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Review of methods to determine chain-breaking antioxidant activity in food

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Abstract

The beneficial influence of many foodstuffs and beverages including fruits, vegetables, tea, red wine, coffee, and cacao on human health has been recently recognized to originate from the chain-breaking antioxidant activity (AOA) of natural polyphenols, a significant constituent of the above products. For this reason, the dietary value of such products is determined to a large extent by their AOA. The latter stimulated the development of effective and reliable methods for determining AOA. Although the kinetic approach provides the basis of the majority of these methods, only a few of them have been analyzed from the viewpoint of chemical kinetics. This review is intended to close down this gap, at least partly. The most popular methods for determining chain-breaking AOA of food are considered with the aim to estimate their reliability and limitations. The main requirements imposed on these methods have been suggested. The main attention has been paid to the repeatability of the data obtained. Along with the methods that are currently popular among researchers working in food chemistry and biomedical sciences, perspectives of the application of the methods used to studying industrial antioxidants have also been considered. The review consists of two main sections. In the first general section, definitions of the main parameters used to characterize AOA are given and the kinetic basis of the methods applied is considered in some detail. The second section is devoted to particular methods including some technical details. In conclusion the data on AOA obtained by various methods are correlated with each other.

1. Introduction

The chain-breaking antioxidant activity (AOA) of many natural products is now a hot point. The interest in this problem quickened since about 1990 when it was widely recognized that the beneficial influence of many foodstuffs and beverages including fruits, vegetables, tea, red wine, coffee, and cacao on human health is associated to a large degree with AOA of natural polyphenols, an essential constituent of the above products. These days, AOA of many natural products is considered as a significant parameter determining their dietary value.

The problem of AOA of food has at least two sides. First, AOA per se, i.e. the antioxidative potential, which is determined by the antioxidant composition and antioxidative properties of constituents. Second, the biological effects that depend, among other things, on antioxidant bioavailability. The first is the subject of food chemistry; the second is a medico-biological problem. Only the first will be the subject of the review. As for the biomedical side of the problem, this has been a subject of numerous reviews (for instance, Mukhtar & Ahmad, 2000; Ursini & Sevanian, 2002; Yang & Landau, 2000) and will be excluded from further consideration in this work. At the same time, many works comprise the mixture: AOA potential and biological effects are considered together. Although characterizing AOA of food via their biological effects seems to be generally not fruitful,

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some works of such a kind will be mentioned in the review.

The explosive interest in AOA of food in the early 1990s was accompanied by the development of new methods for the determination of AOA. Although many of these new methods are conceptually kinetic ones the majority of these methods have no reliable theoretical kinetic basis. Meanwhile, the determination of AOA is one of the traditional subjects of the kinetics of free radical reactions and the theoretical basis for such a determination has been well developed many years ago (Barclay & Vinquist, 2003 and references therein). It seems to be rational to consider the validity of these methods for reliable and repeatable determining AOA of food. from the viewpoint of chemical kinetics. In addition, some ideas common for chemical kinetics of free-radical reactions may be suggested to improve the existing methods for determining AOA of food and to develop new ones. Both will be the subject of this review. There is no comprehensive overview of this problem yet. It is possible to mention a few rather short surveys on the methods to determine in AOA of biological samples (Frankel & Meyer, 2000; Llesuy, Evelson, Campos, & Lissi, 2001; Prior & Cao, 1999).

2. General

2.1. Definitions

By definition, the antioxidant activity is the capability of a compound (composition) to inhibit oxidative degradation, e.g. lipid peroxidation. Phenolics are the main antioxidant components of food. While in plant oils and fats these are basically monophenolics, first of all tocopherols (vitamin E), water-soluble polyphenols are more typical in water-soluble products (fruits, vegetables, tea, coffee, wine). Although AOA of polyphenols is associated with various mechanisms, the elevated reactivity of phenolics towards active free radicals is considered as the most principle mechanism. We should distinguish between the antioxidant capacity and the reactivity. While the antioxidant capacity gives the information about the duration of antioxidative action, the reactivity characterizes the starting dynamics of antioxidation at a certain concentration of an antioxidant or antioxidant mixture.

When we deal with the inhibition of chain oxidation, e.g. with lipid peroxidation, an antioxidant working thanks to its reaction with a chain-carrying free radical is named a chain-breaking antioxidant. One should distinguish between the antiradical activity and AOA. The former is completely determined by the reactivity of an antioxidant to active free radical, which, in turn, may be characterized by the rate constant for the corresponding reaction. Meanwhile, by AOA we mean the capability to retard the oxidative degradation. The high antiradical activity does not always go together with high AOA. In particular, some synthetic phenolics possessing a rather high reactivity to active free radicals show only a moderate chain-breaking antioxidant activity because of the rather high chemical activity of the derived phenoxy radical and/or semiquinones (see for instance Roginsky, Barsukova, Loshadkin, & Pliss, 2003). Besides, a real AOA is sometimes partly determined by the involvement of products of oxidative transformation of original antioxidant into inhibition (Roginsky, 2003).

As for the biologically relevant active free radicals, which may be scavenged to retard the oxidative destruction, a peroxy radical, $LO_2^{\bullet}$, superoxide anion, $O_2^{\bullet-}$, the hydroxyl radical, $HO^{\bullet}$, and active forms of nitrogen are most frequently considered. The methods for the determination of AOA of agents active to free-radical forms of nitrogen have not been well developed yet and these will not be reviewed in this work. As for "specific" scavengers of $HO^{\bullet}$, this rests on misunderstanding. On one hand, in many cases $HO^{\bullet}$ plays a key role in the oxidative destruction; on the other hand, $HO^{\bullet}$ is so reactive that the reaction between $HO^{\bullet}$ and the majority of organic substrates occurs during the first encounter (diffusion-controlled reactions) (Ross et al., 1994). Thus, any speculations on "specific" acceptors of this species seem to be unreasonable. At the same time, it is rational to say about the inhibition of the processes resulting in the formation of $HO^{\bullet}$, but this is beyond the scope of this review. So the attention will be concentrated on the mechanisms associated with scavenging $LO_2^{\bullet}$ and partly $O_2^{\bullet-}$. It is pertinent to note that $LO_2^{\bullet}$ is the main chain-carrying radical during lipid peroxidation.

2.2. Direct and indirect approaches to AOA determination

Two approaches are applied to determine chain-breaking AOA, direct and indirect. When the indirect approach is applied, they study most frequently the ability of antioxidant to scavenge some free radicals, which is not associated with the real oxidative degradation, or effects of transient metals. For instance, some stable colored free radicals are popular due to their intensive absorbance in the visible region. In this case we change determining chain-breaking AOA for determining H-donating activity. However, many authors doing so state that they determine the chain-breaking AOA. Generally this is not correct, although in special cases the H-donating activity may correlate with AOA. Clearly, preference should be given to direct methods, with all other factors being equal. While the direct methods associated basically with the studies of chain peroxidation have a general theoretical background, each specific indirect method has essential features that do not allow their general consideration. They will be discussed in the

second part of the review, when particular methods will be considered.

As already mentioned, direct methods are based on studying the effect of a tested food containing antioxidants on the oxidative degradation of a testing system. As for the substrate of oxidation, this may be individual lipids, lipid mixtures (oils), proteins, DNA, or lipid-containing biologically relevant species, such as blood plasma, LDL, biological membranes, etc. Lipid peroxidation seems to be the most convenient for this purpose. Depending on the solubility of a sample, the preference should be given to the oxidation of homogeneous lipids or to aqueous microheterogeneous systems, such as micelles and liposomes. Among other things, the kinetics of lipid peroxidation in homogeneous solutions and in micelles and liposomes are well studied, both experimentally and theoretically (Barclay & Vinquist, 2003).

The methods suggested for determining AOA are basically the same for individual antioxidants and for complex mixture. We shall start with individual antioxidants and then consider how the methods can be applied for testing food samples.

2.3. The theory of lipid peroxidation

The most promising methods suggested for the determination of AOA are based on the kinetic studies of lipid peroxidation. This is a reason why the kinetics of lipid peroxidation should be considered in some detail. The kinetic theory of the chain oxidation was basically developed many yeas ago. A reader can find this in many specialized publications, for instance, Barclay and Vinquist (2003); Burton and Ingold (1986); Niki (1987); Roginsky (1988). In the most simple form the chain lipid peroxidation may be described by the kinetic Scheme 1, where LH is a substrate of the oxidation, QH_2 is a phenolic antioxidant (polyphenol).

(0)	$Y\ (+LH + O_2) \longrightarrow LO_2^{\bullet}$	R_{IN}
(1)	$LO_2^{\bullet} + LH \longrightarrow L^{\bullet} + LOOH$	k_1
(1a)	$L^{\bullet} + O_2 \longrightarrow LO_2^{\bullet}$	
(2)	$LO_2^{\bullet} + LO_2^{\bullet} \longrightarrow$ products	$2k_2$
(3)	$LOOH^{\bullet} \longrightarrow$ free radicals and molecular products	k_3
(4)	$QH_2 + LO_2^{\bullet} + \longrightarrow QH^{\bullet} + LOOH$	k_4
(5)	$QH^{\bullet} + LO_2^{\bullet} + \longrightarrow$ products	

Scheme 1. The peroxidation of LH initiated by Y and inhibited by antioxidant QH_2.

