.5352	0.5816	0.5887	0.5946	0.6000
$B$	0.2371	0.2052	0.1971	0.1913	0.1859

solution of the equation results in eqn. (18A).

$$f = 2 - \frac{CW_{in}}{[\text{PhOH}]_{00}} \int_0^\infty \frac{1-i}{\sqrt{i}} dt \quad (18A)$$

While in the classic case  $f$  is always equal to 2, in the 'non-classic' variant  $f < 2$ ; furthermore, with other conditions being equal,  $f$  decreases with  $C$ . Eqn. (18A) may be used for the determination of  $C$  and hence  $k_{17}$  from the kinetic trace of  $i$ .

If we now introduce the parameter in eqn. (19A) and RH is

$$D = \frac{k_3[\text{RH}]}{k_3[\text{RH}] + k_5[\text{PhOH}]} \quad (19A)$$

sufficiently active towards  $\text{ROO}^*$  ( $k_3$  is high) and  $[\text{PhOH}]$  is small, the following relation is valid. In consequence the value

$$k_3[\text{RH}] \gg k_5[\text{PhOH}]$$

of  $D$  approaches unity and eqns. (12A) and (13A) may be simplified, with eqn. (12A) approximating eqn. (6A). Combining eqns. (2A) and (15A) results in eqn. (20A). To find the relation

$$\frac{1-i}{2\sqrt{i}} = \frac{k_5}{\sqrt{k_4W_{in}}} \frac{\sqrt{i}}{C + \sqrt{i}} [\text{PhOH}]_0 \quad (20A)$$

between  $di/dt$  and  $d[\text{PhOH}]/dt$ , eqn. (20A) must be differentiated. Substitution of the relation into eqn. (6A) yields eqn. (21A). Integration of eqn. (21A) gives eqn. (22A). The way to

$$di/dt = \frac{k_5\sqrt{W_{in}}}{\sqrt{k_4}} \frac{2i\sqrt{i}(C + \sqrt{i})}{2C + \sqrt{i}(1+i)} \quad (21A)$$

$$\ln \frac{1+\sqrt{i}}{1-\sqrt{i}} + \frac{1}{C} \ln \frac{C+\sqrt{i}}{\sqrt{i}} - \frac{1}{\sqrt{i}} = \frac{k_5\sqrt{W_{in}}}{\sqrt{k_4}} t + \text{const} \quad (22A)$$

determine  $i_b$ , the point of inflection of the kinetic trace of  $i$ , that has been used for the classic case is valid for the non-classical variant as well, as it is necessary to differentiate eqn. (21A) and to set the second derivative equal to zero. To find a value of the coefficient  $B$  in the expression for the maximum slope of the

$$(di/dt)_{\max} = B \frac{k_5\sqrt{W_{in}}}{\sqrt{k_4}} \quad (23A)$$

kinetic trace of  $i$ , the value of  $i_b$  obtained has to be substituted into eqn. (21A). Now both  $i_b$  and  $(di/dt)_{\max}$  depend on the value of  $C$ , as shown in Table 1A. It is evident that these parameters change only slightly passing from the classic antioxidant ( $C = 0$ ) to the non-classical one with  $C \rightarrow \infty$ .

## References

- B. J. F. Hudson and J. I. Lewis, *Food Chem.*, 1983, **10**, 47.
- J. Torel, J. Cillard and P. Cillard, *Phytochemistry*, 1986, **25**, 383.

