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Natural phenolic antioxidants in bioanalytical chemistry: state of the art and prospects of development

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Published data of the last decade on the chemical analysis and related problems of investigation of phenolic antioxidants as components of complex biomedical and food objects are generalized. The physicochemical properties of these compounds are presented in accordance with the up-to-date classification of their structures. Particular attention is paid to the development of new and advancement of traditional methods for determination of natural phenolic antioxidants in relation to public health protection problems. The interdisciplinary character of the considered problems is emphasized and the ways for problem solution are outlined.
The bibliography includes 380 references.

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I. Introduction

The turn of the 21st century witnesses avalanche-like growth of the number of publications devoted to synthetic and natural compounds containing phenolic groups in the molecules. These compounds are biologically active to one or another extent. Among synthetic structures, most attention is attracted by sterically hindered phenols.[1,2] In the broad range of their biological activities, authors of most publications distinguish, first of all, the antioxidant action, which depends on the structure of the compound, the chemical form of its existence in the body, the concentration and the natural object containing this compound.[3] In the general case, the object composition is complex and this cannot but influence the biological properties.

Apart from antioxidant properties, phenol derivatives exhibit anti-inflammatory, antitumour and other types of action, for example, capillary strengthening action, which is beneficial for human health.[4–6] In the context of the role of antioxidants, the emphasis is placed on the prevention and treatment of socially significant diseases such as cardiovascular diseases and cancer.[7] Since the 1960s, natural and synthetic antioxidants have been attracting the attention of specialists engaged in human ageing and related body problems.[8]

The International Society of Antioxidants in Nutrition and Health (ISANH) was established for dealing with the human health care with the use of this group of biologically active compounds. From time to time, the Society holds conferences on antioxidants, in particular, on the natural phenol derivatives. The international meetings on antioxidants cover, among other issues, studies of tea, coffee and cocoa, which are known to contain a bunch of components possessing antioxidant properties. It is pertinent to note that the overall number of identified phenolic antioxidants in natural objects (fruits, vegetables, plants and so on) has approached ten thousand,[9] this diversity being a result of evolution of the living nature, first of all, the plant kingdom.

In recent reviews [10–13] and experimental papers devoted to phenolic compounds, particularly the antioxidant properties attract attention of not only chemists but also specialists in related life sciences. In Russia, these problems are addressed at a number of institutes of the Russian Academy of Sciences (in Moscow, at the N N Semenov Institute of Chemical Physics, the N M Emanuel Institute of Biochemical Physics and K A Timiryazev Institute of Plant Physiology; in Novosibirsk, at the Research Institute of Antioxidant Chemistry and so on).

The growing interest in these compounds is evidenced by the ever increasing number of publications retrieved upon

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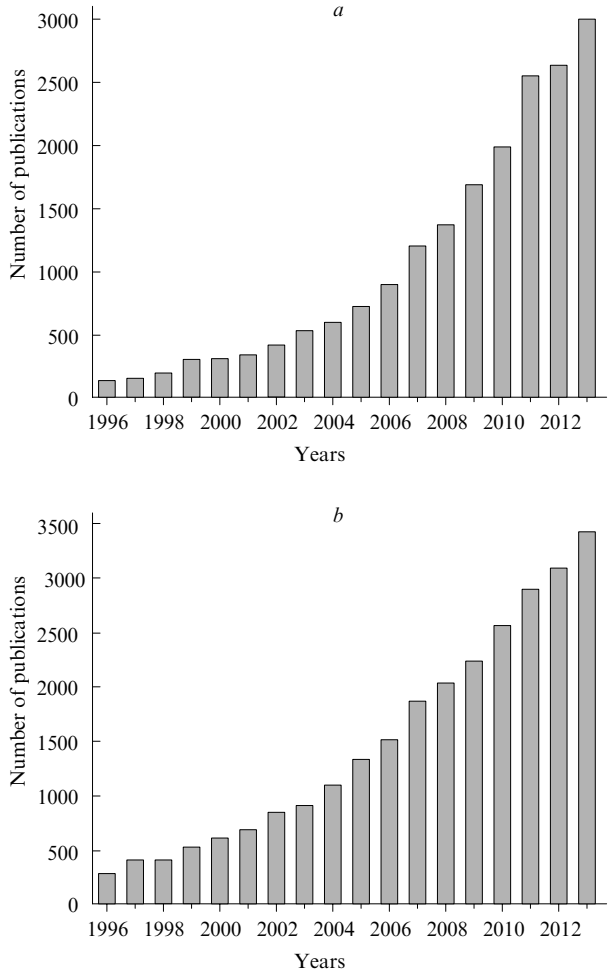


Figure 1. Number of papers in the Web of Science database for the keywords 'phenolic antioxidants' (*a*) and 'polyphenols' (*b*).

search for the key words 'phenolic antioxidants' and 'polyphenols' (Fig. 1 *a*,*b*, respectively) in the Web of Science database. Ample and often contradictory information on this topic can be found on the Internet. Googling the words 'phenolic antioxidants' and 'biomedicine' gives references to $\sim$ 100 000 sources. On the one hand, phenol derivatives are components of special plant formulations, biologically active supplements (BAS) and medicines, which, according to mass media, are to be taken on a regular basis for preventing premature ageing. On the other hand, excessive intake of antioxidants can be harmful. For example, it is known that under certain conditions, antioxidants can induce apoptosis of healthy cells.[14] Thus, the problem of using antioxidants, in particular phenols, has not yet been unambiguously solved in modern health care. The administration of such drugs is theoretically substantiated only at certain diseases. Therefore, it is not by chance that this problem is addressed by specialists who work in various fields of medicine, food technology, chemistry and related disciplines (bioanalytical chemistry, biochemistry, biophysics, medicinal chemistry, pharmaceutical chemistry, *etc.*).

Currently, the free radical oxidation processes involving reactive oxygen species (ROS) and nitrogen species in a living body are considered as the main cause for body ageing and for development of pathological states such as ischemic heart disease, atherosclerosis, cataract, cancer, Alzheimer's disease and so on.[15–17] It was also found that free radicals, in particular, ROS, affect the human genome, being responsible for a series of autosomal-recessive hereditary diseases.[18]

In biological systems, radicals mainly arise *via* reactions involving xanthine oxidase, mitochondrial and microsomal electron transport chains and haemoglobin, one of the most abundant proteins in the body. Simultaneously, some of these components also serve as sources of endogenous hydrogen peroxide. The cytotoxic action of hydrogen peroxide is caused by the fact that it can be reduced by transition metal ions (iron, copper, manganese, *etc.*) to give highly chemically reactive hydroxyl radicals:

$$H_2O_2 + Fe^{2+} \longrightarrow HO^{\bullet} + OH^{-\bullet} + Fe^{3+} \quad (1)$$

Reaction (1), which is known as Fenton's reaction, is the major process generating $HO^{\bullet}$ radicals in biological systems.

Free radicals are reactive species with a short lifetime, which depends on the external conditions. At 37 °C in water, the half-life (τ) of the superoxide radical anion is 10^{-6} s; that of hydroxyl radical is 10^{-9} s; that of alkoxyl radicals is 10^{-6} s, and that of the peroxyl radical is 10^{-12} s.[19] These radicals are constantly formed in the course of normal metabolism in the cells of aerobic organisms upon oxygen reduction and can generate new free radicals upon reactions with non-radical compounds.[20,21] The term ROS embraces the proper oxygen free radicals and their derivatives (Table 1).[22]

Due to the cascade type of the radical formation process, the radicals can damage any cell components despite rather short radius of action and short lifetime. The reactive oxygen species cause oxidative modification of biopolymers: proteins, lipids, nucleic acids and carbohydrates,[21,23,24] which may give rise to various disorders. Free-radical oxidation continuously proceeds, although at a very low rate, in the tissues of any living organism to give reactive products — free radicals (including peroxide radicals), hydroperoxides, aldehydes, ketones — and can be accompanied by radical polymerization.[23,24]

A healthy body is known to maintain a balance between oxidants and antioxidants controlled by homeostasis. A disruption of this balance in favour of oxidants gives rise to so-called oxidative stress.[21] Provision of the balance

Table 1. Reactive oxygen species (R = Alk).[22]

Formula	Name	Origin
$^3O_2$	triplet oxygen	stable species
$^1O_2$	singlet oxygen	excitation of $^3O_2$
$O_2^{-\bullet}$	superoxide radical anion	$O_2 + e^-$
$HO_2^{\bullet}$	hydroperoxide radical	$O_2^{-\bullet} + H^+$
$HO^{\bullet}$	hydroxyl radical	H_2O_2
H_2O_2	hydrogen peroxide	$O_2^{-\bullet}$, metabolism
$R^{\bullet}$	alkyl radicals	RH
$RO^{\bullet}$	alkoxyl radicals	ROOR, ROOH
$ROO^{\bullet}$	peroxyl radicals	$R^{\bullet} + O_2$, ROOH
ROOH	alkyl hydroperoxides	$ROO^{\bullet}$, $^1O_2$
ROOR	peroxides	peroxidation

implies therapeutic correction and monitoring of the abovementioned processes, and the success of these operations depends on the possibility of determining the concentration of the antioxidant used. It is noteworthy that depending on the concentration, structure and mechanism of action, antioxidants, first of all, phenols, can behave as prooxidants, which is manifested as an increase in the yield of ROS. Although these antioxidants are not xenobiotics, their beneficial action depends on their concentration: beyond the therapeutic dose, not only prooxidant but also toxic properties can be manifested in some cases. For this reason, the problem of control over the state of the human antioxidant system at various types of pathologies for the subsequent therapeutic correction is often related to the need to quantify both single and overall antioxidant contents.

Phenolic antioxidants are active components of some pharmaceuticals; therefore, an important task is to determine their contents in the basic substance and in the dosage forms. The extension of the range of medicines and biologically active supplements based on natural plant raw materials containing phenolic antioxidants necessitates the development of methods for assessment of the biological activities and the influence of extracts on a living body and for control over the quality of such products. As has already been noted, the content of the phenolic antioxidants in specimens is considerably affected by the geographic factor, *i.e.*, the place where the plant material was collected (climatic region, soil characteristics, *etc.*). Hence, study of bioantioxidants demonstrates the intertwining of two scientific disciplines — chemistry and pharmacognosy.

As has already been noted, the interest in the natural phenolic antioxidants in Russia has a rather long history. A large contribution to the studies of the structure and properties of compounds of this class has been made in the last 50 years by the school of N M Emanuel and his followers.[25–29] The activity of this school includes traditional conferences devoted to antioxidants and to various aspects of their application. The researchers pay attention to the issues of prophylactic and therapeutic medicine involving the use of antioxidants to prevent and treat disorders caused by unbalance of free radical and lipid peroxidation levels in the body.

Recently, Russian Chemical Reviews published two comprehensive papers[30, 31] devoted to studies of phenolic antioxidants. One review deals with application of chromatographic methods for determination of biologically active phenolic compounds in various objects. Particular attention is devoted to techniques used for sample preparation and detection of chromatographic peaks of antioxidants and to advantages of high performance liquid chromatography over spectrophotometry. The other review is devoted to theoretical estimation of the antioxidant reactivity of this group of compounds .

It is noteworthy that, while there is ample literature on phenolic antioxidants in the world (interdisciplinary books and survey articles concerning the knowledge areas allied to chemistry are especially numerous), the number of publications on this topic in the Russian literature is moderate, especially as regards the chemical analysis aspects. The topicality of such studies is beyond doubt and there is good reason why the development of methods for analysis of phenolic antioxidants has become the objective of some candidate and doctoral theses[32–37] successfully defended in the last decade.

The diversity of chemical forms of phenolic antioxidants in living systems necessitates the search for new, reliable, simple and fast methods to quantify these compounds over a broad concentration range. This is an interdisciplinary problem being of interest for a large number of specialists whose research is related to one or another extent to the chemistry and biochemistry of these compounds.

II. Classification, structure and physicochemical properties of phenolic antioxidants as important components in natural objects

Development of methods for determination of antioxidant concentrations is usually based on their structural details and the related physicochemical properties, which are found empirically.

II.1. Structure and classification of phenolic antioxidants

There exist several types of classification of phenolic antioxidants, which can be based on the nature of the aromatic nucleus, the number of hydroxyl groups, the number of $Ar(OH)_n$ moieties, the number of *ortho*-substituents, the solubility in water and lipids, the mechanism of antioxidant action and, finally, the origin.[38] The classification we use in this review is based on the biogenetic principle. All phenolic antioxidants are grouped according to the number of benzene rings present in their molecules and types of moieties connecting these rings.[9] Five key classes can be distinguished, the most extensive class comprising flavonoids, or arylbenzopyrans (Fig. 2). The other classes are

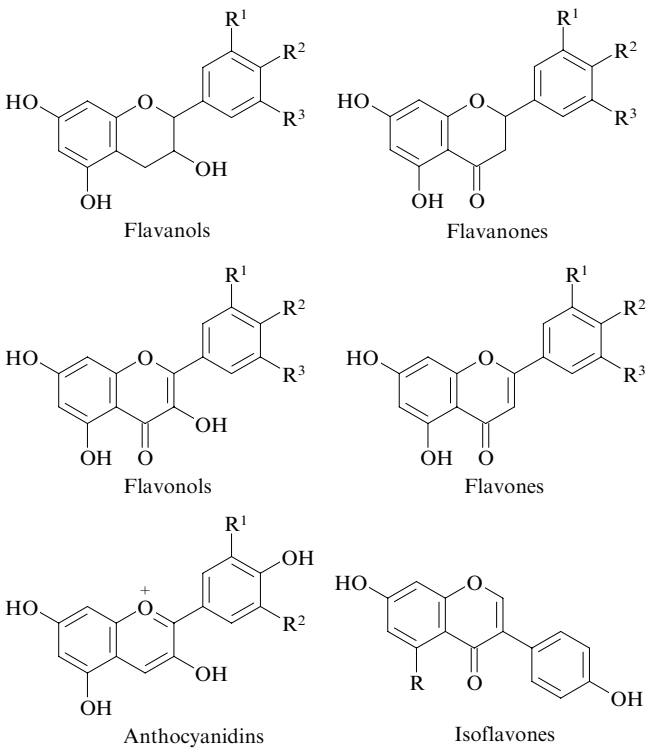


Figure 2. Flavonoids — the main class of natural phenolic antioxidants (R^i = H, OH, OMe).

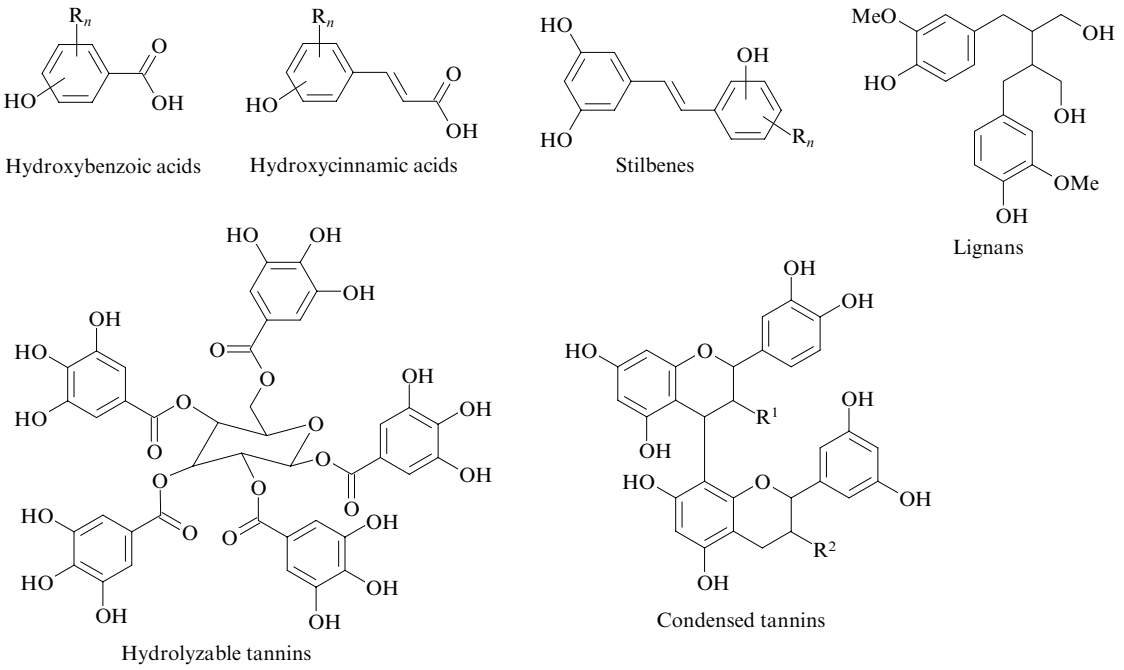


Figure 3. Classes of less abundant natural phenolic antioxidants (R, R^i = H, OH, OMe).