2.3.1. Non-inhibited oxidation

A non-inhibited chain process consists of three main stages: initiation, chain propagation, and chain termination. All the primary reactions resulting in the formation of active free radical may be assigned to the initiation, some ways to produce active free radicals will be considered below. Hydroperoxide, LOOH, a principle product of lipid peroxidation, can be also a source of active free radicals due to its thermolysis or catalytic destruction. Being conceptually the initiation, the destruction of LOOH with formation of an active free radical (reaction (3)) is classified as a special stage – degenerate chain-branching. When oxygen concentration is high enough, the radicals formed by reaction (0) are rapidly converted into $LO_2^{\bullet}$. This is also true for stage (1). The primary product of reaction (1), a C-centered radical $L^{\cdot}$, is rapidly converted into $LO_2^{\bullet}$ due to the very fast reaction $L^{\bullet} + O_2 \rightarrow LO_2^{\bullet}$. As a consequence, $LO_2^{\bullet}$ is the only principle chain-carrying free radical. Independent of the way of initiation, the rate of non-inhibited chain oxidation, R_0, is given by the equation:

$$R_0 = k_1[LH]\sqrt{\frac{R_{IN}}{2k_2}}. \quad \text{(v)}$$

which shows that R_0 is directly with [LH] and proportional to the root of R_{IN}. The length of kinetic chain for the non-inhibited oxidation, v, is

$$v = \frac{R_0}{R_{IN}} = \frac{k_1[LH]}{\sqrt{2k_2R_{IN}}}. \quad \text{(vi)}$$

For many testing systems of practical significance v is very high being equal to several tens and even several hundreds. Although this theory was developed originally for the oxidation in homogeneous solutions, this was later testified also for the oxidation in micelles and liposomes.

2.3.2. Inhibited oxidation

The chain oxidation can be retarded via scavenging the chain-carrying radical $LO_2^{\bullet}$ by a donor of H-atom (reaction (4)). The essential feature of functioning of polyphenols as an antioxidant is that the primary OH-substituted phenoxy radical $QH^{\cdot}$ is converted in aqueous phase at neutral pH into semiquinone $Q^{\cdot -}$ due to fast deprotonation

$$QH^{\bullet} \rightarrow Q^{\bullet -} + H^{+}$$

Contrary to $QH^{\cdot}$ derived from polyphenols, phenoxy radicals produced from monophenolics exist even in aqueous medium as non-charged species. Many natural phenolics including tocopherols in plant oils, flavonoids in many fruits and vegetables, catechins in tea, red wine, etc. belong to the most potent H-donors.

The antioxidant activity of QH_2 (as well as monophenolics) may be characterized by two independent parameters, rate constant for reaction (4), k_4, and

stoichiometric coefficient of inhibition, f, which shows how many kinetic chains may be terminated by one molecule of QH_2. While the first parameter characterizes antioxidant reactivity, the second gives antioxidant capacity. For monophenolics including "classical" chain-breaking antioxidants, such as BHT, α-tocopherol, and Trolox, $f = 2$ (Burton et al., 1985). At the same time, for many polyphenols, f differs considerably from two. Furthermore, f depends on the conditions of oxidation (see for instance, Loshadkin, Roginsky, & Pliss (2002)). In the frame of kinetic Scheme 1, for the case $f = 2$, the rate of inhibited oxidation, R, is

$$R = \frac{k_1[\mathrm{LH}]R_{\mathrm{IN}}}{2k_4[\mathrm{QH_2}]}. \quad \text{(vii)}$$

Eq. (vii) corresponds to the simplest kinetic scheme. Meanwhile, some side reactions, which are not included to the above scheme, can significantly alter both Eq. (vii) and the f value. However, this simplest scheme and Eq. (vii) is quite suitable to consider the most general approaches to AOA determination.

Various methods for determining the rate constant for the key reaction (4), k_4, have been reported in the literature (Barclay & Vinquist, 2003; Roginsky, 1988). When choosing a certain method we should account for certain features of the system applied such as the nature of the antioxidant tested, instrumentation available, and other factors. This procedure is the best developed for the case when the antioxidant is a monophenolic. The key point of this approach is that the process is studied under condition of the steady R_{IN}, which may be easily controlled and altered. This is commonly achieved by using thermo-labile azocompounds. When the testing experiment is conducted at moderate temperatures, the consumption of initiator as well as the contribution of LOOH into initiation may be almost neglected. This is so-called the mode of the controlled chain reaction. This mode provides a rather easy and reliable determination of k_4 and f. The reactivity of antioxidant is originally determined as the k_4/k_1 ratio; as for the absolute value of k_4, this may be calculated from k_4/k_1 provided that k_1 is known.

Let us consider in outline the protocol for the case when the kinetics of oxidation are monitored via oxygen consumption following works by Loshadkin et al. (2002); Roginsky (1990a). There are two ways to determine k_4/k_1 from the kinetics of inhibited oxidation: from the starting value of R (static method) and from the whole kinetic curves (dynamic method). In the frame of Scheme 1, the starting value of R is determined by the following parameter (for the case $f = 2$)

$$F_1 = \frac{R_0}{R} - \frac{R}{R_0} = \frac{2K_4R_0}{k_1[\mathrm{LH}]R_{\mathrm{IN}}}[\mathrm{QH_2}], \quad \text{(viii)}$$

k_4/k_1 can be calculated from F_1 determined at a certain concentration of QH_2 or better from the plot of F_1 vs. $[QH_2]$. The calculation of k_4/k_1 from experiment by using Eq. (viii) requires the knowledge of R_{IN}. R_{IN} can be determined in a separate experiment by using a reference antioxidant (see below). The dynamic method is based on the study of the change in R with time due to consumption of an antioxidant. While the static method for determining k_4/k_1, is rather universal, the dynamic one is applicable in the frame of the kinetic Scheme 1 only. For many individual natural polyphenols and complex mixtures the kinetic scheme is evidently more complex. When going from a simple testing system (oxidation of model hydrocarbons in organic solvent) to more complex systems, such as the oxidation of homogeneous lipids or the oxidation of lipids in microheterogeneous systems (micelles, liposomes) the principle of determination remains the same. The problem is that k_4 determined in more complex systems cannot be assigned to the rate constant for elementary reaction. In this case it is an effective parameter only, which is governed not only by the reactivity of QH_2 in reaction (4), but also by some physical factors including formation of H-bonds between QH_2 and lipid and water as well as localization and molecular dynamics of QH_2 in microheterogeneous system. Some examples of such a kind a reader may find in the literature (for instance, Barclay & Vinquist, 2003; Roginsky, 1990b). Anyway, k_4, more exactly k_4/k_1, gives an objective information on the capability of QH_2 to retard lipid peroxidation in any testing system.

2.3.3. Induction period and stoichiometric coefficient of inhibition

There is no any theoretically based general definition of the induction (lag) period. In chemistry, the induction period (t_{IND}) is most frequently defined as the moment when the rate of a chemical process, for instance, the lipid peroxidation, drastically increases. So defined t_{IND} is a rather uncertain value, which is affected by the way of monitoring, sensitivity of the equipment used, and some other factors. The situation becomes more definite when we deal with the chain oxidation. In the frame of the simplest kinetic Scheme 1, t_{IND} for the case of an individual antioxidant may be given by the equation

$$t_{\mathrm{IND}} = f[\mathrm{QH_2}]_0/R_{\mathrm{IN}}, \quad \text{(ix)}$$

where R_{IN} is the rate of free radical generation (rate of initiation), $[QH_2]_0$ is the starting concentration of inhibitor. Eq. (ix) may be also used to calculate the stoichiometric coefficient of inhibition provided that $[QH_2]_0$ is known.

Despite the simplicity of determining t_{IND} in theory, in reality this presents some difficulties. Commonly, t_{IND} is determined as an intersect between the tangent of the kinetic curve during the propagation phase and the time axis as shown in Fig. 1. However, this "graphical" procedure has actually no reliable theoretical basis and it

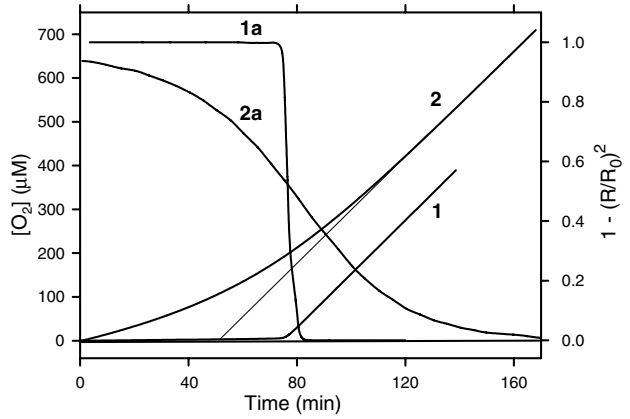

Fig. 1. Kinetics of oxygen consumption during the oxidation of 5 mM LH inhibited by 4×10^{-6} M QH_2 with various reactivity to $LO_2^{\bullet}$ as simulated on the basis of Scheme 1. Plot 1: $k_1 = 1 \times 10^6$ $M^{-1} s^{-1}$; plot 2: $k_1 = 3 \times 10^3$ $M^{-1} s^{-1}$; the other parameters are: $R_{IN} = 1.8 \times 10^{-9}$ $M s^{-1}$; $k_1 = 70$ $M^{-1} s^{-1}$; $2k_2 = 2.1 \times 10^4$ $M^{-1} s^{-1}$; $k_5 = 2 \times 10^8$ $M^{-1} s^{-1}$ (Roginsky, 2003).

generally is not correct (Loshadkin et al., 2002). More correctly, the value of t_{IND} may be determined by integration

$$t_{IND} = \int_{\infty}^{0} \{1 - (R/R_0)^2\}\, dt, \qquad (x)$$

where R and R_0 are the rate of chain oxidation in the presence and the absence of an antioxidant, respectively. Eq. (x) was deduced on the base of Scheme 1 (Loshadkin et al., 2002). Two approaches to determining t_{IND} for the case when the oxidation is inhibited by an individual antioxidant are compared in Fig. 1. While with the most active antioxidants, the "graphical" procedure gives t_{IND} that is not much different from the expected (theoretical) value (83.3 min under the conditions of Fig. 1), t_{IND} determined "graphically" with less active antioxidants is significantly underestimated. Meanwhile, the determination of t_{IND} by using integral Eq. (x) gives exactly the same theoretical value t_{IND}, regardless of antioxidant reactivity. The latter procedure may be also recommended to determine t_{IND} during testing complex mixtures including food samples. At the same time, t_{IND} itself is hardly suitable for characterizing AOA. The matter is that t_{IND} depends on many factors, which sometimes are difficult to reproduce. To do this, the parameters of a testing system, first of all R_{IN}, should be known.

