Russian Chemical Reviews **76** (1) 79 – 90 (2007) © 2007 Russian Academy of Sciences and Turpion Ltd

DOI 10.1070/RC2007v076n01ABEH003632

Determination of biologically active phenols and polyphenols in various objects by chromatographic techniques

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Abstract. Chromatographic techniques for determination of biologically active phenols and polyphenols are considered. Various methods for sample preparation and detection are compared. The advantages of high performance liquid chromatography with spectrophotometric detection for determination of antioxidants are demonstrated. Data on determination of biologically active phenols and polyphenols published in the period from 1995 to 2005 are analysed. The bibliography includes 270 references.

I. Introduction

During the last decade, the number of publications devoted to chromatographic studies of natural and synthetic biologically active compounds has sharply increased.[1–8] The studies of the antioxidant activity of, in particular, phenol and polyphenol derivatives attract special attention of researchers. The environment deterioration and the increase in the number of stress situations decrease the defense forces in humans. This, in turn, increases the risk of pathological states (cardiovascular diseases, inflammatory processes, atherosclerosis, *etc*.). Studies of the active forms and the mechanisms of action *in vitro* and *in vivo* of natural and synthetic biologically active compounds is a topical problem related to preservation of human health.

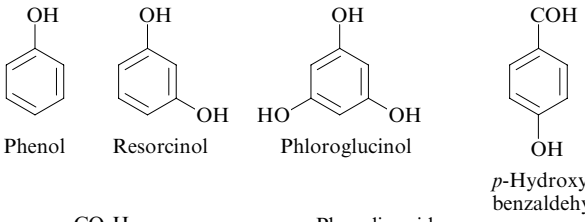
Phenols and polyphenols possess a broad spectrum of biological activities: they exert antioxidant,[9–11] antiinflammatory,[12] antitumour,[13] capillary strengthening [14, 15] and hepatoprotector [16] actions. They are able to form chelates with metals. These chelates can exhibit both antioxidant and prooxidant † activities.[17–19] This accounts for the use of biologically active compounds in medicine, pharmacology, biochemistry and food industry. Despite the wide use of polyphenols as antioxidants, the mechanisms of their action *in vitro* and *in vivo* and the cases where these compounds behave as prooxidants [20] are still not entirely clear. This is due, on the one hand, to the existence of numerous natural derivatives of these compounds and the diversity of their oxidised forms and, on the other hand, to the complexity of composition of the extracts from which these compounds are isolated and determined. Therefore, to analyse biologically active compounds, one needs information on their transformations during all stages of sample preparation.

This review considers the studies devoted to chromatographic methods for determination of biologically active phenols and polyphenols published over the last 10 years. The attention is focused on the most widely used procedures.

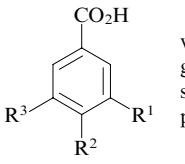
II. General characteristics of biologically active phenols and polyphenols

Phenol derivatives constitute a widely encountered and abundant class of natural products exhibiting biological activity. They are distinguished by the presence of free or bound OH group as a substituent in the benzene ring.

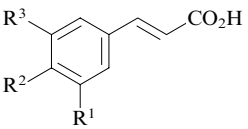
Simple benzene derivatives



Phenol Resorcinol Phloroglucinol *p*-Hydroxybenzaldehyde



Phenolic acids
vanillic acid: $R^1 = OMe$, $R^2 = OH$, $R^3 = H$;
gallic acid: $R^1 = R^2 = R^3 = OH$;
syringic acid: $R^2 = R^3 = OMe$, $R^2 = OH$;
protocatechuic acid: $R^1 = R^2 = OH$, $R^3 = H$.



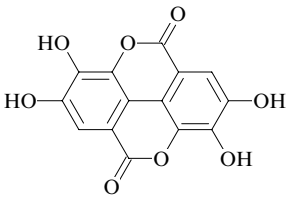
Hydroxycinnamic acids
cinnamic acid: $R^1 = R^2 = R^3 = H$,
p-coumaric acid: $R^1 = OH$, $R^2 = R^3 = H$; caffeic acid: $R^1 = R^3 = OH$, $R^2 = H$; ferulic acid: $R^1 = OH$, $R^2 = H$, $R^3 = OMe$; sinapic acid: $R^1 = OH$, $R^2 = R^3 = OMe$.