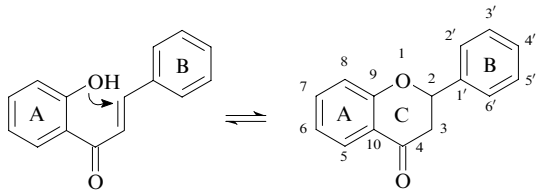
phenolic acids (benzoic and cinnamic acid derivatives), tannins (hydrolyzable and condensed), stilbenes and lignans (Fig. 3).[39]

II.1.a. Flavonoids

Currently, more than 8000 flavonoid derivatives have been isolated and identified.[40, 41] They perform a broad range of functions in the vital activity of plants, for example, in the colouring of flower petals, in seed (pollen) sprouting, in activation of bacterial genes responsible for nitrogen fixation and in the protection from pathogenic microorganisms and UV radiation. Flavonoids constantly enter the human body as components of food, especially, berries, fruits and vegetables. Most of them are synthesized by higher plants, mosses and ferns or, more rarely, by microorganisms.[42] In the above-ground part of the plants, these compounds are concentrated in leaves, flowers and fruits, as well as in the stems (bark and wood).

The biogenetic precursors of flavonoids are chalcones. The intramolecular Michael reaction — addition of the phenol hydroxyl to the activated double bond of chalcone — affords the flavonoid ring system (Scheme 1).

Scheme 1



Flavonoids are considered as compounds with three rings consisting of 6, 3 and 6 carbon atoms ($C_6-C_3-C_6$ structure), *i.e.*, two benzene rings (A and B) are connected by a three-carbon moiety, which forms the pyran ring (C). They are divided into monomeric flavanols (or catechins), flavanones, flavonols, flavones, isoflavones and anthocyanidins[43] (Table 2). Flavonoids are classified in terms of the degree of unsaturation of the three-carbon moiety, the number and positions of hydroxyl groups in the molecule, the site of attachment of the phenyl side group and the type of heterocycle.[41] Note that flavones and flavonols possess high structural diversity and are most abundant in nature.[46]

Flavonols have a double bond between the C(2) and C(3) atoms, a hydroxyl group in position 3 and a carbonyl group in position 4. These compounds occur in foodstuffs

Table 2. Flavonoid classification and natural sources.[9, 44, 45]

Class	Representatives	Natural sources
Flavanols	catechin, gallocatechin	red wine, grapes, green and black tea
Flavanones	naringin, eriodictyol, hesperitin	citrus fruit peel, tree bark
Flavonols	quercetin, rutin, myricetin, kaempferol	larchwood, onion, cranberry, apple peel, fleshy fruits and berries, olives, black tea, red wine, grapes
Flavones	chrysin, apigenin, luteolin	fruit peel, celery, parsley
Anthocyanidins	malvidin, cyanidin, pelargonidin, delphinidin	red grapes, red wine, fleshy coloured berries
Isoflavones	daidzein, genistein	soybean, soymilk and cottage cheese

as *O*-glycosides (most often, with a D-glucose residue or, more rarely, D-galactose, L-rhamnose, L-arabinose, D-xylose or D-glucuronic acid residues, which are preferably attached at positions 3 or 7).[47, 48]

Flavones also contain a double bond between the C(2) and C(3) atoms and a carbonyl group in position 4. They are encountered in plants rather rarely, usually as polymethoxylated derivatives, which are present in high amounts in the fruit peel.

Flavanols have a saturated three-carbon chain with the hydroxyl group in position 3 and exist in either monomeric (catechins) or polymeric form (proanthocyanidins or condensed tannins). Unlike other flavonoids, they are not glycosylated in foodstuffs. The main representatives of this class are catechin and epicatechin, while gallocatechin and epigallocatechin occur mainly in tea.[39]

Flavanone molecules contain a saturated three-carbon chain and a carbonyl group in position 4. Most often, they exist in the glycosylated form, in particular, as disaccharide at position 7.[49]

Structurally, isoflavones resemble oestrogens, *i.e.*, they contain hydroxyl groups in positions 4 and 7. These compounds are encountered in many plants and plant products both as aglycones and as acetic or malonic acid derivatives, or as β-glycosides. Their physiological action is similar to that of oestrogens; therefore, isoflavones are often called phytoestrogens.[50] Isoflavones are sensitive to temperature and are often hydrolyzed to give off the glycoside residue both during industrial processes (*e.g.*, manufacture of soybean products) and during storage.[51]

Anthocyanidins are the basic unit of anthocyanins. They consist of aromatic rings A and B connected *via* an oxygen-containing heterocycle C. In nature, they occur most often as glycoside forms called anthocyanins, existing as either monosaccharides (with glucose, galactose, rhamnose and arabinose residues) or di- and trisaccharides formed by combinations of these four monosaccharides.[52] As a rule, the glycoside residues are attached to position 3 of ring C or positions 5 and 7 of ring A. In rare cases, glycosylation at positions 3′, 4′ and 5′ of ring B is possible.[53] Many anthocyanins contain glycoside residues acylated with aromatic and aliphatic acids. Anthocyanins extracted from plant material are unstable and are rapidly destroyed[54] under the action of temperature, acidic or alkaline medium, light, air oxygen and so on.[55]

Thus, flavonoids can occur in natural objects in the free state but, more often, they exist as mono-, di-, tri- and even tetraglycosides, and, in some cases, acyl residues derived from hydroxycinnamic, hydroxybenzoic or other organic acids (acetic, malonic, oxalic or succinic acid) are attached to the sugar moieties.[56] The diversity of flavonoids is, in particular, due to the presence of asymmetric carbon atoms in the pyran heterocycle and various options for hydroxylation, alkylation and O- and C-glycosylation of aromatic nuclei A and B and acylation of these derivatives.[44, 57]

II.1.b. Phenolic acids

Phenolic acids represent a significant portion of the total number of phenolic compounds present in plants both in the free and in the bound state (acylation to give ester bonds with various plant components).[58] They are represented by two types of compounds: hydroxybenzoic and hydroxycinnamic acids. Hydroxybenzoic acids with the general C_6-C_1 structure are benzoic acid derivatives containing hydroxyl and methoxy groups in various positions of the aromatic ring. The main representatives of this class of compounds are *p*-hydroxybenzoic, vanillic, syringic and protocatechic acids. In the soluble form, they are usually conjugated with sugars or organic acids and with cell wall components.[59] Gallic (trihydroxbenzoic) acid is involved in the formation of hydrolyzable gallotannins.[60] The product of its dimerization (hexahydroxydiphenic acid) and the related dilactone (ellagic acid) are plant metabolites. Ellagic acid is present in ellagotannins as diphenic acid esters.[61]

Hydroxycinnamic acids have an aromatic ring with a three-carbon side chain (C_6-C_3 structure). The main representatives of this class are caffeic, ferulic, *p*-coumaric and sinapic acids. In nature, these compounds are usually conjugated with quinic, shikimic and tartaric acids or exist as glycosides. Free acids are formed upon chemical or enzymatic hydrolysis.[62]

II.1.c. Tannins, stilbenes and lignans

Tannins are compounds with rather high molecular mass that are divided into two types: hydrolyzable and condensed. Hydrolyzable tannins are derivatives of gallic acid, which is connected to the polyol by an ester bond. The pyrogallol moiety can, in turn, be further esterified or undergo oxidative cross-linking to give more complex hydrolyzable tannins. The major sources of hydrolyzable tannins are pomegranate and raspberry. Proanthocyanidins (condensed tannins) are flavonoid dimers, oligomers and polymers, the mechanism of their formation being still unclear. Condensed tannins based on flavan-3-ols, in particular, (−)-epicatechin and (+)-catechins, have been studied to a higher extent.[49] Among the plant sources of condensed tannins, noteworthy are apples, grapes, peaches, plums, mangostan fruits and pears.

Stilbenes have the $C_6-C_2-C_6$ structure and are similar to flavonoids but, unlike the latter, they are present in foodstuffs in minor quantities. A typical representative of this class is resveratrol, which exists as *cis*- and *trans*-isomers and is usually glycosylated.[63] It is synthesized by plants in response to the action of some pathogens and in stresses. More than 70 plant species serve as sources of resveratrol; the major sources are some berries and peanuts. The keen attention to resveratrol is related to its anticarcinogenic effect.

Lignans are formed upon biooxidative dimerization of two molecules of substituted phenylpropane. In nature, they are encountered in the free state or, more rarely, as glycosides. These compounds are of interest as they may possibly be used as chemotherapeutic agents, in particular, to treat cancer.[64]

II.1.d. Properties of phenolic antioxidants

The physicochemical characteristics of natural phenols published in recent years have been surveyed by Denisov and Denisova.[31] The O–H bond dissociation energies for 71 natural phenols were considered. The kinetic characteristics of reactions of peroxyl, alkyl and thiyl radicals with natural phenols and reactions of phenoxyl radicals with phenols, lipids, hydroperoxides, cysteine and ascorbic acid were summarized and described systematically. The reactivity of phenols in radical transformations was estimated with regard to the factors determining the reactivity (reaction enthalpy, triplet repulsion, electronegativity and atomic radii of the reaction centre, the presence of π-elec-

trons in the vicinity of the reaction centre, steric hindrances and force constants of the reacting bonds).

The important role of intermolecular hydrogen bonds involving OH groups for the decrease in the reactivity of natural phenols was noted.

The ability of phenolic compounds to absorb UV light has been known for a long time.[65] The absorption spectra usually contain two bands between 200 and 360 nm: the band at shorter wavelength is due to absorption of the groups of ring B and the longer-wavelength band is due to ring C groups. Both bands shift to longer wavelengths in an alkaline medium. The UV spectra and the extinction coefficients of phenolic antioxidants in solutions depend to a considerable extent on the solvent nature, the donor-acceptor properties of substituents in the aromatic rings, intra- and intermolecular hydrogen bonds, steric effects and the form of existence of phenolic antioxidants depending on the pH. Indeed, the resonance and tautomeric forms, hydrogen bonds and the colour of anthocyanins are determined by the pH. For example, the red wine anthocyanin (malvidin-3-glucoside) is red-coloured at pH 1, colourless at pH 4–5, violet at pH 6–7 and yellow at pH 7–8. At pH 8–10, this compound exists as intensely coloured ionized base, which is rapidly hydrolyzed at pH 12 to give ionized chalcone.[66] Caffeic, chlorogenic and gallic acids are unstable in alkaline media: their molecules and, hence, the UV spectra undergo irreversible changes. (−)-Catechin, (−)-epigallocatechin, ferulic acid, rutin and *trans*-cinnamic acid are stable to the pH change.[67]

Important properties of phenolic antioxidants are stability to high temperature and UV radiation. Catechin, gallic and vanillic acids and spruce bark or grape seed extracts are fairly stable to heat treatment, UV radiation or photooxidation in the presence of citric or ascorbic acid, or sodium chloride or nitrate.[68]

Characteristics of the absorption spectra of phenolic antioxidants are correlated with their electrochemical activities. These compounds exhibit reducing properties both in electrode transformations and in reactions with oxidants in solutions, which is also of analytical significance.

II.2. Antioxidant and prooxidant behaviour of phenolic compounds and its relationship with the structure

The notion 'antioxidant' is widely used in scientific literature. It is interpreted in different ways depending on the method used to assess the antioxidant properties. In order to develop a unified terminology, Halliwell and Gutteridge[20] defined an antioxidant as 'any substance that, when present in low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits the oxidation of that substrate'. This definition covers both radical and non-radical oxidation processes. In accordance with the above definition, the physiological role of antioxidants is to prevent the damage of cell components resulting from chemical reactions involving free radicals.[69]

Note that in the general case, the term 'antioxidant' is not strict if the neutralizable oxidant is not taken into account.[70] Furthermore, the applicability of the term depends on the medium in which the compound functions, in particular, *in vitro* or *in vivo* experiments are considered. In this context, the accurate description of the reactions and conditions in which the antioxidant action is studied becomes the key factor. Otherwise, the assignment of a compound to the class of antioxidants is not biologically significant.[71]

The phenolic compounds considered in this review are classical antioxidants that function by various mechanisms. These derivatives participate in the following vital activity processes:[72]

— direct absorption of free radicals, in particular, ROS;

— activation of enzymatic antioxidant systems [quinone oxidoreductase and NADPH (NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate), glutathione transferase and uridine 5-diphosphate glucuronosyltransferase];

— reduction of α-tocopherol radicals in which phenolic antioxidants act as hydrogen atom donors;

— increase in the uric acid concentration;

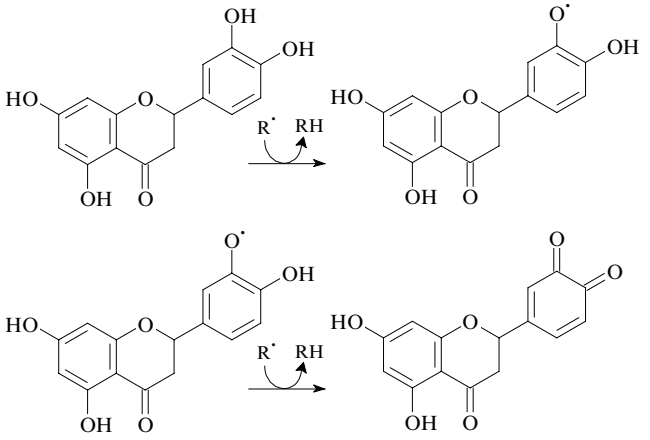
— inhibition of the activity of oxidases (xanthine oxidase, protein kinase C, cyclooxygenase, lipoxygenase, microsomal succinoxidase and the reduced form of nicotinamide adenine dinucleotide oxidase) producing the superoxide radical anion;

— increase in the antioxidant action of other low-molecular-mass antioxidants due to synergistic effect.

In addition, chelation with transition metal ions (iron, manganese, copper, *etc*.) may take place through the pyrocatechol moiety of ring B, 3-hydroxy and 4-oxo groups of ring C or 4-oxo- and 5-hydroxy groups of rings A and C. Finally, it is necessary to mention that flavonoids suppress the formation of $NO^{\bullet}$ species, thus mitigating the intensity of the oxidative stress they induce. This mechanism is implemented due to the presence of the C(2)=C(3) double bond and the 4-oxo group in ring C and the OH groups in positions 3, 5 and 4′ of rings A and B, respectively.[72]

For the analytical chemistry of phenolic antioxidants, of most interest is the above-mentioned ability to absorb free radicals, which is due to the presence of free or bound OH groups in the benzene ring (responsible for the reactions accompanied by hydrogen atom transfer in which the antioxidant molecule functions as the hydrogen donor). The radicals are deactivated according to Scheme 2. The flavonoid radical formed in the first step can react with a second radical, being converted to a stable quinoid structure.[73]

Scheme 2



The antioxidant activity exhibited in experiments *in vitro* depends on the arrangement of functional groups in the phenolic molecule. Since most phenolic antioxidants are

flavonoids, it is pertinent to consider their antioxidant properties in more detail.

The mechanism of antioxidant activity of flavonoids depends considerably on the arrangement and the total number of hydroxyl groups. For absorption of ROS, the hydroxyls of ring B play the key role,[74] whereas substituents in rings A and C make only a minor contribution to the rate constant for the reaction with the superoxide radical anion.[75] The antioxidant activity of phenolic compounds increases upon polymerization of flavonoids, *i.e.*, upon the formation of condensed tannins (anthocyanidins), in particular, catechin polymers, due to the large number of hydroxyl groups in their molecules. The efficiency of antioxidants depends on the length of the oligomer chain and the type of ROS they react with. Phenolic glycosides possess a lower antioxidant activity than aglycones. Generally, the key structural requirements for flavonoids to be able to absorb ROS can be formulated as follows:[76]

— *ortho*-dihydroxy moiety in ring B providing electron delocalization;

— C(2)=C(3) double bond, which is conjugated with the oxygen atom of the 4-substituent of ring C and provides electron delocalization in ring B;

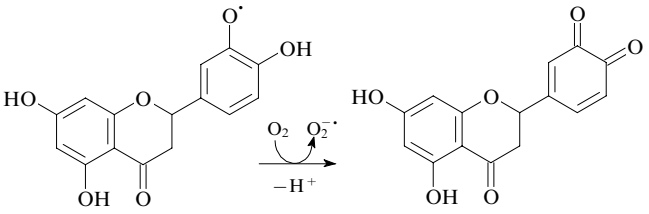
— hydroxyl groups in positions 3 and 5 of rings C and A, respectively, capable of intramolecular hydrogen bonding with the oxo group in position 4.