2.3.4. Percentage of inhibition and IC_{50}

Percentage of inhibition and IC_{50} (the concentration of antioxidant which provides 50% inhibition) are used very frequently as parameters characterizing the antioxidant power. Originally, both parameters have been derived from biochemistry to characterize the capability of some substrates to inhibit enzyme activity. In some cases, this approach remains acceptable when we determine the inhibition of non-chain processes, such as the oxidative degradation of DNA, reactions with stable free radicals, etc. However, this approach becomes almost unsuitable when we deal with the inhibition of chain processes. In this case, percentage of inhibition and thus IC_{50} are governed not only by the reactivities of QH_2 and substrate of oxidation, but they also depend on variable parameters, such as the length of kinetic chain and R_{IN}.

Consider this in more detail based on the Scheme 1. Percentage of inhibition may be calculated from combination of Eqs. (v) and (vii)

$$R/R_0 = \frac{k_1[LH]\sqrt{2R_{IN}k_3}}{2k_4[QH_2]k_2[LH]} = \frac{\sqrt{2R_{IN}k_3}}{2K_4[QH_2]}. \qquad (xi)$$

The expression for IC_{50} may be obtained from Eq. (xi) taking $R/R_0 = 0.5$

$$IC_{50} = \frac{\sqrt{2k_3R_{IN}}}{k_4} = \frac{k_1[LH]}{k_4 v_0}. \qquad (xii)$$

As it may be seen from Eq. (xii), IC_{50} increases when [LH] increases and v_0 decreases. Eqs. (xi) and (xii) are approximate as they have been deduced without regard to reaction (3). Fig. 2 depicts the exact solution for the problem obtained through computer kinetic simulation. It is evident that both the position of the inhibition percentage plot and absolute value of IC_{50} depend dramatically on the parameters of the system applied to testing, R_{IN}, v_0, and [LH]. Among other things, Eq. (xii) and Fig. 2 demonstrate that the use the chain reaction as a testing system provides the higher sensitivity for antioxidant determination – the higher v_0, the lower the concentration of antioxidant at which the inhibition can be detected.

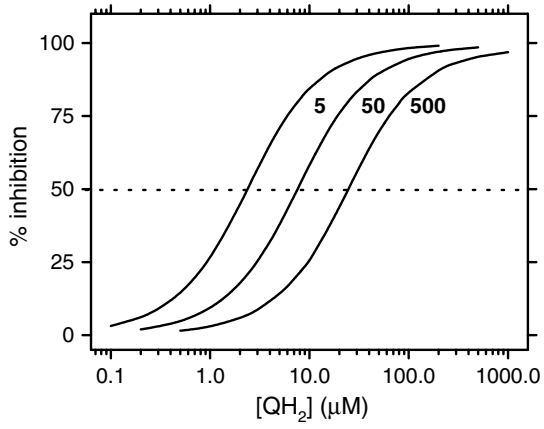

Fig. 2. Plots of % inhibition vs. QH_2 concentration at various R_{IN} (given in nM/s) simulated on the basis of Scheme 1. Parameters used are: [LH] = 1 M; $k_1 = 100$ $M^{-1} s^{-1}$; $2k_2 = 2 \times 10^7$ $M^{-1} s^{-1}$; $k_4 = 1 \times 10^5$ $M^{-1} s^{-1}$; $k_5 = 3 \times 10^8$ $M^{-1} s^{-1}$.

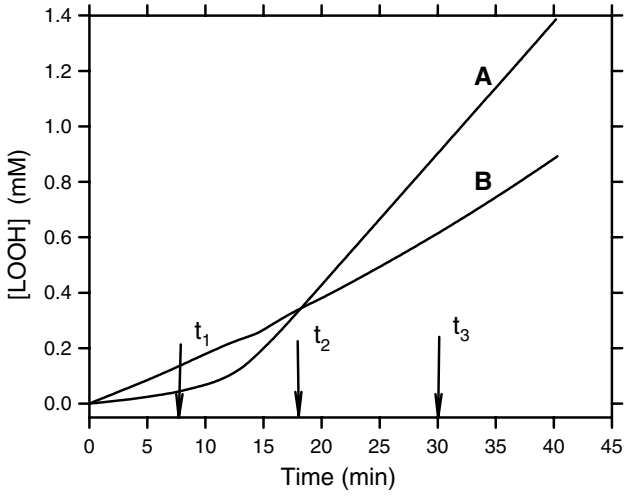

Fig. 3. Kinetics of the oxidation of 0.5 M LH inhibited by QH_2 of various reactivity to $LO_2^{\bullet}$ as simulated on the as simulated on the basis of Scheme 1. (a) 2×10^{-6} M QH_2; $k_4 = 1 \times 10^6$ $M^{-1}s^{-1}$. (b) 8×10^{-6} M QH^2; $k_4 = 5 \times 10^4$ $M^{-1}s^{-1}$. The other parameters used are: $R_{IN} = 5 \times 10^{-9}$ nM/s; $k_1 = 100$ $M^{-1}s^{-1}$; $2k_2 = 2 \times 10^7$ $M^{-1}s^{-1}$; $k_4 = 1 \times 10^5$ $M^{-1}s^{-1}$; $k_5 = 3 \times 10^8$ $M^{-1}s^{-1}$.

One more reason why inhibition percentage and IC_{50} are not suitable to characterize AOA should be mentioned. Fig. 3 depicts the kinetics of the oxidation inhibited by two antioxidants, A and B. A is very reactive and provides a very significant inhibition, but only during a short period of time; B is less reactive and provides less pronounced inhibition even being taken at concentration higher by a factor of three. It may be seen the range of A and B depends on the moment when AOA is compared. While at t_1 AOA(A) > AOA(B), at $t_2 > t_1$ AOA(A) = AOA(B), and at $t_3 > t_2$ AOA(A) < AOA(B). It should be concluded that inhibition percentage and IC_{50} reported in one work may be compared directly with those determined in another work only provided that all other parameters are the same. It is most typical to arrange the compounds (food samples) tested in the order of antioxidant activity ($A > B > C$ and so on). However, even the range of a certain individual antioxidant in such a series may change when going from one work to another (see below).

2.4. Going from determination of AOA of individual compounds to that of complex mixtures

The above considerations concerned individual antioxidants. The majority of the parameters under consideration can be also applied to characterize AOA of food samples. To do this, the mentioned kinetic parameters should be changed for effective values. In particular, instead of f it is possible to use N, the parameter showing the number of kinetic chains, which can be terminated by a food sample. N is conceptually the sum of $f[QH_2]$ product. The rate constant for reaction (4) may be changed for the effective parameter $\sum k_4[QH_2]$ or for inhibition percentage determined under well-defined and standardized conditions.

2.5. The requirements imposed on the rational methods (testing systems) for determining AOA of food samples

A rational method for determining AOA of food samples must meet, among other things, the following requirements:

1. The evident physical meaning of the parameter determined. To provide this, the determination should be based on a well-developed theory, which, in turn, requires that the processes occurring in a testing system are known in detail and can be described by a definite kinetic scheme.
2. The reproducibility of determinations not only within a single work, but also the repeatability of the assay at any other laboratory. This objective can be accomplished, first of all, by using a rather simple testing system and commercially available chemicals. Besides, results of the determination should be independent of the variation in parameters of the test experiment within reasonable ranges.
3. The steady monitoring,which makes it possible to automate the determination and to observe fine kinetic details of functioning of antioxidants containing in tested samples. In almost all the cases the methods that allow steady monitoring are more preferential as compared to those required taking aliquots.
4. The high enough productivity to make it possible to perform routine determinations. This is not obligatory when is designed for more academic studies.
5. The relative simplicity of the procedure to allow the determination in any laboratory specialized in food chemistry and related fields.

None of the suggested methods actually meet all the mentioned requirements. Each time we are forced to think about a compromise between various requirements. At the same time, fulfillment of the requirements (1) and (2) seems to be imperative. However, as this will be demonstrated below, some reported methods do not meet even requirements (1) and (2).