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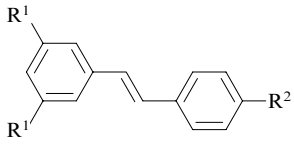
Received 13 February 2006
Uspekhi Khimii **76** (1) 88 – 100 (2007); translated by Z P Bobkova

† Prooxidant activity of a compound usually implies an increase in the yield of active oxygen-centred radicals in the presence of this compound.

Aromatic compounds containing two or more non-fused aromatic rings

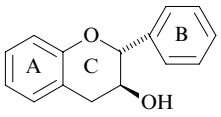


Ellagic acid

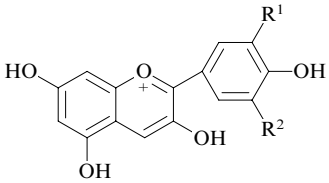


Stilbenes
stilbene: $R^1 = R^2 = H$;
p-hydroxystilbene: $R^1 = H$, $R^2 = OH$; 3,5,4′-trihydroxystilbene (resveratrol): $R^1 = R^2 = OH$.

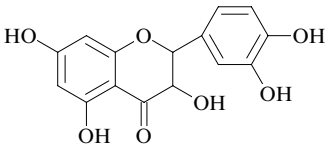
Flavonoids (arylbenzo[*b*]pyrans) and coumarins



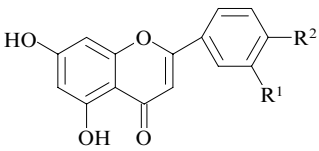
(+)-Catechin



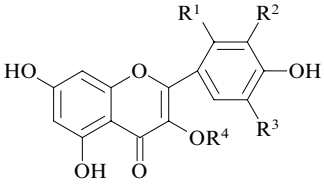
Anthocyanidins
pelargonidin: $R^1 = R^2 = H$;
cyanidin: $R^1 = OH$, $R^2 = H$;
delphinidin: $R^1 = R^2 = OH$;
malvidin: $R^1 = R^2 = OMe$;
petunidin: $R^1 = OMe$, $R^2 = OH$.



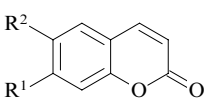
Taxifolin (dihydroquercetin)



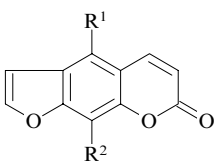
Flavones
chrysin: $R^1 = R^2 = H$;
apigenin: $R^1 = H$, $R^2 = OH$;
luteolin: $R^1 = R^2 = OH$.



Flavonols
kaempferol: $R^1 = R^2 = R^3 = R^4 = H$;
morin: $R^1 = OH$, $R^2 = R^3 = R^4 = H$;
quercetin: $R^1 = R^3 = R^4 = H$, $R^2 = OH$;
myricetin: $R^1 = R^4 = H$, $R^2 = R^3 = OH$;
quercetrin (quercetin rhamnoside): $R^1 = R^3 = H$, $R^2 = OH$, R^4 = α-L-Rha*p*; rutin (quercetin rutinoside):
$R^1 = R^3 = H$, $R^2 = OH$, R^4 = β-D-Glc*p*-(6←1)-α-L-Rha*p*.



Coumarins
esculetin: $R^1 = R^2 = OH$,
umbelliferone: $R^1 = OH$, $R^2 = H$.



Furocoumarins
bergapten: $R^1 = OMe$, $R^2 = H$;
xanthotoxin: $R^1 = H$, $R^2 = OMe$;
isopimpinellin: $R^1 = R^2 = OMe$.