According to these requirements, the flavonols quercetin and myricetin are the best radical traps among flavonoids in an aqueous medium, and this has been confirmed experimentally.[56]

Most low-molecular-mass antioxidants, including phenol derivatives, can function as prooxidants, inducing oxidation of other compounds. The prooxidant activity of flavonoids is directly proportional to the total number of hydroxyl groups in their structure.[77] It was shown that mono- and dihydroxyflavonoids do not exhibit noticeable prooxidant activity, whereas a larger number of OH groups, especially in ring B, substantially enhances the generation of hydroxyl radicals according to Fenton's reaction.[78, 79] In addition, the presence of the C(2)=C(3) double bond and the 4-oxo group may promote the formation of ROS by the reaction of copper(II) ions with oxygen.[80] The prooxidant effect of flavonoids depends on their structure and concentration. For example, the prooxidant effect of quercetin, naringin, hesperidin and morin in human lymphocytes assessed from the intensity of generation of superoxide radical anions and lipid peroxidation products is manifested only at high flavonoid concentrations.[81]

Flavonoids absorb ROS to give the corresponding phenoxyl radicals the lifetime of which is ~200 μs. They have fairly high reactivity and can be further oxidized to a stable quinoid structure[82] (see Scheme 2). The quinones thus formed participate in the subsequent chemical reactions being stabilized upon conjugation with nucleophiles (glutathione, cysteine or nucleic acids). These reactions are responsible for the prooxidant behaviour of flavonoids.[73] Indeed, apigenin, naringenin and naringin can rapidly oxidize NADH, which results in a single-electron reduction of oxygen to give the superoxide radical anion.[83] The other reaction responsible for the prooxidant behaviour of phenolic antioxidants is the reaction between the phenoxyl radicals derived from these compounds and oxygen in the presence of high concentrations of transition metal ions to give the superoxide radical anion and the quinone (Scheme 3).[75]

Scheme 3



In addition, phenoxyl radicals can arise in auto-oxidation reactions. The rate of quercetin and myricetin auto-oxidation largely depends on the pH of the medium[84] and the presence of transition metal ions. Quercetin is barely oxidized at physiological pH values. The rates of auto-oxidation of quercetin and myricetin increase in the presence of iron(II) and copper(II) ions.[85] Meanwhile, monohydroxyflavonols (*e.g.*, kaempferol) and glycosides (*e.g.*, rutin, which is the glycoside form of quercetin) are incapable of auto-oxidation. This confirms once again that the antioxidant and prooxidant behaviours of phenolic antioxidants are mainly due to the presence of at least two hydroxyl groups in ring B and a free OH group in position 3 of ring C.

Some flavonoids can suppress mitochondrial respiration through auto-oxidation resulting in the formation of ROS.[86] Flavonols with a pyrocatechol moiety in ring B (quercetin and fisetin) form *o*-semiquinones and oxidize the cytosolic and mitochondrial NADH in mammal cells in the dose-dependent fashion, thus changing the $NADH/NAD^+$ ratio and shifting the redox equilibrium in the cells.[87, 88]

Yet another mechanism of the prooxidant activity (in some cases, toxicity) of flavonoids comprises the action of peroxidases, which catalyze the oxidation of phenolic antioxidants to give reactive radicals, which are in turn involved in peroxidation of lipids and glutathione.[89] However, some of these processes are an integral part of normal functioning of a living body, the toxic effect being manifested only when the production of ROS is excessive.

Flavonoids containing a pyrocatechol moiety (catechin, luteolin, eriodictyol and quercetin) can oxidize ascorbic acid, NADH and glutathione.[83] The oxidized forms of antioxidants react *in vivo* with other antioxidants forming a sort of cascade accompanied by synergistic effects.[90] For example, a phenolic antioxidant is oxidized to *o*-quinone, which is reduced *via* a reaction with glutathione or ascorbic acid.

In the presence of transition metal ions [iron(II) and copper(II)], phenolic antioxidants, in particular, quercetin, luteolin and kaempferol, can damage the structures of single- and double-stranded DNA, which is attributable to enhanced generation of ROS under these conditions of complexation.[91, 92]

To summarize the foregoing, it can be stated that the antioxidant and prooxidant behavious of phenolic antioxidants appreciably depend on their concentration. Considering the prooxidant properties of phenolic antioxidants as toxic is not quite correct because these compounds are linked to the cell signalling systems and are involved in regulation of their functioning. The toxic effect of phenolic antioxidants is manifested only *in vivo* at high concentrations. Furthermore, the prooxidant behaviour can also be

beneficial when inducing low-intensity oxidative stress. This results in activation of the cellular antioxidant protection and biotransformation of some enzymes, *i.e.*, brings about the cytoprotective effect.[93]

II.3. Bioavailability and bioaccessibility of phenolic antioxidants in complex objects

As has already been noted, phenolic antioxidants are important components of food. Their biological activity noticeably depends on the origin and the nature of the object in which they are contained and the presence of particular macrocomponents and accompanying biochemical processes that occur in a living body. In order to predict the biological activity, information on the bioavailability, reactivity and transformations of phenolic antioxidants is required. The existing published data on this problem were surveyed in a review.[94]

In the context of the problem we consider, the terminology concerning bioavailability should be discussed in more detail. According to the US Food and Drug Administration definition, bioavailability is 'the rate and extent to which the active substances or therapeutic moiety is absorbed from a drug product and becomes available at the site for action'.[95] This term has a similar meaning when applied to food nutrients. As a rule, this characteristics is used to determine the nutrient content *in vivo*, in particular, in human biological fluids.[96]

On the other hand, the amount of a nutrient liberated from a food product and capable of being absorbed in the intestine is defined as bioaccessibility. The amount of the nutrient bioaccessible for further transformations can be equal to or lower than the amount liberated from the initial product (due to incomplete conversion to absorbable forms or inability to be absorbed, or due to interaction with other components during digestion, including processes in the mouth cavity).[97] The bioaccessibility is determined *in vitro*.

In the vast majority of cases, both the bioavailability and bioaccessibility of nutrients are conditioned by the properties of the foodstuff, which affect the physical, enzymatic and chemical processes during digestion.[98]

Having entered the human body in the form they are present in initial sources, most phenolic antioxidants are metabolized in small or large intestine. Detailed metabolism of phenolic antioxidants in the human body and variation of the *in vivo* bioavailability in the biological fluids and human bodies have been discussed in the literature.[99, 100]

The key factors affecting the *in vivo* bioavailability of phenolic antioxidants in the human body, which were considered in detail in a review,[101] are summarized in Fig. 4. The bioavailability of phenolic antioxidants largely depends on the structure and the chemical form of existence. Bioavailable *in vivo* are $< 1\%$ of anthocyanins, from 1% to 5% of quercetin, from 10% to 30% of flavanones and flavanols and 30%–50% of isoflavones and gallic acid.[102]

The absorption of flavonol glycosides varies over a broad range, from very fast (< 0.5 h) to very slow (~ 9 h). The different *in vivo* bioavailabilities of various quercetin glycosides indicate that the nature of the glycoside residue is the crucial factor. The pharmacokinetic studies demonstrated that quercetin glucoside is absorbed in the small intestine, while rutinoside is absorbed in the large intestine after deglycosylation.[99]

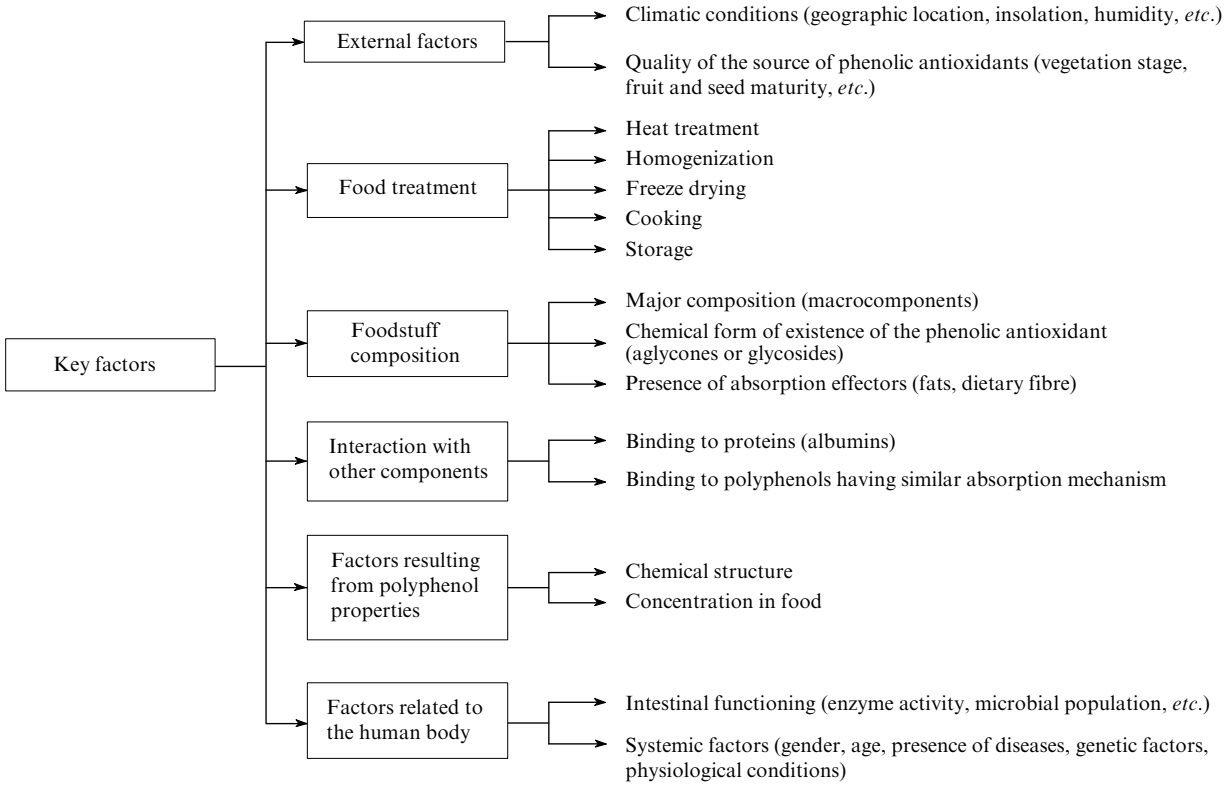


Figure 4. Key factors affecting the *in vivo* bioavailability of phenolic antioxidants in foodstuffs.

Catechins are absorbed rather fast in the small intestine. The presence of gallate residues does not affect the *in vivo* bioavailability, which is approximately the same for all catechin monomers. However, for dimers, this value decreases.[99]

Isoflavones have the highest *in vivo* bioavailability among all flavonoids, which does not depend on the form of existence. Aglycones and glycosides are absorbed rather slowly, *i.e.*, the process takes place in the large intestine. This is in good agreement with the low activity of the lactase-phlorizin hydrolase enzyme toward isoflavone glycosides.[103]

Anthocyanins are absorbed relatively fast but have the lowest *in vivo* bioavailability among all flavonoids, which is confirmed by their low concentrations in human biological fluids.[104] This may be a result of insufficient sensitivity of determination of anthocyanins in blood plasma and urine, on the one hand, and the diversity of anthocyanin structures, resulting in the large number of metabolites in the objects, on the other hand.[105] Also, one cannot rule out the possibility of formation of unstable derivatives, which are destroyed during the sample preparation.[106]

Phenolic acids present in many food products are also bioavaliable. As aglycones, they are usually absorbed in the upper gastrointestinal tract. It was shown that gallic, caffeic, ferulic, coumaric and chlorogenic acids can be absorbed in the stomach as soon as 1–2 h after the meal.[107, 108] Other areas that absorb phenolic acids are located in the small intestine. The absorption of aglycones can have different intensity; for example, for caffeic and ferulic acids, the *in vivo* bioavailability is 19.1% and 56.1%, respectively.[109] Phenolic acid esters have very low bioavailability ($\sim$0.3%–0.4% of the amount having entered the body), because they first need to be converted to aglycones, and the intestinal cell enzymes do not ensure sufficiently effective hydrolysis of the ester bonds.[110] Most of the phenolic acid esters enter the large intestine where they are metabolized. The metabolites can be determined in the blood plasma 7–8 h after the meal. It is noteworthy that for various caffeic acid esters, namely, chlorogenic, caftaric and rosmarinic acids, the metabolic pathways are similar and afford the same metabolites (*m*-coumaric, phenylpropionic and benzoic acids). Thus, different types of esterification of caffeic acid do not affect the metabolic pathway induced by the intestinal microflora.[111] Other issues concerning the *in vivo* bioavailability of the hydroxybenzoic and hydrocinnamic acids including the analytical applications were considered in a review.[109]

When discussing the bioavailability, one should pay attention to the possibility of direct interaction of phenolic antioxidants with lipids, polysaccharides and proteins.[112, 113]

II.3.a. Interaction of phenolic antioxidants with lipids

Phenolic antioxidants interact with cell membrane lipids, which changes both their biological activity and the properties of membranes.[114, 115] The type of interaction depends on the structural characteristics of phenolic antioxidants, in particular, the presence of hydrophobic and hydrophilic moieties. Adsorption on the polar heads of lipids leads to arrangement of the phenolic antioxidant on the bilayer surface, while interaction with the hydrophobic lipid chain results in the incorporation into the bilayer.[116–118]

Studies of the interactions of some flavonoids and liposomes with various polar groups demonstrated[119, 120] that hydrogen bonding between the flavonoid hydroxyl groups and the polar head groups of phospholipids is preferable for adsorption. A positive correlation was found between the intensity of interaction of lipid polar groups and the number of hydroxyl groups in the phenolic antioxidant molecule.[121] Nevertheless, a large number of hydroxyl groups in the monomer flavonoid molecules substantially increases the hydrophilicity, which hampers the adsorption on the lipid membrane surface.[122] The spatial structure of phenolic antioxidants also affects the degree of interaction with the lipid bilayer. In particular, the planar structure of the phenolic antioxidant molecule typical of flavanols (morin and quercetin) is better for the interaction with a lipid membrane than a non-planar structure typical of flavone (naringenin, hesperetin and eriodictyol).[123] In addition, the presence of methoxy groups in the phenolic antioxidant molecule should also be taken into account, as they also reduce its ability to interact with the membrane, which was demonstrated in relation to substituted epigallocatechin gallate. The presence of one or two methoxy groups in ring B and in the gallate residue considerably decreases the affinity of epigallocatechin gallate to the lipid bilayer; upon further methoxylation of positions 4′ of ring B and 4″ of the gallate residue, the affinity completely disappears. These data confirm the key role of the 4′- and 4″-hydroxyl groups in the binding of epigallocatechin gallate to cell membranes.[124] The involvement of gallate residues in binding to lipids was also shown for tannins.[125]

The inclusion of the phenolic antioxidant into the lipid bilayer is due to the hydrophobic interactions in which the key role is played by the relative hydrophobicity of the phenolic molecule. The hydrophobicity sequence for phenolic antioxidants in terms of their partition coefficients in the water–olive oil system was established. According to this sequence, flavans and flavanones are more hydrophobic than flavanols. At the same degree of hydroxylation, flavones are more hydrophobic than flavanones.[120] The ability of cinnamic acid to increase the lipid membrane permeability was established, whereas the 4-hydroxy derivative of this acid (*p*-coumaric acid) having lower relative hydrophobicity does not exhibit such behaviour.[126] The interaction of quercetin with the lipid membrane depends on the pH value. In acidic medium, it is strongly incorporated into planar lipid bilayers,[127] while at physiological pH values, it interacts with the polar groups at the water/lipid layer interface.[127–129] The flavanones naringenin and rutin and some isoflavones are distributed in an ordered manner in the hydrophobic region of liposome bilayers.[130]

Apart from hydrogen bonding and hydrophobic contacts, it is necessary to take into account the possibility of electrostatic interaction between the π-electrons of the aromatic rings of phenolic antioxidants and the Me_3N^+ groups in the phosphatidylcholine structure of the lipid layer.[131]

Phenolic antioxidants affect cell functions by changing the structure and physical characteristics of plasmatic membranes, in particular, fluidity and electrical properties. These effects are observed both upon antioxidant adsorption on the lipid membrane surface and upon antioxidant inclusion into the lipid bilayer. Modification of the membrane results, in turn, in the change of membrane-related biological processes, for example, the enzyme activity and

ligand–receptor interactions, and modulates the signal transduction.[114] Phenolic antioxidants adsorbed on the lipid surface create a barrier for penetration of hydrophilic radical species toward the membranes,[119] while phenolic antioxidants incorporated into the lipid bilayer absorb lipid, peroxylipid and other lipophilic radicals.[119, 132] In this way, phenolic antioxidants protect the membranes and membrane components from radical oxidation.