3. Particular methods

3.1. Direct methods based on the kinetics of lipid peroxidation

3.1.1. Two modes of lipid peroxidation

There are two modes of lipid peroxidation that may be used for testing. The first one is the autoxidation

mode when the process is progressing spontaneously, with self-acceleration due to accumulation of LOOH. This approach has several shortcomings, first of all, the fact that R_{IN} changes with time and remains out of control. Besides, the kinetics of autoxidation are highly sensitive to admixtures of transition metals and to the starting concentration of LOOH. As a result, the repeatability of experiments based on the autoxidation is a big problem. The other problem is that it is complicated to suggest a well-defined parameter characterizing AOA. The parameters usually used in the works applying the autoxidation mode, t_{IND} and percentage of inhibition, are actually not repeatable and can provide the comparison in the frame of a single work only. In conclusion, the methods using the autoxidation mode are hardly suitable for systematic determinations of AOA of food. The second, much more promising approach is based on the use of the kinetic model of the controlled chain reaction(see above). This mode offers to obtain reliable, easily interpretable, and repeatable data. The kinetic theory of the controlled chain reaction was discussed in outline in Section 2. Some more practical aspects will be considered below. Several versions of the kinetic models of controlled chain reaction were widely applied to determining AOA of synthetic phenolic antioxidants starting in the 1950s (Barclay & Vinquist, 2003; Roginsky, 1988), while biologically relevant substances and natural antioxidants began to be studied systematically only in the 1980s. One of the reasons for that was the difference in solubility between synthetic and natural phenolics. While the most synthetic antioxidants designed for stabilization of polymers and other organic materials are lipid-soluble, the majority of natural antioxidants are water-soluble. To test natural water-soluble antioxidants, microheterogeneous systems, micelles, liposomes, lipoproteins (basically LDL), biological membranes as well as blood plasma were suggested. Starting works in this area have been reviewed by Burton and Ingold (1986) and Niki (1987).

3.1.2. Substrate of oxidation

When choosing a substrate of oxidation, preference should be given to individual compounds. The use of commercially accessible individual compounds provides the repeatability of determination in any laboratory. Among individual lipids, methyl linoleate and linoleic acid seem to be the most convenient. These compounds are relatively cheep and their oxidation is quite representative of the most essential features of biologically relevant lipid peroxidation. As for the use of lipid mixtures such as plant oils and biologically relevant substrates, they cannot be recommended, as it is impossible to provide the identity of substrate. Besides, biologically originated substrates usually contain endogenous chain-breaking antioxidants (vitamin E, etc.), which can intervene in the testing procedure. The search of a specific method is determined to a large degree by the character of the sample tested. For lipid-soluble samples, plant oil, fats, lipid extracts, etc., the kinetic models based on the oxidation of homogeneous lipid or a model hydrocarbon, e.g. styrene, are preferential. As for water-soluble samples, aqueous systems seem to be more suitable. At the same time, several ''universal'' testing systems suitable for testing both lipid-soluble and water-soluble samples have been suggested (Barclay, Baskin, Dakin, Locke, & Vinquist, 1990; Pryor, Strickland, & Church, 1988; Roginsky, 1990b; Roginsky & Barsukova, 2001). A proper search of the testing system makes it possible to circumvent any preliminary procedure (extraction and so on).

3.1.3. Initiation

The use of thermolabile azo-compounds, most typically water-soluble 2,2′-azobis(2-amidinopropan) dihydrochloride (AAPH) and lipid-soluble 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN), is very convenient. The above azo-compounds decompose and produce active free radicals at moderate temperatures at any desired rate, which can be easily altered and controlled. The advantage of these initiators is that the rate of free radical production is proportional to initiator concentration and independent of other components of testing system. Meanwhile, redox initiation by using transition metals, such as Fe(3+)/ascorbate; Cu(2+) etc., is still very popular among researchers working in food chemistry and in biomedical sciences. However, this way of initiation is usually unsuitable for quantitative tests for several reasons. First, the chemistry and kinetic regularities of such systems are rather complex and have not been studied in detail yet. Some components of these systems (for instance, ascorbate) react with free radicals and hence interfere with the inhibition. Second, the rate of initiation, R_{IN}, usually remains indeterminate; what is more, R_{IN} changes with time due to interaction of transition metals with LOOH. Third, the application of transition metal-containing systems does not allow the discrimination of the inhibition mechanisms different from chain-breaking. The matter is that the reason for the inhibition by natural phenolics may be explained by, along with chain-breaking, chelating of catalytically active transition metals. A reader can find numerous examples of such studies in the literature. The repeatability of the testing systems using a redox initiation is commonly far from been perfect.

3.1.4. Monitoring of lipid peroxidation

Two kinds of monitoring are possible: the continuous monitoring and that via the collection of a number of aliquots. The first way seems to be preferable in all the cases. Continuous monitoring not only offers a higher productivity of assay, but also allows the observation of fine details of the process under study. Measurement

of LOOH determined as conjugated dienes (absorbance at 234 nm) and the TBARS assay (tiobarbituric acid reactive substances) are the most popular. Among suggested methods, the determination of conjugated dienes and oxygen consumption is most suitable for continuous monitoring. It is possible to find the data in the literature allowed the correlation between various ways of monitoring lipid peroxidation in PC liposomes (Yamamoto, Niki, Kamiya, & Shimasaki, 1984) and LDL (Noguchi, Gotoh, & Niki, 1993). Commonly, the quantity of oxygen consumed far exceeds that of LOOH and TBARS accumulated. For instance, during LDL oxidation LOOH and TBARS account for 25% and 5% of oxygen consumed, respectively. This difference is less significant when lipid oxidation occurs in homogeneous solution (Yamamoto et al., 1984) and disappears completely when linoleic acid derivatives are oxidized in bulk (Yamamoto et al., 1984). As for the TBARS assay it is possible to perform via the collection of aliquot only. Besides, TRABS is representative of a rather late stage of lipid peroxidation, when substrate of oxidation has undergone substantial transformation and is not identical to the original substrate. The latter complicates interpretation of data obtained. Hence it follows that oxygen consumption is not only more adequately represents the oxidation, but also provides a higher sensitivity of determination. Unfortunately, in works related to food chemistry, oxygen consumption is rarely used for monitoring oxidation.

3.1.5. The determination of the rate of initiation

The rate of initiation, R_{IN}, is one of the most essential parameters, which should be determined in every assay using the model of the controlled chain reaction. R_{IN} is commonly determined by using the inhibitor methods. The principle of the methods is as follows. When the oxidation occurs in the presence of a reference antioxidant for which the parameter f is known, R_{IN} can be calculated from t_{IND} by using the Eq. (ix). A reference antioxidant (inhibitor) must to be very effective to provide a well-pronounced induction period. Trolox and 6-hydroxy-2,2,5,7,8-pentamethylbenzochroman (HMPC), the synthetic analogs of α-tocopherol, have such a property (f is as much as 2 for both (Burton et al., 1985)) and may be recommended as a reference antioxidant.

3.1.6. Represenrative examples of the application of various models based on lipid peroxidation

(1) (Mosca, Marco, Visioli, & Cannella, 2000). AOA of virgin oil samples was determined by using the model of the oxidation of LDL induced by Cu(2+) with TBARS assay as well as several assays including the Folin–Ciocalteu method, the DPPH test, and the enzymatic determination of phenolics based on the tyrosinase-NADH system. Polyphenols from oils were preliminarily extracted to make samples water-soluble. The enzymatic assay was suggested as the most productive and representative. The data obtained with the LDL model are presented as plots of TBARS accumulated vs. time.

(2) (Guilot, Malnoe, & Stadler, 1996). AOA of green, light-roasted, and dark-roasted samples of coffee were tested with the model based on the oxidation of rat liver homogenate induced by Fe(2+)/ascorbate followed by the TBARS assay. It was shown that AOA of coffee increased with heat treatment (dark-roasted > light-roasted > green). However, quantitative and repeatable parameters of AOA have not been reported.

(3) (Toschi et al., 2000). AOA of green tea extracts solubilized with alcohol was determined by using forced oxidation of refined peanut oil at 98 °C with the OSI assay and the oxidation of rat heart cell suspension induced by Fe(3+)-transferrin or by xanthine oxidase with measuring absorbance at 235 nm in aliquots. AOA was found to correlate with the total concentration of catechins determined by HPLC.

(4) (Abu-Amsha, Croft, Puddey, Proudfoot, & Beilin, 1996). AOA of several beverages, wines, beers, and grape juices was determined with the models based on the oxidation of LDL and serum induced by Cu(2+) and AAPH. The kinetics of LDL oxidation were monitored by the change in absorbance at 234 nm (conjugated dienes) and accumulation of LOOH. AOA was characterized by t_{IND}. Interestingly, the beverages showed a significantly higher AOA when the oxidation was induced by Cu(2+) than that induced by AAPH. The latter suggests that chelating Cu(2+) by natural phenolics contributes to the total AOA.

(5) (Roginsky & Barsukova, 2001; Roginsky, Barsukova, Hsu, & Kilmartin, 2003a). AOA of various beverages including wines, teas, coffee, and beer was determined with the model based on the oxidation of methyl linoleate in Triton X-100 micelles induced by AAPH. The kinetics of oxidation were followed by oxygen consumption determined with a computerized Clark electrode. The antioxidant capacity was characterized in concentration units by the number of kinetic chains terminated by one liter of a beverage, N. N was calculated from t_{IND} by using equation

$$N = R_{IN} \cdot t_{IND}/(v/V)(xv), \qquad (xv)$$

where v and V are the volume of the added sample and that of the testing system, respectively. In turn, t_{IND} was determined from $[O_2]$ traces by using Eq. (x); R_{IN} required to calculate N was determined by the inhibitor method. To the first approxima-

tion, N may be considered as the sum $\Sigma f[QH_2]$. N is a well repeatable value, in particular, N determined by such a way is not sensitive to parameters of the testing system. N allows comparison of not only among water-soluble products, but also lipid-soluble samples with water-soluble ones. Thanks to using the steady monitoring, the following unexpected effect was firstly observed by Roginsky and Barsukova (2001) during the oxidation inhibited by red wine, tea, and coffee: during the first part of induction period the rate of inhibited oxidation decreases with time (i.e., the inhibition increases). R begins to increase only after some period of time (the inflection point). This effect is not specific for beverages only, it was also observed during testing some individual natural polyphenols, catechin, epicatechin, quercetin, caffeic acid (Roginsky, 2003). The effect can be partly explained by the elevated reactivity of products of the oxidative transformation of some polyphenols to the peroxyl radical.