- 3 N. P. Das and A. K. Ratty, in *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationship*, eds. V. Cody, E. Middleton and J. B. Harborne, Alan R. Liss Inc., New York, 1986, p. 243.
- 4 A. Affany, R. Salvayre and L. Dousté-Blazy, *Fundam. Clin. Pharmacol.*, 1987, **1**, 451.
- 5 A. K. Ratty and N. P. Das, *Biochem. Med. Metab. Biol.*, 1988, **39**, 69.
- 6 A. Mora, M. Paya, J. L. Rios and M. J. Alcaraz, *Biochem. Pharmacol.*, 1990, **40**, 793.
- 7 Y. Chen, R. Zheng, Z. Jia and Y. Ju, *Free Radical Biol. Med.*, 1990, **9**, 19.
- 8 N. P. Das and L. Ramanathan, in *Lipid-soluble Antioxidants: Biochemistry and Clinical Applications*, eds. A. S. H. Ong and L. Packer, Birkhäuser Verlag, Basel, 1992, p. 295.
- 9 R. A. Larson, *Phytochemistry*, 1988, **27**, 969.
- 10 Eds. V. Cody, E. Middleton, J. B. Harborne and A. Beretz, *Plant Flavonoids in Biology and Medicine II: Biochemical, Cellular and Medicinal Properties*, Alan R. Liss, New York, 1988.
- 11 Eds. V. Cody, E. Middleton and J. B. Harborne, *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure-Activity Relationship*, Alan R. Liss, New York, 1986.
- 12 R. W. Teel and A. Castonguay, *Cancer Lett.*, 1992, **66**, 107.
- 13 B. Stavric and T. I. Matula, in *Lipid-soluble Antioxidants: Biochemistry and Clinical Applications*, eds. A. S. H. Ong and L. Packer, Birkhäuser Verlag, Basel, 1992, p. 274.
- 14 I. Morel, G. Lescoat, P. Cogrel, O. Sergent, N. Paseloup, P. Brissot, P. Cillard and J. Cillard, *Biochem. Pharmacol.*, 1993, **45**, 13.
- 15 W. Bors, W. Heller, C. Michel and M. Saran, *Meth. Enzymol.*, 1990, **186**, 343.
- 16 W. Bors, W. Heller, C. Michel and M. Saran, in *Free Radicals and the Liver*, eds. G. Csomós and J. Fehér, Springer-Verlag, Berlin, 1992, p. 77.
- 17 S. R. Husain, J. Cillard and P. Cillard, *Phytochemistry*, 1987, **26**, 2489.
- 18 M. Erben-Russ, C. Michel, W. Bors and M. Saran, *Int. J. Radiat. Biol.*, 1987, **52**, 393.
- 19 W. Bors, C. Michel and M. Saran, *Meth. Enzymol.*, 1994, **234**, 420.
- 20 V. Ja. Slyapintoch, O. N. Karpuchin, L. M. Postnikov, I. V. Zacharov, A. A. Vitcutinskii and V. F. Chepalov, *Chemiluminogenic methods for studies of slow chemical processes* (in Russian), Nauka, Moscow, 1966.
- 21 V. A. Roginsky, *Phenol antioxidants. Efficiency and reactivity* (in Russian), Nauka, Moscow, 1988.
- 22 V. A. Beljakov, E. L. Shanina, V. A. Roginsky and V. B. Miller, *Izv. Akad. Nauk USSR*, 1975, **12**, 2685 (in Russian).
- 23 V. A. Roginsky, *Biol. Membr.* (transl. from Russian), 1990, **4**, 437.
- 24 V. A. Beljakov, unpublished results.
- 25 V. A. Roginsky and H. B. Stegmann, *Chem. Phys. Lipids*, 1993, **65**, 103.
- 26 E. Niki, T. Saito, A. Kawakami and Y. Kamiya, *J. Biol. Chem.*, 1984, **259**, 4177.
- 27 V. A. Roginsky, *J. Org. Chem.* (transl. from Russian), 1990, **25**, 403.
- 28 V. W. Bowry and R. Stocker, *J. Am. Chem. Soc.*, 1993, **115**, 6029.
- 29 M. Matsuo, S. Matsumoto, Y. Iitaka and E. Niki, *J. Am. Chem. Soc.*, 1989, **111**, 7179.
- 30 R. Yamauchi, *Vitamins (Japan)*, 1991, **65**, 47.
- 31 K. Kano, T. Mabuchi, B. Uno, Y. Esaka, T. Tanaka and M. Inuma, *J. Chem. Soc., Chem. Commun.*, 1994, 593.
- 32 Y. Shinagawa and Y. O. Shinagawa, *J. Am. Chem. Soc.*, 1978, **100**, 67.
- 33 W. Bors and M. Saran, *Free Radical Res. Commun.*, 1987, **2**, 289.
- 34 A. B. Ross, W. G. Mallard, W. P. Helman, G. V. Buxton, R. E. Huie and P. Neta, *NDRL-NIST Solution Kinetics Database—Version 2*, NIST Standard Reference Data, Gaithersburg, Maryland, 1994.
- 35 K. U. Ingold and C. W. Burton, *Acc. Chem. Res.*, 1986, **19**, 194.
- 36 V. A. Roginsky, T. K. Barsukova, A. A. Remorova and W. Bors, *J. Am. Oil Chem. Soc.*, 1995, in the press.

Paper 5/02486B

Received 19th April 1995

Accepted 22nd June 1995