Although the interaction of phenolic antioxidants with membranes is considered as non-specific, recent investigations showed that some phenolic compounds selectively interact with particular regions of the membrane, in particular, with lipid rafts.[133–135] The latter are microdomains of the cell lipid bilayer membrane enriched in cholesterol and sphingolipids and in specific membrane proteins involved in the cell signal transduction.[136] Tannins and other phenolic antioxidants containing gallate moieties can specifically interact with the Me_3N^+ groups of sphingomyelin. The high reactivity of gallates with respect to the nucleophilic moieties of the membrane proteins, especially in the presence of peroxidases, promotes the formation of bridges between neighbouring cells. This induces the intercellular interactions important for information transmission and formation of cellular neural networks.[131] Phenolic antioxidants, for example, tea catechins, are involved in the intercellular interactions responsible for anticarcinogenic activity.[137, 138]

The interaction of some classes of phenolic antioxidants and their representatives with the lipid membranes has been considered in detail.[115, 120, 131, 139]

II.3.b. Interaction of phenolic antioxidants with polysaccharides

Currently, there are few published data about the interactions of phenolic antioxidants with polysaccharides. Most of the data are based on studies of model systems involving cyclodextrins or polysaccharides in the solid state. The mechanism of interaction is similar to that described above for lipids and comprises hydrophobic contacts and hydrogen bonding.[140, 141] When considering non-covalent interactions of phenolic antioxidants with polysaccharides, particular attention is paid to wine and related products.

Soluble polysaccharides (pectins, hemicellulose, starch, *etc.*) can be associated with phenolic compounds in solutions. The polysaccharides of wine do not prevent aggregation of tannins but affect the size of the resulting particles. For example, rhamnogalactouronan II causes the diameter of tannin aggregate particles to increase, while the polysaccharide dimer induces precipitation, which implies co-aggregation of tannin and the polysaccharide. This behaviour is related to the polysaccharide structure, in particular, the presence of the hydrophobic moiety, which interacts with the hydrophobic moieties of tannin aggregates.[142]

The specific interactions between the hesperidin and pectin cause turbidity of orange juices. Pectins containing $\sim$80% of neutral sugars and $>$10% of rhamnose form stable particles in the presence of hesperidin. For pectins with a different composition (40% of neutral sugars and $\sim$2% of rhamnose), unstable particles are formed although the molecular mass and the degree of esterification of pectins almost do not change.[143] This difference is attributable to the interaction of neutral sugars of pectin with the hesperidin molecule *via* hydrogen bonding. The stability of complexes formed by the phenolic antioxidant with pectin depends on the pectin molecular mass, the decrease in the molecular mass resulting in a decrease in the complex stability.

(−)-Epigallocatechin 3-*O*-gallate is trapped by the tamarind seed xyloglucan *via* non-covalent interactions to give a gel-like network.[144] The phenolic antioxidant molecules act as bridges between the xyloglucan chains.[145] The yeast polysaccharides (mannoproteins), which are partly liberated in wine during fermentation, bind anthocyanins and tannins,[146] resulting in a decrease in the polyphenol index of wines. Mannoproteins inhibit the aggregation of the grape tannins thus preventing turbidization and precipitation during wine ageing.[142] Mannoproteins with low and medium molecular masses stabilize the aggregates of phenolic compounds, while high-molecular-mass polysaccharides do not affect the aggregate particle size, as was shown in relation to model wines. The most probable mechanism implies the steric stabilization of aggregates.[147] The effect of mannoproteins on phenolic antioxidants depends on the yeast strain (race).[146, 148, 149] The crucial factors for the adsorption are mannoprotein characteristics: the total charge, charge distribution and the accessible surface area.[150]

Procyanidins can be bound by polysaccharides of various classes. The apparent affinity constant varies in the following series: pectins $\gg$ xyloglucan > starch > cellulose.[151] Higher affinity to pectin is due to its ability to form gel-like networks with the hydrophobic regions in which procyanidin can be encapsulated. Fibrous and globular polysaccharides (cellulose and xyloglucan) weakly bind procyanidins. The interactions of phenolic antioxidants with macromolecules including polysaccharides and methods for investigation of these interactions have been surveyed in more detail.[152]

The size and conformational mobility of phenolic antioxidant molecules are important factors influencing the strength of their interaction with polysaccharides. The binding of phenolic antioxidants to polysaccharides can considerably affect the bioavailability and bioaccessibility of natural phenols and requires further investigation.

II.3.c. Interaction of phenolic antioxidants with proteins

The interaction between proteins and phenolic antioxidants changes the properties of both components. On the one hand, it decreases the protein bioavailability in the body and protein binding, including that giving precipitates, and inhibits the enzyme activity. In other words, the interaction of proteins with phenol antioxidants affects the properties of proteins, which changes the protein functions in living systems.[153, 154]

On the other hand, proteins present in food products can form complexes with phenolic compounds.[155] This changes the biological activity of the latter,[156] in particular, the bioavailability and bioaccessibility.[157] In the presence of proteins, the antioxidant properties of natural phenols also change.[158] Nevertheless, today the question of influence of proteins on phenolic antioxidants is still open. On the one hand, it was found that the addition of milk reduces the antioxidant capacity of cocoa by 30%, which is attributable to binding of phenols to milk proteins.[157] On the other hand, the *in vivo* bioavailability of monomeric flavonoids (*e.g.*, epicatechin) does not decrease upon intake of cocoa with milk.[159] Meanwhile, the saliva proteins bind to phenolic compounds, which results in precipitation of insoluble complexes and in the astringent taste.[160] High-molecular-

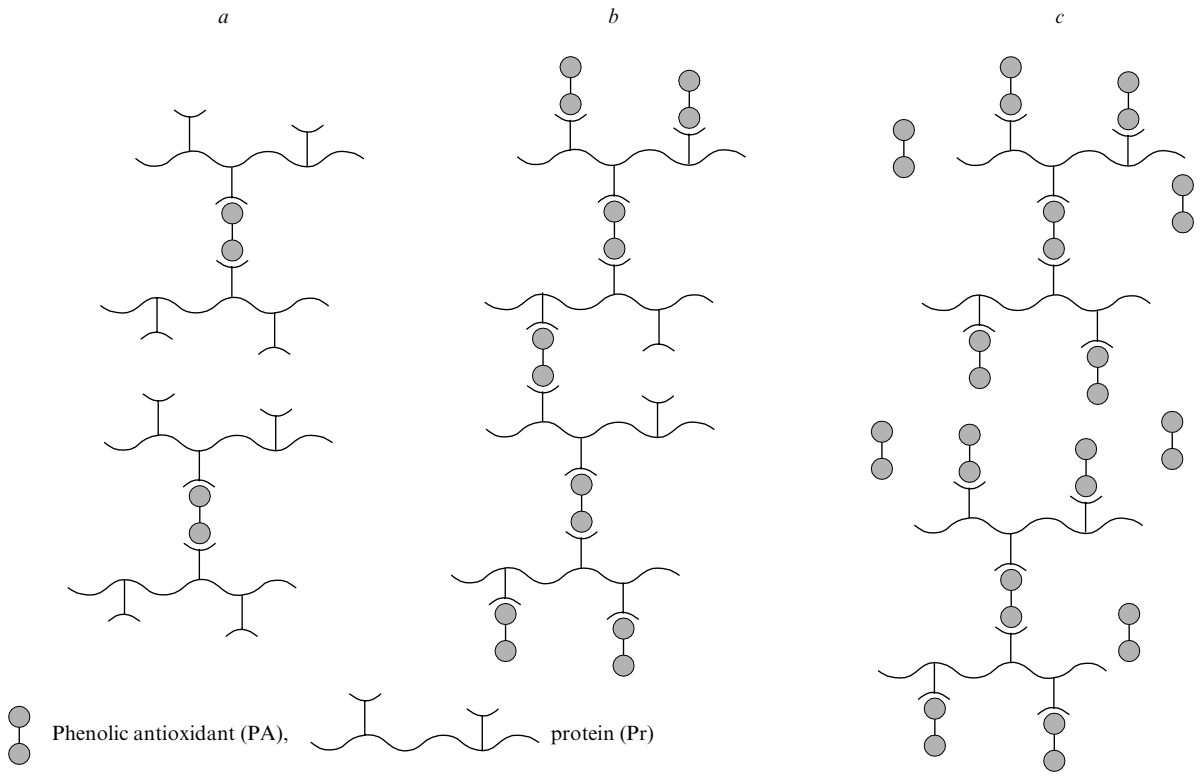


Figure 5. Binding of phenolic antioxidants (PA) and proteins (Pr) *via* hydrogen bonds depending on the component ratio: [PA] ≪ [Pr] (*a*), [PA] = [Pr] (*b*), [PA] ≫ [Pr] (*c*).[164]

mass derivatives can undergo strong interactions with proteins. Generally, phenolic antioxidants bind not only to saliva proteins but also to the food proteins as well as to the digestive enzymes, which, in turn, can affect their transportation and absorption activity.[161] Thus, the presence of proteins in foodstuff and in the digestive system determines both the bioavailability and bioaccessibility of phenolic antioxidants.

The mechanism of binding of phenols to proteins depends on the protein structure. Most often, they form covalent or intermolecular bonds. Non-covalent binding includes hydrogen bonding and hydrophobic interactions between phenolic antioxidant molecules and various functional groups of proteins.[152] In some cases, ionic bonds can form.[162] In the hydrogen bonding between the carbonyl group of the peptide bond and the hydroxyl groups of a phenolic antioxidant, the protein structure and mobility play the crucial role.[163]

The size and the stoichiometry of aggregates formed by phenols with proteins depend on the component ratio (Fig. 5).[164] If the number of active groups of the phenolic antioxidant is equal to the number of protein binding sites, large particles are formed. In the presence of excess protein, each phenolic antioxidant molecule forms hydrogen bonds with two protein molecules, while the proteins do not interact with one another to form dimers or small aggregates. When the phenolic antioxidant is present in excess, all of the protein active sites are occupied, and, hence, small aggregates with different solubility are formed. The ability of phenolic antioxidants to cross-link the protein molecules is manifested to a higher extent at low concentrations of phenol.[165]

Hydrophobic interactions involve the aliphatic and aromatic moieties of proteins and the aromatic rings of phenolic antioxidants.[166] The key role belongs to the amino acid residues: proline, histidine, arginine, phenylalanine, tryptophan, lysine, cysteine and methionine (Fig. 6). The process intensity is caused by the surface charge.

The protein structure also affects the mechanism of interaction with phenolic antioxidants. Linear proteins are attached in the face-to-face fashion, while globular proteins [*e.g.*, bovine serum albumin (BSA) and α-lactalbumin] are bound only through amino acid residues located on the protein globule surface.[167] Depending on the phenolic antioxidant structure and the protein nature, the contribution of each particular interaction can vary. For instance, quercetin is attached to BSA mainly *via* hydrophobic interactions.[168]

Thus, in the case of non-covalent binding to proteins, phenolic antioxidants act as multivalent ligands able to form intermolecular bonds with two or more molecules. The stoichiometry and the size of aggregates formed depend on the reactant concentrations and ratio.

The ability to form covalent bonds is affected by the phenolic antioxidant polarity.[169,170] Either multicentre (several antioxidant molecules are bonded to one protein molecule) or polydentate (one antioxidant molecule reacts with several active sites or protein molecules) binding is possible. The type of binding depends on the protein and phenolic antioxidant natures and on their ratio.[171,172] Generally, the reaction may be specific, like binding of tannins to gelatin, or non-specific, like interaction of tannins with BSA. In addition, the cooperative effect may take place,

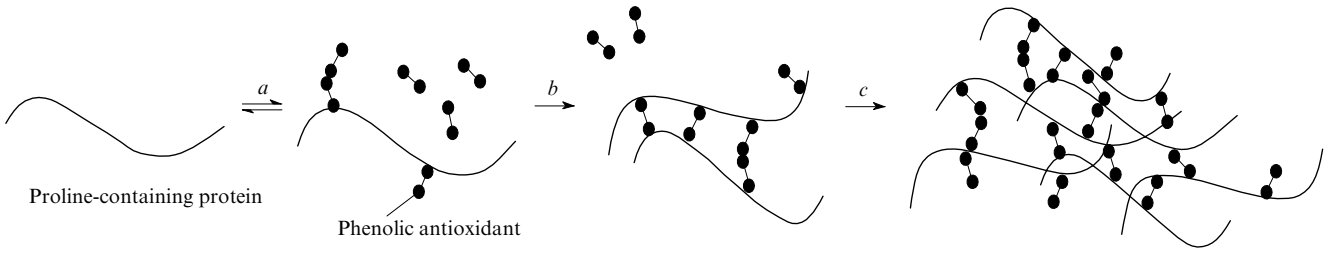


Figure 6. Binding of phenolic antioxidants by proline-containing proteins *via* hydrophobic interaction: (*a*) reversible binding to give a soluble complex; (*b*) cross-linking of two peptides upon increase in the amount of phenols (the complex becomes insoluble); (*c*) subsequent aggregation of the complex.[166]

which means that tannins that have already linked to the protein affect further interaction of the protein with new antioxidant molecules.[163]

The complexation between phenolic antioxidants and proteins can be reversible or irreversible, which affords soluble and insoluble complexes, respectively.[173, 174] It is noteworthy that an increase in the concentration of the phenolic antioxidant promotes the formation of insoluble aggregates.[175] For the formation of a protein–tannin complex, it is necessary that a particular number of active groups capable of cross-linking with tannins be present in the protein.[104]

As noted above, protein binding is caused by the affinity of tannins to proteins, polypeptides and polymers with high proline contents.[176] The absence of secondary and tertiary structure of the protein is the principal factor favourable for the binding.

Several publications [164, 177, 178] are devoted to the interaction of phenolic antioxidants with BSA. Phenols are shown to function as multivalent ligands able to form intermolecular bonds with protein molecules. The stoichiometry and the size of the resulting complexes depend on the reactant concentrations and ratio.

The results of studies devoted to the tannin complexes with saliva proteins, which contain many proline residues, were summarized by Bennick.[179] It was found that these complexes are large molecular associates.[180] The size of phenolic antioxidant molecules affects the binding of proteins: the smaller the size, the less efficient the complexation.

The interaction of the phenolic compounds isolated from olive oil with various food proteins (sodium salts of casein, BSA, β-lactoglobulin and gelatin) was studied by gel filtration, HPLC and some physical methods. It was found that the constants of this binding are rather low as compared with those for tannic acid.[181]

Epigallocatechin gallate binds to various proteins, especially to non-globular extended proteins with high percentage of proline.[182] One of such proteins is β-casein — the most important milk protein. It was found that upon the addition of epigallocatechin gallate, some β-casein molecules decrease in size and become less mobile. The components interact *via* non-covalent intermolecular binding. By atomic force microscopy, it was shown that single protein molecules surround the epigallocatechin gallate and exert a pronounced effect on the bioavailability of phenolic antioxidants in tea with milk and on the astringency of tea caused by the presence of tannins.[183]

The main types of interaction between various phenolic compounds and proteins, including the proline-containing saliva proteins and lysozyme are presented in Table 3.