(6) The models based on the controlled chain reaction have probably never been reported to determine AOA of lipid-soluble products such as plant oils, lipid extracts, etc. Meanwhile, a very elegant approach to determine AOA of lipid-soluble samples may be suggested. Conceptually it is almost identical to that applied to determine AOA of individual phenolic antioxidants (AO). The theoretical basis for that was given above in Section 2. Now some more practical aspects will be discussed. Several alkyl-aromatic hydrocarbons with elevated oxidizability, for instance, styrene, or methyl linoleate, may be suggested as a substrate of oxidation. Lipid-soluble azocompounds, i.e. AMVN, may be used as an initiator. As for the way of monitoring, the kinetics of oxygen consumption may be recommended as the most rational. This can be performed by using a transducer (Barclay & Ingold, 1981) or capillary volumometer (Loshadkin et al., 2002). Inasmuch as natural products contain a complex mixture of antioxidants, only effective values of reactivity and antioxidant capacity can be determined. The starting rate of inhibited oxidation can be used to calculate the parameter $\Sigma k[AO]$ (using Eq. (viii)) characterizing antioxidant reactivity. As for the antioxidant capacity, N, this may be calculated from t_{IND} by using Eq. (xv).

Automatic assays of lipid oxidation in bulk by using enzymatic sensors to record the consumption of phenolic components were suggested by Campanella, Faferi, Pasotorino, and Tomasetti (1999) and by Capannesi, Palchetti, Mascini, and Parenti (2000). These assays have never been applied for systematic testing of lipid-soluble food samples.

In conclusion of this Section, the values of IC_{50} for several natural polyphenols obtained by using various experimental models based on the kinetics of lipid peroxidation are correlated Table 1. The cited works are pooled by a general feature: the rate of free radical generation was not controlled. As it may be expected, the IC_{50} values determined in various works are in a rather poor agreement with each other. In particular, IC_{50} reported for green tea catechins, catechin, epicatechin,

Table 1
Comparison of IC_{50} for individual natural polyphenols (in μM) determined during lipid peroxidation by various methods

Polyphenol	IC_{50} (μM)							
	A	B	C	D	E	F	G	H
Quercetin	7.7	9.6			3.0	11.0	7.7	
Epicatechin			39.1	0.19			5.30	28.1
Catechin		12.2		0.67	4.5	16.0	3.4	32.3
Rutin		22.3			15.7	17.0		
Epigallocatechin		8.3	42.2	0.10				20.8
Epicatechin gallate		4.8	14.6	0.14			25.2	9.4
Epigallocatechin gallate		3.5	21.3	0.08				15.6
Myricetin		7.9			3.4	18.0		
Gallic acid				1.25			68.9	
Caffeic acid	3.9							
Kaempferol	12.6	12.4						
Trolox	12.6		36.2				12.6	

A: oxidation of liposomes (1 mg/ml phospholipid) induced by 200 μM Fe(3+) and 50 μM ascorbate at 37 °C; TBARS assay (Plumb et al., 1997); B: oxidation of rat liver microsome homogenate (4 mg protein per mL) induced by 10 μM Fe(2+) and 200 M ascorbic acid at 37 °C; TBARS assay (van der Sluis et al., 2000); C: oxidation of human blood plasma (diluted 4-fold) induced by 400 μM Cu(2+) at 37 °C; the assay based on determining the fragmentation of apolipoptrotein B-100 (Hashimoto et al., 2000); D: oxidation of LDL + VLDL induced by Cu(2+) (Vinson et al., 1998); E: oxidation of liposomes produced from rat liver microsomes (5 mM of lipid phosphorous induced by 50 μM Fe(3+) and 50 μM EDTA + 150 μM ascorbate at 37 °C; TRABS assay (Silva et al., 2002)); F: oxidation of liposomes produced from rat liver microsomes (5 mM of lipid phosphorous induced by 20 mM AAPH at 37 °C); TRABS assay (Silva et al., 2002); G: oxidation of 1 mg/ml phospholipid liposomes induced by 200 μM Fe(3+) and 50 μM ascorbate at 37 °C; TBARS assay (Plumb et al., 1998); H: oxidation of human plasma induced by 50 mM AAPH at 37 °C; TRABS assay (Lotito and Fraga, 2000).

epigallocatechin, epicatechin gallate, and epigallocatechin gallate vary within two orders of magnitude (Table 1). Moreover, there is a several-fold difference in IC_{50} determined in the same work with the same model (LDL oxidation) but using different initiation (by Fe(3+)/EDTA + ascorbate or by AAPH) (Silva et al., 2002). The data presented in Table 1 confirm the above statement that the application of the kinetic models, in which the rate of initiation is not controlled, is hardly suitable for repeatable testing of food samples.

3.2. Direct competition methods

Along with the direct methods based on the studies of chain lipid peroxidation, several methods based on studying the kinetics of non-chain processes have also been suggested. In these kinetic models, natural antioxidants compete for the peroxy radical with a reference free radical scavenger.

3.2.1. Free-radical induced decay of fluorescence of R-phycoerythrin

In this assay, a fluorescent natural protein R-phycoerythrin (PE) is applied as a reference scavenger. The principle of the assay is as follows. The intensity of fluorescence of PE decreases with time under the flux of the peroxyl radical formed at the thermolysis of APPH in aqueous buffer. In the presence of a tested sample containing chain-breaking antioxidants, the decay of PE fluorescence is retarded. A rather intricate history of the method reported by Prior and Cao (1999) distinguishes between the PE-based assay (Ghiselli, Serafini, Maiani, Azzini, & Ferro-Luzzi, 1995) and the so-called ORAC protocol (Cao & Prior, 1997). In reality, both methods use the same testing system, the difference is in shapes of kinetic curves observed and in the way, which is used to calculate AOA from the kinetic curves. While Ghiselli et al. (1995) reported that fluorescence decay occurred at a constant rate up to 80% transformation, Cao and Prior (1997) found that the intensity of flourescence decreased with time with progressive slowing down from the very beginning. Most likely, the reason for this discrepancy is the difference in the initial concentrations of the fluorescent protein (Pino & Lissi, 2001). In the assay suggested by Ghiselli et al. (1995), AOA is characterized by TRAP (total radical-trapping antioxidant parameter), which, in turn, is calculated from t_{IND} by using the "common graphical" procedure. In the ORAC protocol AOA is characterized by the Oxygen Radical Absorbance Capacity (ORAC), which is determined by using the area-under-curve technique. Both TRAP and ORAC are expressed in Trolox equivalents and Trolox is used as a standard antioxidant. Although the above areas are conceptually similar to the induction period, their physical meaning is not always clear. The detailed kinetic analysis of the testing system under consideration has been reported by Lissi, Pizarro, Aspee, and Romay (2000) and by Pino and Lissi (2001). It has been found that the mechanism of chemical process occurring in the testing system is likely more complex than this was assumed in earlier studies. In particular, it has been proposed that some portion of peroxyl radicals derived from AAPH reacts with each other instead of the attack on PE and added antioxidants. These cast some doubt on the reliability of the ORAC assay. The further studies are wanted. At the same time, the ORAC values for individual polyphenols and related phenolics are in reasonable agreement with their structure (Guo, Cao, Sofic, & Prior, 1997).

Originally, both versions of the method were designed to determine the antioxidant status in biological systems. Later, the ORAC protocol was widely used to test natural phenolic antioxidants and food samples, including tea, vegetables, fruits (Caldwell, 2001; Guo et al., 1997; Henning et al., 2003), and herbal extracts (Wang, Cao, & Prior, 1996). Both with individual phenolics (Silva et al., 2002) and tea and spinach extracts (Caldwell, 2001) the ORAC value was found to be proportional to the amount of addition. In subsequent works, the ORAC protocol has been modified and improved. In particular, Guo et al. (1997); Caldwell (2000) and Caldwell (2001) reported automated versions of the ORAC protocol.

3.2.2. Competitive crocin bleaching

Croicin is a natural compound with extremely strong absorbance in visible range. The assay was originally suggested by (Bors, Michel, & Saran (1984) and modified by Tubaro, Gheselli, Rapuzzi, Maiorino, & Ursini, 1998). Crocin undergoes bleaching under attack of the peroxyl radical. The addition of a sample containing chain-breaking antioxidants results in the decrease in the rate of crocin decay. For individual compound A the ratio of the rates of non-inhibited bleaching (R_0) to that of inhibited bleaching (R) is given by the Stern–Volmer-like relation

$$R_0/R = 1 + (k_A/k_C)x([A]/[C]),$$

where [A] and [C] are the concentrations of the tested antioxidant and crocin, k_C and k_A are rate constants for reaction of the peroxy radical with crocin and with A, respectively. Determining R_0/R value at known ratio of [A] to [C], k_A/k_C may be calculated. As for complex mixture of AO, the assay gives probably the sum $\sum k_i x[A_i]$. Thus, the assay allows the determination of total inhibiting effects, but two independent parameters, the reactivity and antioxidant capacity, cannot be determined separately. Later a modified microplate-based version suitable for routine determinations was suggested by Lussignoli, Fracaroli, Andrioli, Brocc, and Bellavite (1999). The assay under consideration has been originally designed for testing blood plasma, however,

there is nothing to prevent from using the crocin assay for testing food samples.