Phenols are present almost in all plants either as glycosides or in the free state in amounts from 0.1% to 7%.[21]

Phenolic alcohols and their glycosides favour the increase in human performance capability and the organism resistance against adverse effects. Hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic and sinapic acids) are present in many plants[22] and fruits[23,24] in various combinations, either in the free state or as glycosides or esters. Caffeic acid and its derivatives (chlorogenic acid and its isomers), which have antiinflammatory and choleretic actions, are found in nature most often. Chlorogenic acid occurs in large amounts in coffee beans, bilberry, mountain tobacco, wild chamomile leaves, *etc*. Ferulic acid is a part of plant cell walls.[25] Caffeic, chlorogenic, ferulic and coumaric acids together have a hypouremic effect, enhance the kidney function and stimulate the detoxifying function of the liver. Hydroxycinnamic acids are also present in echinacea, burdock root, hawthorn and rhubarb.

Ellagic acid is abundant in many fruits and vegetables, especially in raspberry, strawberry and pomegranates. Stilbenes are mainly contained in various kinds of wood (pine tree, gum-tree, *etc*.).

Catechins were found in many fruits[26] (apples, peaches, apricots, queen-apples and plums) and berries (cherry, strawberry, currant, raspberry, gooseberry and cowberry). Large amounts of catechins are present in tea-plant browses[27] (up to 20%–25% of the dry weight) and acacia catechu browses (which gave rise to the name of these compounds), in grapes (mainly in stones and peel),[28] and in cocoa beans.[29] Catechins show high biological activity; in the human body, they control the capillary permeability and increase the wall elasticity and also increase the bioavailability of ascorbic acid. Therefore, catechins are classified as substances having P-vitamin activity and are used in the therapy of diseases related to capillary dysfunction and edemas.[30]

The cell vacuoles of many flowers and fruits contain pigments such as anthocyanidins and anthocyanins [as oligomeric and polymeric forms (proanthocyanidins) or complexes with metals, proteins and polysaccharides], which are responsible for their bright colour.[31,32] Proanthocyanidins were found in most fruits[33] and vegetables[34] and in leaves of many plants. The main source of these compounds on an industrial scale are grape stones and peel and the cluster pine cork. In medicine, they are used to prevent the cardiovascular diseases and to reduce the cholesterol level in blood.[35] Studies of the antitumour activities of anthocyanidins are in progress.[36] The instability and structural diversity of anthocyanidins complicate their qualitative analysis.

Flavonols (dihydroflavonols) are mainly present in coniferous wood (pine tree, larch tree and cedar) and some leaf wood (eucalyptus, beech, cherry). Flavanones are encountered in citrus plants. Hesperetin and its glycoside, hesperidin, are contained in the orange, lemon and citron pulp; the glycoside naringin is responsible for the bitterish taste of grapefruit, and eriodictyol occurs in lemons.[37,38]

Like flavones, flavonols are yellow pigments,[39] which are distributed in plants most widely (almost universally).[40] Compounds of this group encountered most frequently include kaempferol, quercetin, myricetin and their numerous derivatives. 3,5,4′-Trihydroxystilbene (*trans*-resveratrol) present in red wine is an interesting biologically active polyphenol, which has been the subject of a large number of studies in recent years. It was found[41] that *trans*-resveratrol protects the membrane lipids from peroxidation; it quenches free radicals less effectively than flavonoids but it is more effective in chelating copper ions.

Coumarins are also widely distributed in nature.[42–44] They are present in umbelliferous, leguminous and rue plants, being mainly concentrated in roots and fruits.[45] Owing to the availability and the ease of chemical modification, coumarins can be used for the development of medicinal drugs.[46] A meptazinol derivative based on coumarin is more easily uptaken in the body than the starting drug,[47] and the coumarin derivative with *trans*-

resveratrol is a more efficient vasodilator than *trans*-resveratrol itself.[48] Coumarins are used as anticoagulants;[49] they exhibit bactericidal,[50] fungicidal [51] and antitumour [52] activities.