The interaction of proteins with phenolic compounds changes both the bioavailability and bioaccessibility of the latter. Phenolic antioxidants are known to exert a beneficial action on the blood vessels in the human body. Tea is a source of phenolic antioxidants. It was found that taking tea with milk considerably decreases the bioavailability of its phenolic antioxidants and, hence, the action on the vascular system.[184]

The effect of coffee with milk on the urinary excretion of chlorogenic acid and its metabolites has been investigated. It was shown that the amount of chlorogenic acid and the metabolites is lower in all test subjects after taking coffee with milk ($40 \pm 27\%$) than after taking black coffee ($68 \pm 20\%$). The simultaneous intake of milk and coffee decreases the *in vivo* bioavailability of chlorogenic acid.[185]

The difference between the excretions of hippuric acid and 3,4-dihydroxyphenylacetic acid upon taking coffee or coffee with milk was found to be statistically insignificant.[186] However, when the content of milk is high, the *in vivo* bioavailability of these compounds decreases by 28%. Note that the amount of milk used in the experiments was that proposed by Brazil manufacturers of instant coffee. However, the fractions of coffee and milk used by consumers all over the world may be considerably variable depending on the national traditions and personal taste. It is quite possible that lower amounts of milk added to coffee would not affect the bioavailability of chlorogenic acid and the metabolism, which is supported by data for 20% of whole milk.[187]

It was shown that the addition of 26% of whole milk to black or green tea or to bilberry juice changes the antioxidant capacity of the human blood plasma.[188–190] Also, the addition of 10% of whole milk to black tea counteracts the favourable effect of tea catechins on the human vascular system.[184]

However, some studies dealing with the influence of taking milk together with green and black tea [191–193] or bilberry juice [194] did not reveal reliably detectable changes of the antioxidant properties of blood plasma, although the authors admitted the possibility of formation of complexes between phenolic antioxidants and proteins.

The *in vitro* bioaccessibility is assessed, most often, by the change in the concentrations of particular phenolic compounds or integral parameters characterizing the antioxidant properties of the whole specimen in the presence of proteins.

Table 3. Examples of interactions of phenolic antioxidants with proteins.[162]

Proteins	Phenolic antioxidants
Hydrophobic interaction and hydrogen bonds	
Polyamino acids, bovine serum albumin	grape, quebracho and mimosa tannins
Casein, gelatin, proline-containing proteins	penta-, tetra- and trigalloylglucoses, tea phenolic antioxidants
Proline-containing proteins	tannic acid
Peptides	tannic acid, catechin
Bovine serum albumin	pentagalloylglucose, epicatechin$_{16}$(4→8)catechin
Hydrogen bonds	
Gelatin, proline-containing proteins, polyamino acids	sorgo condensed tannins
Sunflower seed proteins	chlorogenic acid
Bovine serum albumin	red wine tannins
Proline-containing proteins	procyanidin B3
Hydrophobic interaction	
Proline-containing proteins	tannins
Saliva histatins	epigallocatechin gallate, pentagalloylglucose, quebracho tannins
Covalent bonding	
Myoglobin	chlorogenic, caffeic and quinic acids, *p*-quinone
Isolated wheat proteins	ferulic, chlorogenic, caffeic, quinic and gallic acids, *p*-quinone, tea, coffee, potato and pea extracts, quercetin, rutin
Lysozyme	*m*-, *p*-, *p*-dihydroxybenzenes, ferulic and gallic acids
Glycine and trypsin inhibitor from soya	chlorogenic, caffeic and gallic acids, flavone, apigenin, kaempferol, quercetin, myricetin
Bovine serum albumin	chlorogenic acid, quercetin
β-Lactoglobulin	quercetin, rutin
Human serum albumin	quercetin
Hydrophobic interaction, hydrogen and ionic bonds	
Sunflower seed proteins	caffeic and quinic acids
Hydrophobic contacts and covalent bonds, hydrogen and ionic bonds	
Rapeseed proteins	phenolic acids, rapeseed condensed tannins
Non-covalent interactions	
Bovine serum albumin, lysozyme, α-lactoalbumin	chlorogenic acid
Hydrophobic and covalent interaction, hydrogen bonds	
Bovine serum albumin	pentagalloylglucose and its oxidized form

Cocoa and cocoa products, specifically, cocoa paste, cocoa powder and milk and plain chocolate contain a broad range of phenolic compounds and possess a variety of antioxidant properties. During the last decade, about thirty studies were performed dealing with analysis of these cocoa products (see, for example, Ref. 195). Apart from phenolic antioxidants and methylxanthines, cocoa also contains proteins, which are responsible for its taste.[196] Cocoa beans contain four types of proteins: albumins, globulins, prolamins and glutenins. Albumins and globulins are the major proteins[197, 198] (52% and 43% of the total protein content in cocoa beans, respectively[199]) to bind phenolic antioxidants.

The addition of milk to tea was shown to markedly decrease the antioxidant action of tea; this provides the conclusion that phenolic antioxidants interact with milk proteins.[188, 200]

The *in vitro* bioavailability of the considered compounds in the presence of milk proteins was measured using electrogenerated bromine as a coulometric titrant. It was shown that proteins bind rutin and quercetin (14% to 90%) at a high rate, thus decreasing the bioavailability of flavonoids. The content of the free flavonoid decreases with an increase in the percentage of proteins in the mixture. The total antioxidant capacity of tea substantially decreases (from 7% to 85%) in the presence of milk in proportion to the percentage of milk in the mixture. The effect of milk addition does not depend on the black tea brand, and the degree of the decrease in its total antioxidant capacity on going from one tea brand to another virtually does not change.[201]

The effect of milk on the voltammetric signal of tea was estimated. Various tea types (green, black and oolong teas) and instant coffee were studied. It was found that milk (skimmed or whole) decreases the cyclic voltammetry response of tea to a larger extent than it decreases the signal of coffee. The considerable decrease of the response of a beverage upon the addition of milk attests to a decrease in the bioaccessibility of phenolic antioxidants in solution upon the oxidation on a glassy carbon electrode. The

addition of 0.5 ml of milk to 20 ml of the beverage reduces the response by about 25% and 10% for tea and coffee, respectively. When 2.0 ml of milk is added, the signal decreases by 40%–55% for tea and ~25% for coffee. This is attributable to the fact that tea catechins are more strongly bound to milk components than the coffee hydroxycinnamic acids, which have less HO groups in the molecules. No substantial influence of the fat content of milk on the signal was identified, which indicates that phenolic compounds of the beverages interact with the protein components rather than with the lipid fraction of milk.[202]

The electrogenerated hexacyanoferrate(III) ions were proposed as the coulometric titrant to determine the total content of free phenolic antioxidants in tea and coffee in the presence of proteins. It was found that proteins (casein and bovine serum albumin) bind 5% to 76% of phenolic antioxidants into complexes. The effect of milk proteins on the total content of free phenolic compounds in tea and coffee was estimated based on the ability to reduce $Fe(CN)_6^{3-}$ ions as a measure of their content. The addition of milk decreased this ability by 10%–70%, which reflected high content of phenolic antioxidants in the inactive form.[203]

The influence of milk caseins on the level of free catechins in tea responsible for its antioxidant action was studied. It was shown by micellar electrokinetic chromatography that the addition of milk into tea decreases the level of free catechins as they are bound to proteins.[204]

Both bioavailability and bioaccessibility of phenolic antioxidants in the presence of proteins are important parameters characterizing the biological activity. The recent achievements concerning this problem are summarized in the review.[205]

Thus, the biochemical aspects of the reactions of phenolic antioxidants with lipids, polysaccharides and proteins are of high theoretical and applied value. They determine the chemical form of existence and properties of the substance; this information is necessary to develop analytical methods for determination of these compounds, in particular, in biological objects, especially in food products having complex composition.

III. Problems of analytical chemistry of phenolic antioxidants

In complex natural objects, phenolic antioxidants occur, most often in the bound state and in a rather wide concentration range. In view of the large diversity of phenolic antioxidants, it should be borne in mind that currently there is no unified analytical approach for quantification of these compounds. In each particular case, the method that is most adequate to the designated tasks should be selected.

An important aspect of analysis of phenolic antioxidants is the form in which they exist in a real specimen — as aglycone, glycoside or conjugate. For example, in biological fluids (blood serum, blood plasma and urine), flavonoids exist as conjugates with glucuronic acid and sulfates. In most cases, quantitative analysis gives the total content of aglycones; therefore, preliminary hydrolysis is required. However, in plant raw materials, medicines and food products, phenolic antioxidants are determined in the native form, in some cases, as conjugates. This complicates the procedure of analysis and calls for the development of new highly selective and sensitive methods.

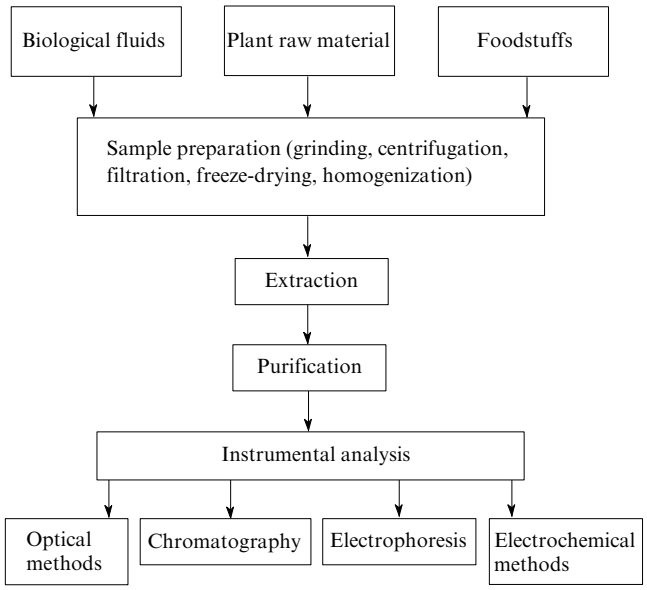


Figure 7. Flow diagram of determination of phenolic antioxidants in real objects.

The general flow diagram of analysis of biological fluids, food products and plant raw materials for the content of phenolic antioxidants is shown in Fig. 7. It is noteworthy that the samples are usually purified by solid phase extraction or column chromatography but this stage may be absent in some cases.

Phenolic compounds are quantified using reactions of various types (complexation, oxidation, *etc.*) in combination with physicochemical methods. Most popular are chromatographic and spectrophotometric methods. However, in recent years, the contribution of electrochemical methods, including electrochemical detection in a liquid flow, has increased considerably. Flow methods such as high performance liquid chromatography (HPLC) and capillary electrophoresis are used.

III.1. Methods for selective determination of phenolic antioxidants

As noted above, people receive most of phenolic antioxidants from food, in particular, fruits and vegetables, and beverages (tea, coffee, cocoa, juices, wine, and so on). The sample preparation and extraction of phenolic compounds are performed taking into account the nature of the investigation object and chemical properties of phenolic antioxidants: molecular structure (the number of aromatic rings and hydroxyl groups), polarity and concentration. The concentration ratio of derivatives of different classes (phenolic acids, flavonoids, anthocyanins, tannins, *etc.*) in the specimen also plays a role. Furthermore, quantitative isolation of phenolic antioxidants is hampered as they exist in different chemical forms, and other components (carbohydrates, lipids, *etc.*) can also be present. These circumstances account for the lack of a unified method of sample preparation and extraction of phenolic antioxidants from complex specimens, in particular, plant raw material. In the latter case, the sample preparation can include the drying stage (for example, freeze drying), grinding to a specified particle size, centrifugation, filtration and purification (for

Table 4. Examples of extraction of phenolic antioxidants from plant sources.

Sample	Antioxidant to be extracted	Extractant	Time /min	Ref.
Ultrasonic extraction				
Puerariae lobatae	isoflavones	80% EtOH	55	210
Galla chinensis	tannins	70% EtOH	40	211
Grapes	flavonoids	MeOH	60	212
Sunflower	polyphenols	80% acetone	30	213
Mandarin (peel)	hesperidin	80% MeOH	60	214
Microwave extraction				
Green tea	flavanols	water	30	215
Tea	polyphenols	60% EtOH	10	216
Sweet potato	"	53% EtOH	2.05	217
Sorgo	phenolic acids	2 M NaOH	0.45	218
Spices	the same	50% EtOH	18	219
Angelicae sinensis	ferulic acid	90% EtOH	9	220
Combined ultrasonic and microwave extraction				
Arctium lappa	caffeic acid	ionic liquid	0.5	221
Spatholobus suberectus	flavonoids	70% MeOH	7.5	222
Burdock (leaves)	phenolic compounds	70% EtOH	0.5	223
Anoectochilu roxburghii	quercetin	50% EtOH	15	224
Supercritical liquid extraction				
Psidium guajava (seeds)	phenolic compounds	CO_2 – ethyl acetate and CO_2 – EtOH	120	225
Momordica charantia	flavonoids	CO_2 – EtOH	53.2	226
Mentha spicata	"	CO_2	60	227
Strobilanthes crispus	"	CO_2 – EtOH	60	228
Pomegranate (seeds)	phenolic compounds	H_2O	30	229
Cinnamon	the same	H_2O	60	230
High hydrostatic pressure extraction				
Maclura pomifera (fruits)	polyphenols	H_2O – EtOH – MeOH – acetone – CH_2Cl_2 (1 : 2.5 : 2.5 : 2 : 2)	10	231
Green tea (leaves)	"	50% EtOH	1	232
Anacardium occidentale (juice)	"	5 vol.% of ethylene glycol in H_2O	5	233

liquid samples) and removal of lipids or hydrolysis for fatty materials.

The completeness of the extraction determines the correctness of the final result. Phenolic antioxidants are recovered most often by liquid extraction into organic or inorganic solvents. The extraction ratio depends on the time and the number of extraction procedures, temperature, raw material to extractant ratio and the natures of the solvent and test object. Optimization of these factors is important, because extraction may be accompanied by undesirable chemical processes (for example, enzymatic oxidative destruction, hydrolysis of glycosides, *etc*.). Effective extractants are water, acetone, ethyl acetate, alcohols (methanol, ethanol, propanol) and mixtures thereof.[206]

In the last decade, non-selective solid-phase extraction on alkylated silica gels (most often in the C18 form) has started to be used for isolation of phenolic antioxidants. In this case, the sample solution or the solvent is acidified to prevent ionization of phenolic antioxidants and, hence, to increase the retention time. Of interest also are sorbents based on molecularly imprinted polymers, which are highly selective to the analyte and possess sufficient mechanical and thermal stability.[207, 208] The main drawback of the solid-phase extraction with molecularly imprinted polymers is the necessity to manufacture a specific polymer for each particular analyte. Furthermore, determination is often interfered with compounds of similar structure. For example, morin and (+)-catechin exhibit affinity for the sorbent when quercetin extraction is performed.[207]

The methods for extraction of phenolic antioxidants of various classes from real objects are comprehensively described in reviews.[49, 209] The latest achievements in this field are based on the use of ultrasound, microwave radiation (or both), high-pressure supercritical fluids and other types of physical treatment (Table 4). These approaches are rather experimentally simple, and they reduce the time of extraction and the required volumes of organic solvents, which may be toxic.

III.1.a. Chromatographic methods

Chromatographic techniques, in particular, liquid (LC) or gas (GC) chromatography, including that coupled with mass spectrometry (MS) are the main methods for separation, identification and quantitative determination of phenolic antioxidants.

Currently, LC coupled with tandem mass spectrometry (LC-MS/MS) has actually become the routine method for identification and structure characterization, covering

also previously unknown representatives of various classes of natural phenolic compounds. The phenolic antioxidants of *Swertia chirayita* were identified by LC-MS/MS (ESI) based on the preliminary reaction of the compounds with free radicals (superoxide radical anion, peroxyl and polyaniline radicals) on the surface of functionalized graphene.[234]

Note that for reliable LC-MS/MS identification of phenolic antioxidants, the analysis is in some cases preceded by identification of the class of phenolic compounds by LC with UV diode array detection. Depending on the goals of investigation, tandem mass-spectrometric detection can be replaced by usual MS, which provides better selectivity and more information. Also, additional information is gained from the chromatographic retention times, UV absorption and fluorescence characteristics, and the electrochemical behaviour when other types of detection are used.