3.2.3. Competitive β-carotene bleaching

The assay is based on the competitive bleaching β-carotene during the autoxidation of linoleic acid in aqueous emulsion monitored as decay of absorbance in the visible region (Miller, 1971). The addition of an antioxidant-containing sample, individual antioxidants (von Gadov, Joubert, & Hansmann, 1997), or natural extracts (Moure et al., 2000) results in retarding β-carotene decay. By present, the assay is only poorly quantified as AOA has been given as an inhibition percentage only. This as well as the fact that a testing system is oxidized under non-controlled conditions does not make it possible to obtain repeatable data. In principle, the assay under consideration may be converted into a more quantitative and repeatable one if the regime of autoxidation is changed for the regime of the controlled chain reaction. The latter may be achieved by using a free-radical initiator, e.g. AAPH.

3.2.4. The assay based on the competition between antioxidant and KI for the peroxyl radical (Sano, Yoshida, Degawa, Miyase, & Yoshino, 2003)

The idea of this automated assay is as follows. KI reacts with the AAPH-derived peroxyl radical with formation of molecular iodine. The latter is determined using an automatic potentiometric titrator with sodium thiosulfate. In the presence of antioxidant-containing samples the rate of iodine release decreases. AOA is calculated from the ratio of inhibited and non-inhibited rates of iodine release. The method has been applied for determining AOA of both individual natural polyphenols and vegetable extracts.

3.3. Indirect methods

3.3.1. ABTS test

These days, the ABTS test is likely the most popular among other indirect assays. The method was firstly suggested by Miller, Rice-Evans, Davies, Copinathan, and Milner (1993) to test biological samples and then was widely applied to test food and natural water-soluble phenolics. The idea of the method is to monitor the decay of the radical-cation $ABTS^{\cdot+}$ produced by the oxidation of 2,2′-azinobis(3-ethylbenzothiaziline-6-sulfonate) (ABTS) caused by the addition of a phenolic-containing sample. $ABTS^{\cdot+}$ has a strong absorption in the range of 600–750 nm and can be easily determined spectrophotometrically. In the absence of phenolics, $ABTS^{\cdot+}$ is rather stable, but it reacts energetically with a H-atom donor, such as phenolics, being converted into a non-colored form of ABTS. The authors determined the quantity of $ABTS^{\cdot+}$ consumed due to reaction with phenolic-containing sample, which was expressed in Trolox equivalents (concentration units). This value was designated as TEAC, Trolox equivalent antioxidant capacity. As TEAC for Trolox was reported to be as much as 1 (Campos & Lissi, 1997), TEAC for an individual antioxidant is really the number of the $ABTS^{\cdot+}$ radical-cation consumed per one molecule of antioxidant. Thus, TEAC is conceptually close to the inhibition coefficient *f*. By now the great amount of information on TEAC value for both individual polyphenols (Rice-Evans, Miller, & Paganga, 1996) and many food samples has been amassed.

In the commercial version the ABTS test known as the TEAC protocol, $ABTS^{\cdot+}$ is generated from ABTS by its reaction with the ferrymyoglobin radical produced, in turn, from metamyoglobin and H_2O_2 in the presence of peroxidase. Several modifications of this protocol depending on the way for $ABTS^{\cdot+}$ generation and change in the nature of a reference antioxidant have been suggested. In particular, Romay, Pascual, and Lissi (1996) and Fogliano, Verde, Randazzo, and Ritteni (1999) suggested to use AAPH; Schleisier, Harwat, Böhm, and Bitsch (2002) and De Beer, Jubert, Wentzel, Gelderblom, and Manley (2003) suggested to use $K_2S_2O_8$ to generate $ABTS^{\cdot+}$ from ABTS. Alonso, Dominguez, Gullen, and Barroso (2002) reported the electrochemical generation of $ABTS^{\cdot+}$. Kim, Lee, Lee, and Lee (2002) suggested to use ascorbic acid as a reference antioxidant instead of Trolox.

The advantage of the ABTS test is its relative simplicity that allows its application for routine determinations in any laboratory. As for limitations of the ABTS test, one of them is general for all the indirect methods: the TEAC value actually characterizes the capability of the tested sample to react with $ABTS^{+}$ rather than to inhibit the oxidative process. With many phenolics and samples of natural products the reaction with $ABTS^{\cdot+}$ occurs rather slowly (Campos, Escobar, & Lissi, 1996; Lissi, Modak, Torres, Esocbar, & Urzua, 1999). Thus, the result of determination of TEAC is expected to be dependent on the time of incubation as well as on the ratio of sample quantity to $ABTS^{\cdot+}$ concentration. This remains to be studied in more detail. As mentioned above, TEAC is conceptually close to the inhibition coefficient f, this does not characterize directly the reactivity. In this connections, the attempts to correlate TEAC with the structure of phenolics (Rice-Evans et al., 1996) seem to be unjustified. Poor selectivity of $ABTS^{\cdot+}$ in the reaction with H-atom donors is one more limitation of the method. As it follows from the kinetic study of Campos and Lissi (1997) and from the recent work of Arts, Dallinga, Voss, Haenen, and Bast (2003), $ABTS^{\cdot+}$ reacts with any hydroxylated aromatics independently of their real antioxidative potential. In fact, the ABTS test is reduced to titration of aromatic OH-groups including OH-groups which do not contribute to the antioxidation.

Some examples may testify this statement. The AOA of resorcinol determined with microsomal model (Arts

et al., 2003) was found to be less than that of catechol and *p*-hydroquinone 150- and 10-fold, respectively. At the same time, TEAC for resorcinol (2.49) significantly superiors that for catechol (1.43) and *p*-hydroquinone (1.33). A much higher antioxidant potential of catechol and hydroquinone as compared with that of resorcinol is also evident from the fact that the rate constant for the reaction between the peroxy radical with catechol and hydroquinone superiors that for resorcinol by factor about 100 (Ross et al., 1994). TEAC for chrysin and galangin, flavonoids containing two m-OH-groups in A-ring but no OH-groups in B-ring, were reported to be as much as 2.52 (Ishige, Schubert, & Sagara, 2001) 1.43 (Rice-Evans et al., 1996), and 2.08 (Ishige et al., 2001), respectively. The latter values are comparable with those of many flavonoids with one and even two OH-groups in B-ring (Rice-Evans et al., 1996). Meanwhile, flavonoids that have no OH-groups in B-ring show a moderate if any capability to inhibit lipid peroxidation (Ross et al., 1994).

De Beer et al. (2003) considered two reasons why different works reported different values for TEAC: the difference in the strategy of $ABTS^{\bullet+}$ generation and the difference in the time of incubation. When $ABTS^{\bullet+}$ is generated enzynatically, simultaneously with its scavenging (as this occurs in the TEAC protocol), addition of a phenolic-containing sample may depress the $ABTS^{\bullet+}$-producing enzyme along with scavenging $ABTS^{\bullet+}$. The latter may result in overestimating TEAC. To make the $ABTS^{\bullet+}$ more reliable, De Beer et al. (2003) suggested to separate in time the production of $ABTS^{\bullet+}$ and $ABTS^{\bullet+}$ scavenging as well as to standardize the procedure.

Fogliano et al. (1999) suggested a new version of the ABTS test where $ABTS^{\bullet+}$ was changed for the stable $DMPD^{\bullet+}$ radical cation derived from *N*,*N*-dimethylphenylenediamine. As Fogliano et al. (1999) and Schleisier et al. (2002) reported, this method is simpler, more productive, and less expensive as compared with the traditional ABTS test.

3.3.2. DPPH test

This is the oldest indirect method for determining AOA, it was firstly suggested in 1950s originally to discover H-donors in natural materials. Later the test was quantified to determine the antioxidant potential of both individual phenolics and food as well as of biologically relevant samples. The DPPH test is based on the capability of stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with H-donors including phenolics. As DPPH shows a very intensive absorption in the visible region, it can be easily determined by the UV–Vis spectroscopy, although ESR determination is also acceptable. DPPH is likely more selective than $ABTS^{\bullet+}$ in the reaction with H-donors. For instance, the *f* value for series of tea catechins was found to be almost equal to the number of active OH-groups inherent in catechol and pyrogallol fragments (Nanjo, Mori, Goto, & Hara, 1999). In contrast to $ABTS^{\bullet+}$, DPPH does not react with flavonoids, which contain no OH-groups in B-ring (Yokozawa et al., 1998) as well as with aromatic acids containing only one OH-group (von Gadov et al., 1997).

The DPPH test was suggested in two versions, dynamic and static. In the first version, one measures the rate of DPPH decay observed after the addition of a phenolic-containing sample. In the static version, one determines the amount of DPPH scavenged by a sample tested. While the first assay characterizes the reactivity, the second determines the stoichiometry for the reaction of DPPH with H-donor for individual substance or the quantity of active OH-groups in complex mixture. In the dynamic version the reactivity is commonly characterized by the starting rate of DPPH decay (Da Porto, Calligaris, Celotti, & Nicoli, 2000; Nanjo et al., 1999; von Gadov et al., 1997; Yen & Duh, 1994). Recently, the dynamic version was modified to determine rate constants for the reaction of DPPH with polyphenols (Gaupy, Dufcur, Loonis, & Dangles, 2003). Unfortunately, that was done on the basis of rather arbitrary kinetic schemes. It was shown that not only an original polyphenol, but also products of its transformation may be involved into interaction with DPPH. As for the static version, the H-donating potential of a sample tested is most frequently expressed in IC_{50} (see for instance, Amakura, Umino, Tsuji, & Tonogai (2000); Arnous, Makris, & Kefalas (2001); Standley et al. (2001); Yokozawa et al. (1998)). As the starting concentration of DPPH changes when going from one work to another, the direct comparison of IC_{50} reported in various works is not realistic. It seems to be more rational to express the H-donating capacity as the amount of DPPH, which may be scavenged by a sample tested (the stoichiometric coefficient for individual antioxidants). A good example of such a study was reported by Silva et al. (2002), when the amount of DPPH scavenged was found to be proportional to the concentration of flavonoid added. This approach makes it possible to compare the data obtained in one work with those of another work. It should be taken into consideration that the value of *f* determined increases with the incubation time. For instance, with catechin and epicatechin f, was reported to be ca. 2.2 at 2 min and ca. 3.5 at 15 min (Gaupy et al., 2003). The latter reflects the complex character of the reaction between DPPH and polyphenols.