The relationship between the antioxidant activity and the chemical structure of polyphenols has not been ultimately elucidated as yet. Chromatographic methods can be used to study the relationship between the structures and some properties of phenols.[53] This is done by modelling the retention in various versions of chromatography [54–56] and by numerical methods.[57] It has been established [58] that the antioxidant activity of polyphenols increases in the following cases:

(i) in the presence of a 2,3-double bond of the heterocyclic ring, as this seems to allow electron delocalisation between rings A and B [see the structure of (+)-catechin] and, hence, stabilisation of the aryloxyl radical formed;

(ii) in the presence of hydroxy groups in positions 2 and 4 of ring B, because this apparently also contributes to radical stabilisation;

(iii) on passing from polyphenol glycosides to aglycons, because carbohydrate residues in glycosides markedly decrease the antioxidant activity;

(iv) in the presence of a free phenolic hydroxy group.

It is known [59–65] that vegetables, fruits, red wine, chocolate, and other foodstuffs rich in polyphenols, when eaten on a regular basis, decrease substantially the risk of cardiovascular diseases.

Depending on the type and concentration of the initiator of free radicals, polyphenols can behave as either antioxidants or prooxidants.[66–69] The understanding and interpretation of the dual behaviour of polyphenols is complicated by the fact that despite the large body of data on high biological activity accumulated in recent years, the questions concerning the mechanism of their action and existence in the active forms *in vitro* and *in vivo* are still open. Therefore, development of procedures for determination of phenols and polyphenols in various objects by various analytical techniques, including chromatographic ones, is a topical task.

III. Sample preparation

An important problem that researchers are often faced with when investigating complex mixtures is the need to isolate the analytes from natural sources and thoroughly purify the extracts prior to the analysis.

The strategy of determination of biologically active compounds in plants depends on the nature and structure of the compound under study, the object of analysis (sample) and goals of analysis and cannot be completely unified. The following main stages of the investigation can be distinguished: extraction of the compounds from the sample, purification of the extract from accompanying compounds, separation and identification.

1. Extraction of compounds from natural sources

As a rule, biologically active compounds come from plant sources. Therefore, the primary task of a research is to extract the target compounds from the raw material as fully as possible, better in the native form. Usually, this is done by liquid or supercritical fluid extraction, in some cases, by high-pressure steam extraction. The extraction methods that permit preliminary purification of the extract from accompanying compounds are preferred.

The plant material is crushed and defatted by treatment with light petroleum, hexane or chloroform. If it is necessary to isolate a particular compound or a group of compounds from a complex sample, successive extractions with solvents in the order of increasing polarity is carried out (hexane, diethyl ether, ethyl acetate, butanol, water–ethanol mixtures).[70] In simple cases, one-stage extraction with methanol [71, 72] or dichloromethane is performed.[73] Pinelo *et al.*[74] used ethanol for complete extraction of biologically active phenols from the almond shell and pine sawdust, while polyphenols were selectively extracted with methanol. In another study,[75] phenols were extracted from lablab stems, flowers and fruit coat with 90% methanol and from leaves, with 90% ethanol. Preliminary separation of the flavonoids in plant extracts is carried out using preparative thin layer chromatography or preparative reversed phase chromatography.[76] The isolation of polyphenols from plant material is performed most often using 80% ethanol. The purity of the resulting fractions is checked by thin layer chromatography.[77–79]

Anthocyanins and anthocyanidins are extracted and separated in an acidic medium in order to avoid the destruction of non-acylated derivatives. In addition, the most stable resonance form of anthocyanins, flavylium cation, is formed at pH < 2. The extraction of anthocyanins from the ghost plant (*Graptopetalum paraguayense*) leaves with water and water–ethanol mixtures at different temperatures has been studied.[80] The highest antioxidant activity was found for a 50% ethanolic extract (extraction for 3 h at 75 °C). A mixture of water, acetonitrile and formic acid (1 : 1 : 0.1) [81] or trifluoroacetic acid (1 : 1 : 0.5) [82] was used to extract anthocyanins from liliaceous plant petals. Extraction with water [83] or methanol [84] with addition of an acid is also possible; however, the extraction time in this case is 12–16 h. Water–methanol–organic acid mixtures are more efficient. These extractants were used to isolate anthocyanidins of ornamental plants.[85–87] The extraction efficiency increases when an ultrasonic bath or a magnetic stirrer is used.