The structure of previously unknown phenolic antioxidants can be reliably confirmed by combining LC with NMR detection. These approaches are considered in detail in a review.[235] Also, structure characterization and assignment of phenolic antioxidants to particular classes are performed by combining HPLC and MS with different ionization techniques: electrospray, matrix assisted laser desorption, fast atom bombardment and electron impact ionization.[209]

The preferred method for separation and quantitative determination of phenolic compounds is HPLC.[236] The efficiency of analysis is affected by various factors: degree of sample purification, composition of the mobile phase, the column and detector types. Usually, purified extracts, reverse phase C18 column, a photodiode array detector and acidified polar organic compounds are used. Of high importance are purification and preconcentration of phenolic compounds from objects of complex composition. At the purification stage, side components are removed using partition chromatography on Sephadex LH-20, polyamide, Amberlite resin, solid phase extraction cartridges, styrene–divinylbenzene copolymers (XAD 4, XAD 16, EXA 90, EXA 118, SP70) and acrylic resins (XAD 7, EXA 31).[209] However, in most cases, solid phase extraction is used for purification and preconcentration of such compounds, and only after that, chromatographic separation is performed.

The commonly used mobile phases for HPLC determination of phenolic antioxidants are acetonitrile and methanol or their mixtures with water. In some cases, ethanol, tetrahydrofuran and isopropyl alcohol are also used.[237–239] The key requirement to the mobile phase is to maintain pH in the range of 2 to 4 to prevent ionization of phenolic compounds. Therefore, the mobile phases usually contain acetic acid or, more rarely, formic or phosphoric acid and phosphate, citrate and acetate buffer solutions.[239, 240] Gradient elution is used more often than isocratic elution.[241]

The selection of the column is critical for efficiency of analysis of phenolic compounds and is based on their polarity. HPLC is carried out with normal phase or reversed phase C18 columns (length of 10–30 cm, diameter of 3.9–4.6 mm and sorbent particle size of 3–10 μm) at the ambient temperature. However, in some kinds of HPLC (high-pressure LC, high-temperature and two-dimensional LC), new types of columns are applied: monolithic columns or columns packed with superficially porous particles (length of 3–25 cm, diameter of 1–4.6 mm and sorbent particle size of 1.7–10 μm). Using these columns, analysis can also be performed at elevated temperature.[242] The time required for HPLC analysis is 10 to 120 min depending on the conditions and the test object.

The detection of phenolic antioxidants is carried out, most often, in the UV and visible spectral regions, in particular, using a photodiode array detector at 190–380 nm and a fluorimetric or colorimetric detector.[209] In some cases, electrochemical detection is used;[176, 243, 244] owing to the development of chemically modified electrodes possessing high selectivity and sensitivity, the percentage of such studies gradually increases. Note that the amperometric response of the detector can be linear in a relatively narrow range of concentrations due to the possible change in the mechanism of oxidation of phenolic antioxidants as their concentration increases. Thus, the use of coulometric detectors with response depending only on the amount of the substance is promising.[245]

Relatively new chromatographic techniques employed to determine phenolic antioxidants include hydrophilic interaction HPLC, which serves to determine polar components in complex samples,[246] and two-dimensional LC, which is suitable for separation and identification of structurally related and minor compounds in complex samples as a result of increase in the peak capacity and selectivity.[247] A combination of two-dimensional and hydrophilic interaction LC, and two-dimensional and reversed phase LC[248] allowed for the successful detection of polar and semipolar fractions in traditional Chinese medications, which would be difficult in the case of usual reversed phase LC. The key achievements of LC for determination of phenolic antioxidants in plant specimens and food products are summarized in reviews.[235, 249, 250]

Gas chromatography can be utilized to determine various classes of phenol derivatives, in particular, phenolic acids,[251] condensed tannins[252] and flavonoids.[253] The major drawback of GC as compared with HPLC is the necessity of derivatization to convert phenolic compounds to the volatile form. Determination in foodstuffs can comprise purification (for example, removal of lipids from the extract), release from glycosides or esters in an alkaline or acidic medium or chemical modification, *e.g.*, conversion to more volatile compounds.

The volatile derivarives of phenols are formed upon reactions with various types of reagents. Methyl and ethyl esters are prepared using ethyl and methyl chloroformates, diazomethane and dimethyl sulfoxide in combination with methyl iodate. However, this may bring about the problem of differentiation between the methyl esters formed upon derivatization and those present in the sample beforehand.[254] Yet another type of reagents are trimethylsilyl derivatives, for example *N*-(*tert*-butyldimethylsilyl)-*N*-methyltrifluoroacetamide.[58] The silylation reactions are simple, exclude side processes and afford volatile compounds that do not interfere with the analysis.[241] This type of derivatization is well suited for identification of phenolic antioxidants but requires more time.

In gas chromatography, a flame ionization detector is used most often, but recently mass spectrometry providing higher selectivity and sensitivity of determination has gained popularity.

For example, flavonoid glycosides are determined by high-temperature GC/high-resolution MS.[255] This method

is more efficient, requires less time and provides better separation with minimum co-elution as compared with HPLC.[256]

Other chromatographic methods (thin-layer, paper, supercritical fluid and centrifugal partition chromatography) are rarely aplied for determination of phenolic antioxidants.

III.1.b. Electrophoretic methods

An alternative method for separation and analysis of phenolic antioxidants is capillary electrophoresis. It is especially demanded for low- and medium-molecular-mass polar, including ionized, compounds. In this case, the separation procedure is more efficient and requires less time than in the case of HPLC.[257] This method is suitable for routine determination of a broad range of phenolic antioxidants in various samples with high separation efficacy, high resolution, short time of analysis and low consumption of reagents and the sample material. The main drawbacks of capillary electrophoresis include low sensitivity (regarding the analyte concentration) and low reproducibility compared with GC and HPLC, which depend on the length of the capillary and solution volumes introduced into the system (several nanolitres). In practice, two versions of this method are used: capillary zone electrophoresis and micellar electrokinetic chromatography in a phosphate or borate buffer solution with capillary diameters of 50–100 μm, applied voltage of 10–30 kV and injected volumes of 10–50 nl.

A good example of the simultaneous determination of six antioxidants (glutathione and ascorbic, vanillic, chlorogenic, salicylic and caffeic acids) in fruits and vegetables by capillary zone electrophoresis has been the use of a chemically modified electrode based on electropolymerized vitamin B_{12} immobilized on a carbon material. The analytes are separated in a borate buffer solution with pH 8.4 during 15 min. The dynamic range of concentrations is 0.25–100 μmol litre^{-1} and the detection limit for antioxidants is 0.01 μmol litre^{-1} (Ref. 258).

A review[259] considers the electrophoretic methods for identification and quantification of phenolic antioxidants in various plant and food specimens (tea, wine) and human biological fluids (urine, blood plasma and serum, and saliva).

Micellar electrokinetic chromatography makes it possible to separate neutral analytes under the action of electric field. For determination of phenolic antioxidants, preliminary fractionation of monomeric and polymeric pigments having high molecular mass is performed by gel permeation chromatography, which enhances the analytical characteristics of determination.[260]

Micellar electrokinetic chromatography in the reversed polarity mode (pH < 2.5) was shown to be the method of choice for the simultaneous fast determination of catechins and caffeine in black and green teas. The limit of detection of the analytes was 1 mg litre^{-1} (Ref. 261). New potential was revealed and evaluation characteristics were determined for micellar electrokinetic chromatography in the normal and reversed polarity mode and for microemulsion electrokinetic chromatography for determination of catechins in tea.[262]

Signal detection in electrophoretic methods is carried out by ultraviolet,[263] fluorescent,[264] electrochemical[265–268] and mass spectrometric[55,269] detectors.

III.1.c. Spectroscopic methods

Molecular spectroscopy techniques are widely used for identification and determination of the structures of phenolic antioxidants. Most popular are NMR and near-IR spectroscopy and mass spectrometry.

Currently, NMR spectroscopy has started to be actively used to analyze food products — the main source of natural phenolic antioxidants. The key advantages of the method include simple sample preparation and assay procedures and reliability of the recorded spectrum and signal interpretation. Conventional ^{1}H and ^{13}C NMR spectra and magic angle spinning high resolution NMR spectra provide extensive information on the chemical composition of liquid and semiliquid foodstuffs.[49]

Thus, NMR spectra can be used for fingerprinting of samples to perform their comparison, recognition and classification. This can be done using either the whole spectrum or particular spectral characteristics (peak heights or areas) corresponding to specific features of the samples. The obtained data are treated more and more often using chemometric techniques.

The food sample preparation is rather simple and depends on the physical state of the foodstuff (liquid or solid). Direct analysis without sample pretreatment is also possible. In the case of aqueous liquid samples (fruit juices, beer, wine, *etc.*), 5%–10% of D_2O is added for analysis by high-resolution ^{1}H, ^{13}C and ^{31}P NMR spectroscopy.[270] Deuterated solvents stabilize the signal and improve the peak resolution. In some cases, preliminary extraction and fractionation are carried out. For example, solid samples (fruits, vegetables, tea) are freeze-dried, crushed and extracted into deuterated solvents. Instant coffee or oils are dissolved in an appropriate deuterated solvent to a required concentration. A common procedure must be adhered to in routine analyses in order to ensure reproducibility and comparability of the results.

The major limitation of the use of NMR in foodstuff analysis is high cost of the equipment. The second limitation is relatively low sensitivity compared with HPLC or GC. Nevertheless, these drawbacks are not crucial since the method is versatile and has advantages. First, NMR is a potent tool for elucidation of the compound structures, which is highly important for identification of new phenolic compounds or new chemical forms of their existence. Second, being one of the best versatile analytical methods not targeted to a particular analyte, NMR spectroscopy is suitable for screening of extracts to determine all major metabolites (fatty acids, amino acids and other organic acids, sugars and aromatic compounds) in the same spectrum with minor and non-destructive sample preparation.[270]

To characterize the structures of various natural phenolic antioxidants without preliminary separation, ^{1}H and ^{13}C NMR, correlation NMR (2D homonuclear, heteronuclear and totally), nuclear Overhauser effect, including that in the rotating frame of reference version, are employed.[271] For example, ^{1}H NMR spectroscopy can serve to detect and quantify phenolic compounds in onion samples.[272] Spectra ^{1}H and ^{13}C NMR in deuterated DMSO and methanol were applied for identification of antioxidants in walnut kernels.[273] Recording of these spectra is the preferred method for detecting anthocyanins and the products of their reactions with cinnamic acid derivatives, peroxyl radicals, catechins and flavonols.[55]

Mass spectrometry also holds a considerable place in the studies of structures of phenolic compounds. This method is based on ionization, giving rise to a charged molecule or molecular fragments followed by determination of the mass to charge ratio. The following types of ionization are used in the analysis of phenolic compounds: fast atom bombardment, electrospray ionization, atmospheric pressure ionization including chemical ionization, atmospheric pressure photoionization and matrix assisted laser desorption ionization. An attempt to apply thermospray ionization MS for determination of oligomeric and polymeric phenolic antioxidants [274] was unsuccessful due to thermal degradation of the analytes. Direct flow-injection electrospray ionization mass spectrometry also provides fingerprints of phenolic antioxidants in objects of complex composition.[274] For accurate determination of high-molecular-mass compounds, good results were obtained by matrix assisted laser desorption time-of-flight mass spectrometry, in particular, for procyanidin oligomers and heptamers in the reflection mode and for up to nonamers in the linear mode.[274] Furthermore, using this method, it was possible to characterize the structure (degree of polymerization, substituents in the monomer and the nature of intermolecular bonds) [275] for different-type phenolic antioxidants from cranberry, grape seeds, sorgo and pomegranate using indol-3-ylacrylic acid as the matrix.

One more fast, accurate and non-destructive method for investigation of the structure and determination of phenolic antioxidants is near-IR spectroscopy. It was used to estimate the contents of theaflavin and moisture in black tea [276] and for the simultaneous prediction of alkaloids and phenolic compounds in green tea leaves.[277] Fourier transform near-IR reflectance spectroscopy was proposed for determination of the major green tea catechins and free amino acids, caffeine, the total content of phenolic antioxidants and amylose when present simultaneously.[278]

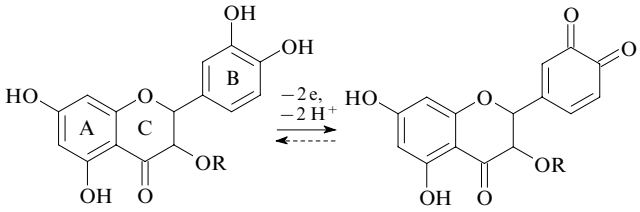
As a rule, spectrophotometry is not used to determine single phenolic antioxidants because the absorption band characteristics of particular compounds are similar; however, it is thus possible to assign the compounds to a definite class. The approaches used to estimate the overall contents of compounds are considered below in Section III.2. Nevertheless, optical sensors for determination of phenolic antioxidants were reported.[279, 280] The response of these sensors is affected by numerous factors, the optimization of which is a labour-consuming procedure. In addition, in some cases, a derivatization stage is required to obtain a compound that absorbs light and the completeness of this stage needs to be verified.

III.1.d. Electrochemical methods of analysis

Electrochemical methods have found use for both batch analysis and flow analysis of phenolic antioxidants. Note once again that antioxidants, including phenols, can be electrochemically oxidized at electrodes of various types. Single compounds are determined without particular difficulties using anodic voltammetry. However, in view of the proximity of energies of the higher occupied molecular orbitals from which the first electron is removed in the potential-determining step of oxidation, the electrochemical resolution of peaks or steps is impossible. Therefore, if antioxidants are contained in a complex sample or in a mixture, the problem of selective determination can be solved by means of preliminary chromatographic separation.

It is noteworthy that electrooxidation of antioxidants can be similar to a reversible process in the first step. This can be followed in the cyclic polarization curves depending on the conditions of electrolysis. Electrooxidation can end in the formation of various final products (corresponding quinones, products of dimerization of the radicals arising in the first stage of oxidation, polymeric film on the electrode surface, which results in surface modification, *etc.*).[281] Finally, the anodic current response would depend linearly on the antioxidant concentration only in a limited range and under definite conditions. In addition, the voltammograms may exhibit several poorly resolved peaks due to stepwise oxidation of a single compound, so that partial signal overlap is expected for a mixture of phenolic antioxidants. Therefore, pulsed polarization technique has advantages over the direct current polarization.[282] These phenomena must be taken into account for obtaining reproducible results for solutions of complex composition, in particular, for detection in a liquid flow. Each phenolic antioxidant possesses its own mechanism of electrooxidation and its own parameters; for flavonoids, the electrode reaction can be written as shown in Scheme 4.

Scheme 4



The stability of the intermediate radical depends on the structure of the initial molecule and detection depends also on the electrolysis parameters, in particular, the time of the process (polarization pulse duration).

A number of studies are devoted to regularities of electrochemical oxidation of rutin,[283] tea catechins,[284] phenolic acids,[285] resveratrol,[286] chrysin and taxifolin,[287] anthocyanins,[288] morin [289, 290] and curcumin [291] on electrodes made of carbon materials or platinum. The structure–antioxidant behaviour relationships for phenolic compounds were elucidated and the antioxidant capacity was evaluated.

The characteristics of the voltammetric determination of phenolic antioxidants on various electrodes are presented in Table 5. Note that for selective determination of phenolic antioxidants in complex objects, preliminary extraction or, as mentioned above, chromatographic separation are often performed.

In recent years, considerable interest has been aroused in the use of modified electrodes for sample analysis for the contents of single phenolic antioxidants. This extends the range of objects and provides better analytical characteristics of determination, which, in turn, opens up new prospects for organic voltammetry.

The electrode surface is modified most often by carbon nanomaterials (tubes, fibres, graphene, fullerenes, *etc.*, the structures of which contain fused six-membered rings, which exhibit a structural similarity to phenolic antioxidants), metal nanoparticles, ionic liquids, polymeric films and so on and their combinations (Table 6). As indicated by

Table 5. Characteristics of the voltammetric determination of phenolic antioxidants.