Sanches-Mareno, Larrauri, and Saura-Calixto (1998) suggested to use the combination of kinetic and static approaches to characterize the antioxidant efficiency (AE). AE was calculated as the parameter $1/EC_{50} \cdot t_{50}$, where t_{50} is the time, which is required to attain 50% transformation. AE has dimensionality similar to that of the rate constant for bimolecular reaction, however, the physical meaning of AE remains unclear. It should

be noted that the data obtained with this method give an uncommon order of AOA: ascorbic acid > caffeic acid > gallic acid > tannic acid > tocopherol > rutin > quercetin. A conceptually similar parameter for characterizing radical scavenging efficiency, RSE, was suggested by De Beer et al. (2003). RSE is calculated as the ratio of the starting rate of DPPH decay to EC_{50}. It is evident that both AE and RSE are hardly repeatable values as they are expected to vary with the changes in reagent concentrations.

A very elegant and promising version of the DPPH test combined this with the HLPC assay was recently suggested by Bandoniene and Mukovic (2002). The HPLC assay with detection at 280 nm is immediately followed by monitoring the decay of DPPH at 515 nm within a single HPLC peak. In the work by Bandoniene and Mukovic (2002), this protocol was applied to determine AOA of apples. The limitation of this method is connected with the general limitation of HPLC assay: only some portion of phenolics (basically of relatively low MM) can be determined during a single run. This limitation is the most significant for determination of AOA of red wines, black tea, coffee, and cacao where the contribution of polymeric phenolics is especially significant.

Solerrivias, Espin, and Wickers (2000) suggested a fast, rather simple, and elegant version of the DPPH test based on thin layer chromatography with chromametric recording of DPPH quenching. However, this assay can hardly be suggested as repeatable quantitative method since results of such a determination are expected to be highly dependent on the protocol. Recently, Polasek and Skala (2004) suggested a new rapid automated version of the DPPH test based on the sequential injection analysis.

3.3.3. Reduction of the Fremy's radical

The assay is based on the capability of the Fremy's stable free radical (potassium nitrosidsulfonate) to react with H-donors (Gardner, McPhail, & Duthie (1998)). The concentration of the Fermy's radical was followed by ESR. In particular, the method was applied by Burns et al. (2001) for testing wines in the process of wine-making.

3.3.4. Methods based on Fe(+3) reduction

The FRAP assay (ferricreducing antioxidant power) (Benzie & Strain, 1996; Pulido, Bravo, & Saura-Calixto, 2000) is based on the ability of phenolics to reduce Fe(3+) to Fe(2+). When this occurs in the presence of 2,4,6-trypyridyl-*s*-triazine, the reduction is accompanied by the formation of a colored complex with Fe(2+). Being expressed in ascorbic acid equivalents, the FRAP value was applied to determine AOA of red wines (Arnous et al., 2001). The work by Schleisier et al. (2002) was designed to determining AOA of juices and tea extracts in Fe(2+) equivalents. The absolute initial rate of ferrylmetmyoglobin reduction determined by spectroscopy in the visible region was suggested to characterize AOA of individual flavonoids (Silva et al., 2002). The question about the relationship between FRAP and AOA remains open.

3.3.5. Methods based on the chemiluminescence of luminol

The general principle of these methods is based on the ability of luminol and related compounds to luminescence under the flux of free radicals (chemiluminescence, CL). CL is brought about due to a reaction of free radical derived from luminol with active free radicals. CL can be easily recorded. The addition of an antioxidant, being a scavenger of an active free radical, results in CL quenching, commonly with a pronounced induction period. The quantity of the tested antioxidant can be estimated from the duration of t_{IND}. As a rule, AOA is given in Trolox equivalents. The attractive feature of CL methods is their productivity; commonly, one run takes a few minutes only; in addition, the assay can be easily automated. As for shortcomings of this group of methods, first of all, the mechanism for chemical processes resulting in CL is not known in detail. The latter may create problems with interpreting data obtained. Different versions of this method differ in the type of active free radical produced and the way of free radical production as well as in details of the protocol. While the majority of assays have been developed for testing biologically relevant samples, they can be easily applied for food testing too.

Lissi, Pascual, and Del Castillo (1992) and Alho and Leinonen (1999) suggested AAPH to excite CL. While this protocol was originally designed to determine AOA of blood plasma and LDL, this can be evidently applied for testing food samples. Lissi, Salim-Hanna, Pascual, and Castillo (1995) have proposed two assays based on CL quenching. One of them (TRAP) measures t_{IND} and hence the stoichiometric factor. The other one (total antioxidant reactivity, TAR) measures the steady state CL decrease following the addition of an antioxidant-containing sample and hence measures the reactivity of incorporated compounds. Both methodologies have been applied to a series of individual polyphenols and beverages (Campos et al., 1996; Perez, Leighton, Aspee, Aliaga, & Lissi, 2000).

Parejo, Codina, Petrakis, and Kefalas (2000) suggested to induce CL by reaction of Co(2+) chelated by EDTA with H_2O_2. Although the authors suggested $HO^{\bullet}$ as an active free radical, which attacks to luminol, it is more realistic that $O_2^{\bullet -}$ plays this role. The method was applied for testing red wines (Arnous et al., 2001). Ogawa, Arai, Tanizawa, Miyahara, and Toyo'oko (1999) suggested to excite luminol CL by the reaction with $O_2^{\bullet -}$, which, in turn, was produced by using the xanthin-hypoxantine-oxidase system. CL detection was

combined with HPLC. The method was applied for testing green tea leaves.

The method based on photosensitized CL of luminol (Popov & Lewin, 2000) seems to be one of the most promising. The idea of the assay is that the production of $O_2^{\bullet-}$ and its reaction with luminol resulting in CL are separated both in space and in time. $O_2^{\bullet-}$ is produced by photolysis of riboflavin in aerated buffer at pH 10-11 to prevent the fast decay of $O_2^{\bullet-}$ due to dismutation. The following CL reaction of $O_2^{\bullet}$ with luminol occurs in the measuring cell in dark. The addition of an antioxidant sample reacting with $O_2^{\bullet-}$ causes the inhibition of CL commonly with a pronounced induction period, with duration of t_{IND} being proportional to the amount of addition. The method is well-instrumented and computerized. The capability of the method was demonstrated by the example of testing several natural products including wines, tea, and medicinal herb extracts. The evident advantage of the method is its very high productivity: the procedure takes commonly a couple of minutes only. At the same time, the kinetic theory of the process underlying the assay is really not suggested.

The enhanced chemiluminescence assay (Robinson, Maxwell, & Thorpe, 1997) is also based on luminol CL. In this case, the testing system also includes, along with luminol, also *p*-iodophenol. Adding *p*-iodophenol provides more intensive, prolonged, and stable light emission as compared to the traditional luminol system. The addition of an individual chain-breaking antioxidant or antioxidant-containing sample results in quenching CL with a pronounced induction period, with t_{IND} proportional to the amount of addition. The method was originally developed for determining antioxidant properties of biological fluids (Whitehead, Thorpe, & Maxwell, 1992) and then was successfully applied to testing tea extracts (Robinson et al., 1997). Although the mechanism underlying the method is known in outline only, similar to other related methods, this seems to be promising for routine testing of natural products. The advantages of the method are its high productivity and relative simplicity of protocol.

4. Other methods useful to estimate AOA

Now we shall consider in outline some methods, which are not destined directly for the determination of AOA, but may be useful for such studies, especially in combination with other methods.

4.1. HPLC

As mentioned above, AOA of food is basically determined by phenolic composition. In the past few years considerable progress has been made in HPLC analysis of many complex natural products including tea (Sakakibara, Honda, Nakagawa, Ashida, & Kanazawa, 2003; Sano et al., 2001), wine (Kennedy & Waterhouse, 2000), fruits and vegatables (Bandoniene & Mukovic, 2002; Guo et al., 1997; Mattila, Astola, & Kumpulainen, 2000; Sakakibara et al., 2003), and cacao (Kennedy & Waterhouse, 2000). However, it is impossible to indicate even one work where all the phenolics have been determined. The most difficult problem is the determination of condensed and oligomerized phenolics typical of red wine and cacao (Kennedy & Waterhouse, 2000) as well as of black tea (Harbowy & Balentine, 1997). Even though the composition of phenolics is completely known, AOA cannot be properly calculated from these data. First, the current information on AOA of individual natural phenolics is rather poor and conflicting. Second, the total AOA may be not the additive sum of AOA of individual compounds owing to complex interaction between them (synergism, antagonism), which has been poorly investigated.

4.2. Determination of total phenols by using the Folin–Ciocalteu reagent

The Folin–Ciocalteu assay (FC) is one of the oldest methods designed to determine the total content of phenolics, so-called total phenols (Singleton, Orthofer, & Lamuela-Raventós, 1999). The testing system is the mixture of tungstate and molibdate in highly basic medium (5–10% aqueous Na_2CO_3). Phenolics are energetically oxidized in basic medium resulting in the formation of $O_2^{\bullet-}$, which in turn, reacts with molibdate with formation of molybdenum oxide, MoO^{4+} having a very intensive absorbance near 750 nm. General phenolics determined by the FC test are most frequently expressed in gallic acid equivalents. The FC assay is not selective, similar to the ABTS test, it determines both polyphenols and monophenolics (Singleton et al., 1999). The advantage of the FC assay over the ABTS test is that the former is associated with the appearance of absorbance rather than with absorbance decrease as this takes place in the ABTS test. Among other things, the procedure associated with the appearance of collared products is more sensitive in principle; besides, this does not require a strict standardization of the assay conditions. Although the FC assay was not supposed to characterize AOA, in reality this method seems to be one of the best for rough estimating AOA of food samples provided that the sample tested does not contain proteins in significant amounts. A reader may find numerous examples of the application of the FC assay to characterize natural products a reader may find in the literature. Some of them are listed in Table 2 where total phenols determined by the Folin–Ciocalteu method are correlated with AOA determined by several methods. In most cases, the correlation is well pronounced, which confirms the value of the Folin–Ciocalteu test. As for the

Table 2
Correlation between AOA and total phenols determined by the Folin–Ciocalteu method

Work	Product	Method for AOA determination	r
A	13 Red and white wines	ABTS test	0.990
B	54 Red and white wines	ABTS test	0.959
C	4 Red wines in the process of wine-making	Reaction with the Fremy's radical	0.818
D	24 Red wines	ABTS test	0.746
E	26 Red and white wines	N (see text)	0.940
F	Several teas and juices	ABTS test	0.859
		DPPH test	0.752
		DMPD test	0.592
G	46 Red wines	ABTS test	0.935
	40 White wines	ABTS test	0.907
H	20 Red and white wines	ABTS test	0.997

A: Simonetti et al. (1997); B: Landrault et al. (2001); C: calculated from the data of Burns et al. (2001); D: calculated from the data of Pellegrini et al. (2000); E: De Beer et al., 2004; F: calculated from the data of Schleisier et al. (2002); G: De Beer et al. (2003); H: Campodonico et al. (1998).

repeatability of data obtained with the Folin–Ciocalteu method, this is expected to be quite acceptable, although the problem of its standardization remains to be solved (De Beer et al., 2003; Singleton et al., 1999).

4.3. Cyclic voltammetry

Among several methods, which have been applied to characterize food samples and individual natural phenolics, cyclic voltammetry (CV) seems to be rather informative and promising (Kilmartin, 2001). The general principle of the CV method in the modern modification is as follows. The electrochemical oxidation of a certain compound on an inert carbon glassy electrode is accompanied by the appearance of the current at a certain potential. While the potential at which a CV peak appears is determined by the redox properties of the tested compound, the value of the current shows the quantity of this compound. Besides, the tested compound may be characterized by the reversibility of the CV traces recorded when potential is altered in both directions. By present, a number of the most widely distributed natural phenolics have been studied by the CV method (Kilmartin & Hsu, 2003; Kilmartin, Zou, & Waterhouse, 2001; Kilmartin, Zou, & Waterhouse, 2002; Zou, Kilmartin, Inglis, & Frost, 2002). These studies made it possible the use of the CV method for testing food samples containing a complex mixture of antioxidants including wine (Kilmartin et al., 2002; Zou et al., 2002) and tea (Kilmartin & Hsu, 2003; Roginsky et al., 2003a). Although the CV test does not allow the determination of individual antioxidants in complex mixtures, this gives a valuable information about the quantity of main groups of phenolics such as catechins, gallates, anthocyanins, monophenolics, etc. The CV method may be considered as a cheep and more productive alternative for HPLC. Because of overlapping of many peaks belonging to a variety of individual substrates, the peaks observed in CV traces display broadening. Under these circumstances, it was suggested to use the integral of current instead of current itself to characterize the quantity of antioxidants in food samples. (Kilmartin et al., 2002; Roginsky et al., 2003; Zou et al., 2002).

5. Comparison of various methods for determining AOA: general conclusions and perspectives

As mentioned above, AOA of food samples may be characterized by two independent parameters: antioxidant capacity and reactivity. For individual antioxidants, this corresponds to the stoichiometric coefficient and the rate constant for reaction between antioxidants and reactive free radicals. There is no single answer to the question which index of AOA is more relevant. The main attention is currently paid to determining antioxidant capacity. The absolute majority of the recently developed methods are designed to solve this problem. Admittedly, the reactivity of food samples may be of interest under certain conditions. Meanwhile, the information on the reactivity of food and individual natural polyphenols is still rather poor and conflicting.

It is very complicated to correlate the data on AOA of natural products reported in various works and measured by various methods. These data are generally poorly repeatable, first of all, because natural products are hardly repeatable in principle. What can we do under these circumstances is to correlate the data on AOA of natural individual antioxidants. The stoichiometric coefficients f for some typical polyphenols are presented in Table 3. When AOA has been determined by the DPPH and ABTS tests as well as by using the ORAC protocol, the f value is expressed in Trolox equivalents. For the case when AOA has been determined by using the model of the chain controlled reaction, f is given in absolute units. As it may be seen, the antioxidant capacities of polyphenols determined by different methods, as a rule, only poorly correlate with each other. In particular, the

Table 3
Comparable parameters characterizing antioxidant capacity determined by various methods for individual natural polyphenols

Polyphenol	*f*			
	DPPH	$ABTS^{\cdot+}$	$LO_2^{\cdot}$ (ORAC)	$LO_2^{\cdot}$ (POL)
Quercetin		4.72[c];4.84[d]; 3.8[e]	3.29[h]; 3.2[i]	5.6[j]
Epicatechin	2.1[a]; 2.96[b]	2.5[c]; 3.16[d]; 3.0[e]	2.36[h]	3.1[k]; 6.3[j]
Catechin	2.0[a]; 2,56[b]	2.40[c]; 3.42[d]; 2.7[e]	2.49[h]; 1.49[i]	5.7[j]
Rutin		2.4[c]; 2.67[d]; 2.9[e]	0.56[h]; 2.9[i]	4.6[j]
Epigallocatechin	4.7[a];2.73[b]	3.82[c]		1.1[k];1.57[j]
Epicatechin gallate	7.1[a]	4.93[c]		
Epigallocatechin gallate	9.9[a]; 4.35[b]	4.75[c]		1.7[k]
Myricetin		3.12[c]; 3.08[d]		2.5[j]
Gallic acid	4.0[a]; 2.30[b]	3.01[c]; 2.69[d]; 2.45[f]	1.76[h]	1.87[j]
Caffeic acid		1.26[c]; 1.33[g]; 1.20[d]; 1.1[e]; 0.99[f]		5.7[j]
Kaempferol		1.34[c]; 1.45[d]	2.67[h]	2.05[j]
Chrysin		1.43[c]; 2.52[d]		No inhibition[l]
Catechol	1.9[a]			3.6[j]

[a] Nanjo et al. (1999).
[b] Gardner et al. (1998).
[c] Rice-Evans et al. (1996).
[d] Ishige et al. (2001).
[e] Baderschneider and Winterhalter (2001).
[f] Pellegrini et al. (2000).
[g] Plumb et al. (1997).
[h] Guo et al. (1997).
[i] Silva et al. (2002).
[j] Roginsky (2003).
[k] Kondo et al. (1999).
[l] Roginsky, unpublished results.

capacity of some tea catechins, epigallocatechin and epigallocatechin gallate, changes significantly when they react with DPPH and $ABTS^{\cdot+}$, on one hand, and with the peroxyl radical, on the other hand. Besides, polyphenols containing no or only one active OH-group in B-ring, which displays a very moderate AOA during lipid peroxidation, show quite a significant ''antioxidant'' capacity, being determining by the DPPH or ABTS tests. The various methods applied to determine AOA of real food samples typically show only moderate correlation even in the frame of a single work (Davalos, Gomez-Cordoves, & Bartolome, 2003; Pellegrini et al., 2003).

Generally, indirect methods are used more frequently than direct methods. The question arises which of the methods, direct or indirect, is better in principle. Each kind of methods has both advantages and disadvantages. The direct methods are more adequate in principle, especially those based on the model of the chain controlled reaction. Besides, they are commonly more sensitive. Disadvantage of the direct methods is that the most of them are rather time-consumed and their application requires a significant experience in chemical kinetics. As a consequence, direct methods are commonly not so suitable for routine testing natural products.

As a rule, well-developed indirect methods, such as the DPPH and ABTS tests, are more productive and easier in handling. The crucial point concerning the application of indirect is their informative capability. The indirect methods commonly provide for the information on the capability of natural products to scavenge stable free radicals, e.g. DPPH and $ABTS^{\cdot+}$. Undoubtedly, the best indirect methods as well as the Folin–Ciocalteu test allow the estimation of AOA to the first approximation. However, it is questionable whether the data obtained with indirect methods give a quantitative information on the capability of natural products to inhibit oxidative processes. The other problem concerning the application of indirect methods is their poor repeatability. The main reason for this is that the results of determination are highly dependent on the protocol, first of all, on reagent concentrations and the time of incubation. The problem can be likely solved by standardization of protocols.

The following way may be suggested to exploit the advantages of direct and indirect methods. Well-standardized indirect methods as well as the Folin–Ciocalteu test may be applied for routine estimation of AOA of natural products. To make determinations more precise and reliable, the data obtained with indirect methods should be regularly correlated with the data obtained by a direct method. In other words, direct methods may be recommended to use to calibrate indirect methods.

In conclusion, it should be remembered that the mentioned above methods are intended for the determination of AOA of food sample per se, i.e. the antioxidative potential of food. As for the antioxidative action of food

substituents in real biological systems, this will depend also on their bioavailability and food antioxidants metabolism. Besides, the difference in the conditions of functioning antioxidants between model systems applied to determine AOA and biological systems (first of all difference in oxygen concentration) as well as possible interaction between food-derived and endogenous antioxidants must be taken into account.

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