Analyte	Sample	Supporting electrolyte (pH)	Electrode	Method	Determination range /μmol litre^{-1}	Limit of detection /μmol litre^{-1}	Ref.
Rutin	tea, pharmaceuticals	0.01 M BRB (6.0)	HMDE	AdSV	$(2.0-85.0)\times10^{-3}$	0.5×10^{-3}	292
	biological fluids	0.1 M PB (7.0)	CPE	SWV	0.15–5	9×10^{-3}	293
	rutoside drug	0.1 M PB (7.4)	CPE	DPV	2–15	–	294
		0.1 M PB (7.4)	GCE	DPV	2–20	–	294
	pharmaceuticals	0.1 M H_2SO_4	Pt	LSV	7.9–200	4.8	295
		0.1 M H_2SO_4	PGE	LSV	24–250	7.9	295
Quercetin	biological fluids	0.1 M PB (7.0)	CPE	SWV	0.15–5	7×10^{-3}	293
	pharmaceuticals	0.1 M H_2SO_4	Pt	LSV	87–680	36	295
Quercitrine	biological fluids	0.1 M PB (7.0)	CPE	SWV	0.15–5	10^{-2}	293
Chrysin	the same	0.1 M PB (7.0)	CPE	SWV	0.15–5	42×10^{-3}	293
Diosmin	"	0.1 M PB (7.0)	CPE	SWV	12.5–200	2.7	293
Hesperidin	orange juice, Helopyrin tablets	–	Hg	SV	0.164–41	–	296
Curcumin	model systems	0.2 M AB (5)	CPE	AdSDPV	$(57.6-4830)\times10^{-3}$	–	297
	spices	0.1 M $LiClO_4$ in EtOH	GCE	CV	9.9–107	4.1	298
Quercetin and its derivatives	onion	50 mM AB (5.5) [a]	GCE	HPLC-ED	–	80.5×10^{-3}	299
3-*O*-β-glycoside	"	50 mM AB [a]	GCE	HPLC-ED	–	108×10^{-3}	299
4′-*O*-β-glycoside	"	50 mM AB (5.5) [a]	GCE	HPLC-ED	–	122×10^{-3}	299
3,4′-di-*O*-β-glycoside	"	50 mM AB (5.5) [a]	GCE	HPLC-ED	–	260×10^{-3}	299
Hydroxyflavone	biological fluids	0.5 M BRB	HMDE	AdSSWV	$(19-242)\times10^{-3}$	4.4×10^{-3}	300
Morin	the same	0.5 M BRB	HMDE	AdSSWV	$(19-193)\times10^{-3}$	7.19×10^{-3}	300
	"	0.5 M BRB (3.25)	PGE [b]	AdSSWV	$(95-133)\times10^{-3}$	25×10^{-3}	301
Hesperidin	"	0.5 M BRB	HMDE	AdSSWV	$(19-654)\times10^{-3}$	7.54×10^{-3}	300

Note. The following designations are used: for electrolytes,: BRB is Britton–Robinson buffer, PB is phosphate buffer, AB is acetate buffer; for electrodes: HMDE is hanging mercury drop electrode, CPE is carbon paste electrode, GCE is glassy carbon electrode, PGE is pencil graphite electrode; for methods, AdSV is adsorptive stripping voltammetry, SWV is square wave voltammetry, DPV is differential pulse voltammetry, LSV is linear sweep voltammetry, SV is stripping voltammetry, AdSDPV is adsorptive stripping differential pulse voltammetry, CV is cyclic voltammetry, HPLC-ED is high performance liquid chromatography with electrochemical detection, AdSSWV is adsorptive stripping square wave voltammetry. [a] In 80% MeOH; [b] pencil graphite electrode.

the data of Table 6, by modification of the electrode surface, it is possible in some cases to substantially decrease the limits of detection of phenolic antioxidants (down to nmol litre^{-1}) and to extend the quantifiable range.

In relation to catechol, flavonoid and hydroxycinnamic acid derivatives, factors affecting the electrochemical response of structurally related phenolic antioxidants on nanostructured hybrid films (gold nanoparticles in chitosan) were established. The key role is played by the chemical structure of antioxidant molecules, in particular, the presence of two *ortho*-hydroxyl groups in the catechol ring.[323]

Yet another approach to modification of the electrode surface is related to the use of biomaterials,[324] in particular, enzymes. It is known that phenolic antioxidants are oxidized under the action of enzymes, which makes possible the manufacture of enzymatic biosensors for determination of antioxidants of this type. As a rule, the biolayer comprises a polyphenol oxidases: laccase, tyrosinase or peroxidase.[325] Combinations of two enzymes, for example, laccase and tyrosinase, are also used;[326] this extends the range of phenolic antioxidants that can be analyzed.[327]

Enzyme immobilization on the electrode surface is accomplished *via* physical adsorption, covalent linking of the enzyme to the carbon material or the metal (gold, platinum, *etc.*) surface and incorporation of the enzyme into a polymeric matrix. The key advantage of redox enzymes is the low potential (usually $-0.2-0$ V) for detecting the substrate reduction or oxidation on the electrode surface, which minimizes the possible interfering influence of other electrochemically active compounds.[328]

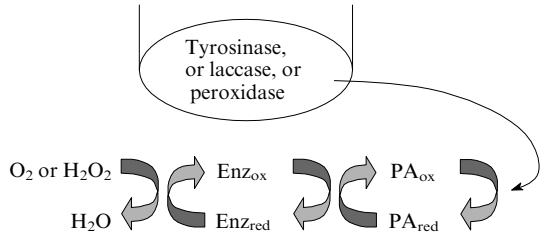


Figure 8. Scheme of amperometric response of biosensors based on peroxidases and oxidases to phenolic compounds (Enz is enzyme, subscripts: ox is oxidized, red is reduced).

Table 6. Characteristics of the voltammetric determination of phenolic antioxidants on modified electrodes.[a]

Analyte	Sample	Electrode	Method	Analytical range /μmol litre^{-1}	Limit of detection /μmol litre^{-1}	Ref.
Catechin	see [b]	GCE/SWCNT – cetyltrimethylammonium bromide [c]	DPV	$(0.372-2.38)\times10^{-3}$	0.112×10^{-3}	302
	tea beverages	GCE/polyaspartic acid	DPV	0.25 – 30	72×10^{-3}	303
	green tea	Au/3-sulfanylpropionic acid/Ni(II) complex	SWV	3.31 – 25.3	0.826	304
	tea	CPE/$Cu_3(PO_4)_2$ in polyether resin	SWV	$(99-1200)\times10^{-3}$	58×10^{-3}	305
	"	CPE/hydroxypropyl-β-cyclodextrin	SWV	up to 70 [d]	1.35 [d]	306
	beverages	CPE/hydroxypropyl-β-cyclodextrin	SWV	0.001 – 7.2 [d]	0.12×10^{-6} (see [d])	306
	"	CPE/hydroxypropyl-β-cyclodextrin	DPV	0.002 – 4.2 [d]	0.30×10^{-6} (see [d])	307
Rutin	pharmaceuticals	GCE/acetylene black nanoparticles	DPV	20 – 50 [d]	10 [d]	308
	tablets	GCE/Pb films	AdSV	$(0.5-10)\times10^{-3}$	0.25×10^{-3}	309
	pharmaceuticals	GCE/polyglutamic acid	SWV	0.7 – 10	–	310
	tablets	CPE/*N*-n-butylpyridinium hexafluorophosphate	CV	0.5 – 100	0.35	311
	"	CPE/1-amyl-3-methylimidazolium bromide	SWV	0.04 – 0.10	10^{-2}	312
	pharmaceuticals	CPE/polyvinylpyrrolidone	LSV	0.39 – 13.0	0.15	313
	green tea	boron-doped diamond electrode	FIA-ED [e]	10 – 250	7.7	314
	tablets	GCE/poly(*p*-aminobenzenesulfonic acid)	DPV	0.25 – 10.0	0.1	315
	pharmaceuticals	CPE/Cu(II)-based composite	CV	0.99 – 807	0.0265	316
	blood serum, pharmaceuticals	GCE/graphene/chitosan/polyamidoamine dendrimer				317
	pharmaceuticals	GCE/MWCNT in 1% sodium dodecyl sulfate [f]	CV	1.4 – 28 28 – 210	0.71	318
Curcumin	milk	GCE/MWCNT	AdSV	2 – 1000	0.5	319
	"	CPE/nanodysprosium	AdSV	0.01 – 1.0	0.005	319
Quercetin	rutin hydrolyzate	MWCNT-based paste electrode	CV	$(2-100)\times10^{-3}$	–	320
	pharmaceuticals	GCE/MWCNT in 1% sodium dodecyl sulfate	CV	2.0 – 220	1.0	318
	medicinal herb extracts	Au/cysteamine/Au nanoparticles	CV	10 – 100	10^{-3}	321
Taxifolin	pharmaceuticals	GCE/MWCNT in 1% sodium dodecyl sulfate	CV	0.52 – 210	0.26	318
Morin	liquorice extract	MWCNT-based paste electrode	CV	$(5-100)\times10^{-3}$	10^{-3}	322
Daizeol	medicinal herb extracts	Au/cysteamine/Au nanoparticles	CV	0.1 – 10	10^{-2}	321
Puerarin	the same	Au/cysteamine/Au nanoparticles	CV	0.5 – 1	10^{-1}	321

[a] For designations of the methods, see Table 5; [b] commercial sample was used; [c] SWCNT are single-walled carbon nanotubes; [d] mg litre^{-1}; [e] FIA-ED flow injection analysis with electrochemical detection; [f] MWCNT are multiwalled carbon nanotubes.

The mechanism of formation of response of the oxidase- or peroxidase-based biosensors is depicted in Fig. 8.[329] It is noteworthy that phenol oxidases and peroxidases have different enzymatic mechanisms in electrochemical biosensors. The main reaction is enzyme oxidation on the electrode surface in the presence of the substrate, namely, oxygen (for phenol oxidase) or hydrogen peroxide (for peroxidase), the latter being reduced to water. The next step is regeneration of the enzyme to its original form *via* electron transfer from a suitable mediator, *i.e.*, the reduced form of the phenol. The measured current is due to the reduction of the mediator to the original form.

Tyrosinase biosensors are meant for monitoring of phenolic compounds that have at least one free *ortho*-position in the aromatic ring. Conversely, the laccase biosensor can detect free *para*- and *meta*-positions but the catalytic cycle is, in this case, complicated, and the structures of the products have not yet been finally clarified.[329]

The key characteristics of biosensors for determination of phenolic antioxidants are summarized in Table 7. As applied to objects of complex composition, for example, wine, the response of biosensors depends on the presence of other phenolic antioxidants, which restricts the scope of applicability of such devices. In addition, preliminary solid-phase extraction is often needed for preconcentration of analytes.[341]

A non-enzymatic sensor operating on biomimetic principles for determination of catechin in green tea deserves attention. The used binuclear copper(II) complex functions like the enzyme catechol oxidase active site. The sensor has a simple design, acceptable cost and high analytical parameters. The calibration plot is linear in the range of 4.95 – 32.7 μmol litre^{-1} of catechin with a limit of detection of 0.28 μmol litre^{-1} (Ref. 342).

One more electrochemical method suitable for determining a broad range of phenolic antioxidants is based on their

Table 7. Analytical characteristics of biosensors for single phenolic antioxidants.

Modifying sensor components	Analyte	Sample	Analytical range /μmol litre^{-1}	Limit of detection /μmol litre^{-1}	Ref.
Laccase/Au nanoparticles encapsulated into dendrimer	catechin	green tea	0.1–10	0.05	330
Laccase/1-n-butyl-3-methylimidazolium hexafluorophosphate/mineral oil–graphite black	rosmarinic acid	plant extracts	0.99–65.4	0.188	331
Laccase/photopolymer based on polyvinyl alcohol/screen-printed graphite electrode	caffeic acid	tea extracts	0.5–130	0.524	332
Laccase/Nafion/carbon paste screen-printed electrode	rosmarinic acid	plant extracts	0.7–1.5	0.119	333
Tyrosinase/diazonium-functionized screen-printed Au electrode	catechin	tea	2.8–29	2.8	334
	caffeic acid	"	0.3–83	0.2	334
	gallic acid	"	2.5–65	2.1	334
Au nanoparticles in 1-n-butyl-3-methylimidazolium hexafluorophosphate/garlic polyphenol oxidase	esculetin	black tea	$(0.40–9.86)\times10^{-3}$	0.11×10^{-3}	335
Laccase– tyrosinase/diglycerylsilane sol-gel matrix/ ferrocene-graphite electrode	gallic acid	wines	–	50	327
	caffeic acid	"	–	24	327
	catechin	"	–	40	327
Laccase–chitosan micrograins/sodium tripolyphosphate cross-linking/ CPE	rutin	pharmaceuticals	0.599–3.92 5.82–13.1	0.0623 0.712	336
Ag nanoparticles in 1-n-butyl-3-methyl-imidazolium-bis(trifluoromethylsulfonyl)imide/laccase immobilized into β-cyclodextrin modified with epichlorohydrin/CPE	quercetin	pharmaceuticals, fruits	0.499–7.407	0.037	337
Laccase from *Trametes versicolor* (or *Trametes hirsuta*) in the polyazetidine prepolymer/MWCNT/ screen-printed electrode	gallic acid	white (red) wine	1.07–49.16 (0.79–19.90) [a]	0.32 (0.24) [a]	338
	caffeic acid	the same	0.03–0.79 (0.03–0.60) [a]	0.01 (0.01) [a]	338
Iridium nanoparticles in 1-n-butyl-3-methyl-imidazolium hexafluorophosphate/ polyphenol oxidase from apple marrow/chitosan	chlorogenic acid	coffee	3.48–49.5	915×10^{-3}	339
Polyphenol oxidase/laponite/azure B	rutin	model systems	10–300	–	340

[a] In mg litre^{-1}.

reactions with electrogenerated oxidants under galvanostatic coulometry conditions.

Coulometry is still the only method that does not utilize the dependence of properties on the analyte concentration, which is typical of any other physicochemical method of analysis. The coulometric methods are based on the Faraday law establishing the relationship between the weight of the electrically converted substance and the quantity of electricity spent. The application of coulometry substantially shortens (as compared with the conventional methods of analysis) the time it takes to acquire the desired information and simultaneously decreases the error and increases the reliability of the results.[343]

The scope of applicability of coulometry has now considerably extended owing to the development of the theoretical grounds of electrochemistry, the use of new electrode materials and mediator systems and preparation of new coulometric titrants. In Russian literature, coulometry has not received adequate attention and it is little used in the analytical practice of industrial or research laboratories. Practical use of coulometric procedures is restricted by the lack of appropriate equipment, despite the fact that instruments for coulometric analysis are manufactured both in Russia and abroad.

Galvanostatic coulometry with electrogenerated titrants has proven itself effective in analysis of medicines for the content of phenolic antioxidants. By varying the nature of titrants, it is possible to cover a broad range of analytes, for example, hypoxen,[344] rutin, quercetin and taxifolin,[345, 346] in dosage forms. Among titrating agents, note oxidants such as halogens (chlorine, bromine) and hexacyanoferrate(III) ions. The reactivities of natural phenolic antioxidants (catechin, flavonols, taxifolin, tannin, luteolin, curcumin, gallic and ellagic acids, syringaldehyde and coniferaldehyde) toward electrogenerated hexacyanoferrate(III) ions have been estimated.[298, 347] Drawbacks of coulometric titration are relatively low selectivity, especially in the case of electrogenerated chlorine and bromine. The problem is solved using preliminary extraction or derivatiztion of phenolic compounds.

Galvanostatic coulometry with electrogenerated titrants is of interest as an alternative to the routine conventional titration used in pharmacy. This method can substantially reduce the time and enhance the accuracy of determination

of phenolic antioxidants. Thus, electrochemical methods have found wide use in analysis of foodstuffs, plant raw materials, pharmaceuticals and so on for the content of single phenol derivatives. A recent review [348] is devoted to electrochemical methods for determination of phenolic compounds in plant materials.

Generally, despite quite a number of studies, the problem of quantitative determination of phenolic antioxidants remains topical. The approaches being developed are mainly based on the reactivity of these compounds caused by the presence of phenol moieties in the molecules.

III.2. Estimation of the total content of phenolic antioxidants

Publications of the recent years show a considerable interest in the estimation of overall indices characterizing the object as a whole. This approach often enables fast screening for the total contents of a number of compounds with specified properties and, hence, rapid control of the amounts of healthy or unhealthy components. The total content of antioxidants, in particular, phenols is among these parameters. Objects of analysis include the key sources of these compounds — food products, pharmaceuticals, plant raw materials, *etc*.

Most often, the total phenolic content is determined, which covers all phenolic compounds that are present in the sample and contribute to the antioxidant properties. This value is determined using the Folin–Ciocalteu reagent (a mixture of phosphomolybdic and phosphotungstic acid salts). In alkaline medium upon the reaction with phenols, these salts are reduced to give blue-coloured complexes, the concentration of which is estimated by spectrophotometry at 760 nm. The optical density is proportional to the phenolic concentration. Gallic acid is usually employed as the reference standard.[349, 350] The drawback of the method is the interfering influence of sulfur dioxide and ascorbic acid or high concentrations of sugars,[350] which react with this reagent.

The total phenolic content can also be found using oxidase-based biosensors (Table 8). This value is usually expressed in equivalents of single phenolic antioxidants (gallic acid, catechin, caffeic or chlorogenic acid) depending on the investigation object.

For example, combination of laccase and tyrosinase with a Clark oxygen electrode can be used to estimate the total phenolic contents in wines by chronoamperometry. The oxygen reduction current is inversely proportional to the total phenolic content.[358]

The total phenolic contents in plant raw materials, beverages and foodstuffs are actively determined using various versions of voltammetry. An assay of the total phenolics in wine by differential pulse voltammetry has been developed.[359] The first oxidation peak of the wine phenols at 360–370 mV (*versus* SCE) serves as the analytical signal; this peak corresponds to reactions of compounds containing a pyrocatechol moiety in ring B, for example, flavonoids, tannins and some phenolic acids (gallic and caffeic). The total phenolic content is expressed in the catechin equivalents.

An 'electronic tongue' based on a battery of six sensing electrodes (gold microelectrode and five modified epoxy-graphite composite sensors based on cobalt phthalocyanine, platinum and copper nanoparticles and electropolymerized polypyrrole), which is able to assess the total polyphenol index of wines in gallic acid equivalents, has been developed.[360] The obtained voltammetric data are subjected to the discrete wavelet transformation in order to compress the amount of information and distinguish the most significant values with the subsequent use of chemometric techniques (artificial neural networks, partial least squares) for quantitative determination of the polyphenol index.

Table 8. Amperometric biosensors for determination of total phenolic contents.

Sample	Equivalent for calculation	Detection limit [a]	Dynamic concentration range /μmol litre^{-1}	Ref.
Enzyme tyrosinase				
Wine	gallic acid	0.1 [b]	1–60 [b]	351
		7	25–900	352
Tea	chlorogenic acid	2.52	70–400	353
Enzyme peroxidase				
Wine and tea	catechin	2	< 25	354
		3	< 15	355
Tea	chlorogenic acid	0.7	1–50	356
Enzyme laccase				
Wine	catechin and caffeic acid	1	2.0–14.0	341
Plant extracts	caffeic acid	0.56	< 10	357

[a] In μmol litre^{-1}; [b] in mg litre^{-1}.

A new electrochemical procedure based on detection on a disk glassy carbon electrode in a flow injection system has been proposed for screening of phenolic antioxidants in foodstuffs.[361] The integral signal is due to the oxidation current of all phenolic antioxidants at a specified potential (0.8 V) in neutral medium (pH 7.5). The phenolic concentration corresponding to this signal was called the electrochemical or polyphenol index. This value is alternative to the total phenolic content determined by the Folin–Ciocalteu method.

Figure 9 shows the key statements of the electrochemical index concept compared to the spectrophotometric approach. It can be seen that the experimental conditions are different. The Folin–Ciocalteu method is implemented in the presence of the reagent, in alkaline medium and at room temperature, while in the case of electrochemical procedure, the conditions are non-selective, as all classes of phenolic antioxidants should be oxidized. In addition, the assays are based on different types of reactions (complexation in the Folin–Ciocalteu method and electrochemical oxidation of the hydroxyl groups in the electrochemical method). On the other hand, both methods are based on measuring the integral signal: absorption at 750 nm and the oxidation current at a specified potential. Finally, in both methods, the total phenolic content is expressed in the equivalents of the chosen standard, the choice being dictated by the phenolic composition of the sample.[361]

However, it should be noted that for samples containing ascorbic acid and α-tocopherol, the electrochemical index

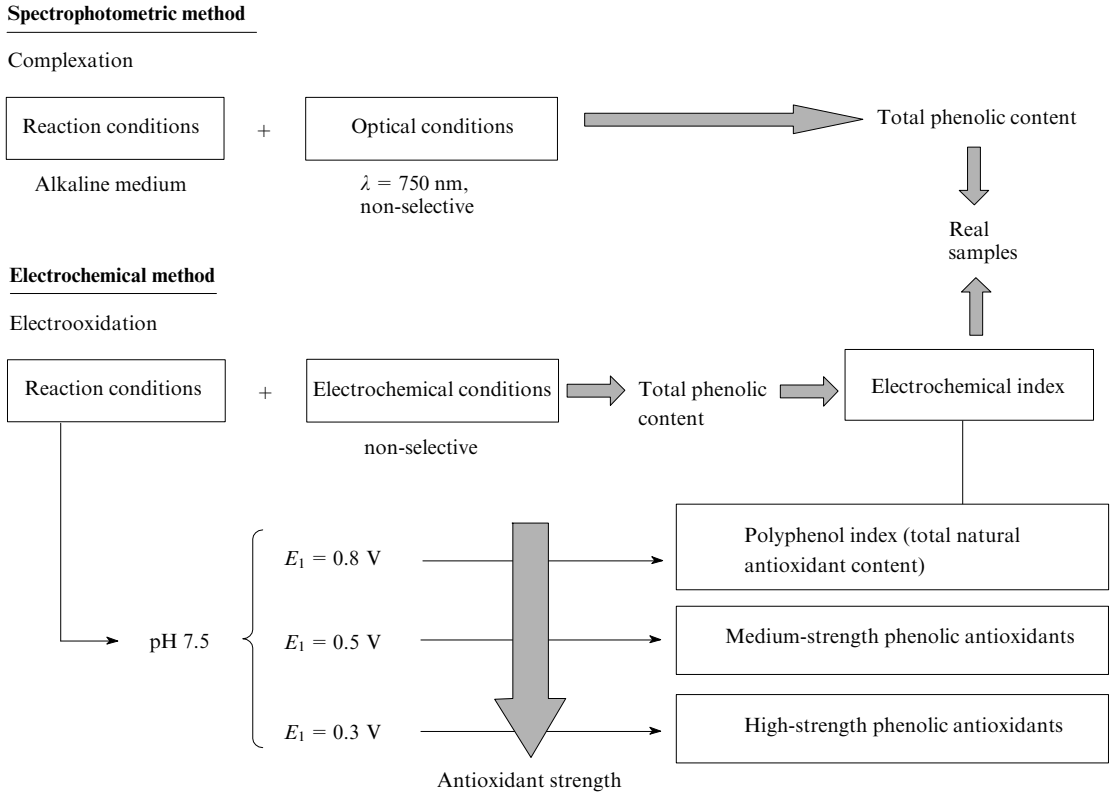


Figure 9. Concept of 'electrochemical index'.[361]

reflects the total content of natural antioxidants rather than only phenolic compounds.

Owing to the change in the detection potential (E), it is possible to select the oxidation processes of phenolic antioxidants of different classes. In other words, by choosing the detection potential at 0.3, 0.5 or 0.8 V, it is possible to estimate the phenolic contents. At 0.3 V, only the strongest antioxidants (*i.e.*, easily oxidizable) are oxidized; at 0.5 V, both strong and medium antioxidants are oxidized; and at 0.8 V, oxidation involves all of the antioxidants present in the sample. As the quantitative measure, the oxidation currents at the mentioned potentials are used (see Fig. 9).[361]

A similar method for determination of phenolic antioxidants is based on their oxidation on a glassy carbon electrode in a flow injection assay with amperometric detection. The peak areas or heights for the analyte and the reference are calculated using dedicated software. Rutin, quercetin, dihydroquercetin, gallic acid and so on are used as reference compounds. The limit of detection of phenolic antioxidants by a amperometric detector is at the nano- and picogramme levels.[362]

The total contents of phenolic antioxidant classes are also determined by spectrophotometry using the ability of products formed in various reactions to absorb light at a definite wavelength. For example, the total flavonoid content in ethanol extracts of plant raw materials is estimated based on reaction of flavonoids with aluminium chloride with subsequent detection at 410–423 nm.[363, 364]

For determination of proanthocyanidins (condensed tannins), derivatizing reagents, vanillin or dimethylaminocinnamic aldehyde, are used;[236] this provides information on the degrees of polymerization and hydroxylation and on the stereochemistry of flavan-3-ol moieties in proanthocyanidins.[365, 366] In the case of reaction with vanillin, catechin usually serves as the reference, which may give overestimated results. The correctness of quantitative determination of proanthocyanidins by the second method also raises questions.[367]

A method for determination of proanthocyanidins in which they are subjected to acid hydrolysis to give anthocyanidins in an n-butanol–HCl system (95 : 5) has been reported.[368] The red-coloured extract formed in the reaction has an absorption maximum at 550 nm. It was shown that the yield of anthocyanidins largely depends on the presence of trace amounts of metal ions, in particular, iron(III).

The ability of tannins to form insoluble complexes with proteins, for example, with BSA, underlies another spectrophotometric determination of proanthocyanidins. The resulting complex is dissolved in an alkaline solution of sodium dodecyl sulfate and triethanolamine and derivatized by treatment with iron(III) chloride to give a violet-coloured complex with an absorption maximum at 510 nm.[369]

Quantification of the total proanthocyanidin content in grape extracts is performed by precipitating the compounds as polymers upon the reaction with methylcellulose.[370] The concentration of proanthocyanidins is measured from the change in the absorbance before and after the reaction.[371]

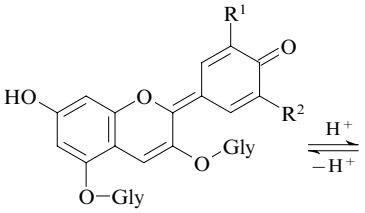
The content of hydrolyzable tannins is determined upon reactions with potassium periodate, sodium nitrite and rhodanine.[372] The first of the mentioned reagents is used most often for large-scale routine assays. It reacts with methyl gallate to give a red-coloured derivative with an

absorption maximum at 500–550 nm.[366] The rhodanine and nitrite methods allow for the determination of hydrolyzable tannins having gallic and ellagic acid structural units, respectively.[373, 374]

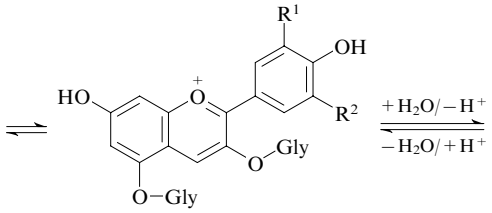
Flavones and dihydroflavonols are determined based on the products they form upon reactions with 2,4-dinitrophenylhydrazine.[375] The flavanone pinocembrin, which absorbs at 486 nm, usually serves as the reference.[376]

The next extensive class of phenolic antioxidants that can be quantified by spectrophotometry is the class of anthocyanins. The total content of monomeric anthocyanins is determined most often by the pH-differential method[377] based on measurement of absorption at two wavelengths (520 and 700 nm) at pH 1.0 and 4.5. At these pH, anthocyanins exist in different forms: the coloured oxonium form at pH 1.0 and colourless semiketal form at pH 4.5 (Scheme 5).[378] The absorption spectra of these forms are considerably different. The determination is not interfered with other components and even by the products of polymerization of phenolic antioxidants, which retain the colour upon the change in the pH. As the reference, the major anthocyanin of the sample (for wines, this is malvidin-3-glycoside) or cyanidin-3-glycoside most abundant in nature are used most often.[377]

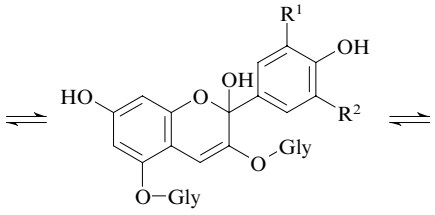
Scheme 5



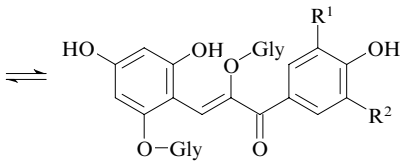
Quinoid base: blue colour (pH 7)



Oxonium form:
orange to purple colour (pH 1)



Semiketal form: colourless (pH 4.5)



Chalcone: colourless (pH 4.5)

R^1, R^2 = H, OH, OMe

The spectrophotometric determination of anthocyanins can be also performed in weakly acidic media at wavelengths of 490–550 nm.[379]

Generally, spectrophotometric techniques for determination of the total contents of phenolic antioxidants of various classes are fairly facile, readily accessible and quite useful for fast screening of real objects. Treatment of the obtained data in terms of chemometric approaches allows for classification, identification and prediction of properties of analytical objects.[380]

IV. Conclusion

At present, humans are permanently in contact with various biologically active substances, which affect their health *via* either the living environment or the food chains. This is why it is necessary to know what is the content of these compounds both before and after they enter the human body. This task is solved by professional analytical chemists who deal with food and pharmaceutical test objects, thus responding to the public mandate for increasing the living standards. One can observe the overlap of the activity areas of various scientific communities, which is demonstrated by recent publications and which gives rise to the field called 'bioanalytical chemistry'. The subject of this interdisciplinary field of knowledge combines the analytical aspects of biology, pharmacy, food chemistry, phytochemistry, *etc*.

Among the investigation objects of bioanalytical chemistry, of special note is the group of compounds exhibiting antioxidant properties and affecting the state of the antioxidant protection system of a living body. The present review discusses the problems related to analysis of the most extensive group of antioxidants, phenolic compounds, which comprises a multitude of several thousand compounds created by Nature during the evolution. Having different structures and exhibiting a broad range of biological activities, these compounds maintain the antioxidant equilibrium, *i.e.*, preserve the appropriate redox balance of the body and support the health. Most likely, the whole set of phenolic antioxidants can participate in radical reactions both individually and cooperatively, exhibiting the kinetic discrimination effect.

Any large scientific problem has its history. The size of this review does not provide room for exhaustive discussion of this issue as regards the evolution of methods and approaches of the proper analytical chemistry, on the one hand, and the permanent extension of the range and diversity of new environmental objects containing phenolic antioxidants, on the other hand. This is related, to an extent, to the increase in the number of biologically active compounds with antioxidant behaviour, many of which have been certified as pharmaceutical drugs with the antioxidant action or are considered as potential active components for the development of new medicines.

The information presented in the review demonstrates that there are still no universal methods for quantification of phenolic antioxidants. The strategy of investigation of natural phenols presented here can currently be regarded as generally accepted. Therefore, the review is restricted to consideration of the achievements of the last decade in which the instrumental methods (*i.e.*, physical, hybrid and combined methods) prevail. The novelty of solutions and approaches is manifested in the context of upgrades and

corrections concerning the sample preparation and other steps of the assay.

Yet another problem in the studies of phenolic antioxidants is related to the large diversity of biotargets that are protected by these compounds. The prooxidant behaviour of their low-molecular-mass derivatives should also be borne in mind. The analytical research teams that actively deal with phenolic antioxidants propose the practical use of various instrumental methods. In the general case, one feature of the analytical object is utilized, being supplemented by other methods. Research in this field needs to be further continued. The important aspects of evaluation of antioxidant properties, in particular, antioxidant activity and capacity are beyond the scope of this review. These issues form a separate topic of interest. One should not forget that the total number of target analytes has reached ten thousand, which proves the persistent interest in these compounds.

Probably, the current surge of interest in the antioxidants will be replaced in course of time by some decline associated with thinking over the information, as it often happens in science. However, the accumulated experience of investigations of real bioanalytical objects having fairly intricate compositions will remain demanded in the future: the problem of quantification of biologically active compounds in natural samples will remain topical for a long period.

The knowledge of the content of biologically active components, including phenolic antioxidants, in foodstuffs and biomedical samples is significant for predicting the influence of these compounds on the human body and the possible therapeutic effects. Studies of the properties of foodstuffs help to compose diets and give recommendations on food rations, and to use the obtained data for the development of new foodstuffs and for the food quality control, in particular, during storage.

As components of complex bioanalytical objects, phenolic antioxidants require approaches from the standpoints of not only biology, medicine and chemistry but also physics. These studies demonstrate that only joint efforts of specialists engaged in life sciences can provide results having practical value. The appearance in the market of medicines, pharmaceuticals and nutrition additives favourable for balancing the antioxidant status of the whole body and prevention of some diseases is a result of scientific research and development performed for a number of years in medical and biological, biochemical, clinical and other laboratories. The new knowledge thus gained and the developed technologies have become property of the society, which illustrates the social function of science.

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