Sample preparation of procyanidins has some specific features. These compounds readily form complexes with metals, polysaccharides and proteins, which hampers their extraction using solid sorbents. The extraction of procyanidins from the seed coats or fruit pulp is complicated by the fact that procyanidins are oxidised during fruit ripening. This yields procyanidin–polysaccharide complexes and the solubility of procyanidins thus decreases.[88]

Rohr *et al.*[89] proposed a procedure for quantitative determination of procyanidins (three dimers and a trimer of epicatechin) in hawthorn leaves and flowers by reversed phase high performance liquid chromatography. The most complete extraction of procyanidin oligomers (yield 99%) was achieved using 70% aqueous acetone. However, during acetone evaporation, polymerisation and precipitation of the target compounds may take place. Extraction with 70% aqueous methanol has been reported.[90, 91] Methanol was used to obtain extracts from olive tree, hawthorn and oregano leaves.[92] The extraction of procyanidins from the cat's claw (*Uncaria tomentosa*) bark by refluxing with water has been reported.[93] Flavonoid glycosides and anthocyanidins can be destroyed by acid hydrolysis carried out at ~90 °C for several hours. The effect of the addition of an acid during extraction on the antioxidant activity of plant extracts has been studied.[94] The activity was found to increase in the vast majority of cases.

The higher the pressure, the higher the efficiency in liquid extraction. Papagiannopoulos *et al.*[95] identified 41 polyphenols in an aqueous acetone extract of algaroba obtained in this way. By varying temperature and pressure, biologically active phenols have been selectively extracted with water from rosemary [96] and other plants.[97] By combined use of solid-phase extraction and high performance liquid chromatography, an automated procedure for determination of procyanidins in malt was developed.[98]

Using supercritical fluid extraction technique, one can substantially increase the yield of labile compounds from biological matrices. As a rule, supercritical carbon dioxide is used. Since the polarity of CO_2 is relatively low, a polar modifying agent is often added. The effects of temperature, pressure and polar additives on the extraction kinetics and antioxidant properties of the ginger oil have been studied.[99] The efficiency of supercritical fluid extraction of phenols from the olive tree leaves with supercritical CO_2 has increased after the addition of methanol.[100] Thermodynamic modelling of quercetin solubility in supercritical CO_2 in the presence of ethanol has been carried out.[101] A scheme for broad-scale supercritical fluid extraction of the rosemary oil followed by liquid chromatography–mass spectrometry analysis of the obtained fractions has been proposed.[102] The use of supercritical

CO_2 for the fractionation of plant extracts has been reported.[103] New extraction techniques such as supercritical fluid extraction and automated tunnelling solid-phase microextraction has been described.[104] The extent of extraction of polyphenols from grape stones with liquid – liquid, solid-phase and supercritical fluid extraction have been compared.[105] The highest yield was achieved by the last-mentioned technique, possibly, due to the fact that the sample was located in a carbon dioxide atmosphere and was protected from light. This minimised decomposition of unstable compounds such as catechins.

A comparison of the efficiency of extraction from natural sources by liquid – liquid, solid-phase and supercritical fluid extraction techniques and by high-pressure extraction using various solvents has been reported.[106]

Usual objects of analysis, *i.e.*, plants, berries, fruits and vegetables, contain diverse phenols and polyphenols. However, substantial portions of these compounds are often lost during extraction, isolation and purification. Therefore, correct choice of the sample preparation method is a key issue in the qualitative and quantitative analysis of complex multicomponent mixtures.[107, 108]

2. Methods for extract purification and preconcentration

A number of techniques for the extraction and purification of phenols from complex mixtures have been reported,[109, 110] specifically, supercritical fluid extraction, semipreparative high performance liquid chromatography, high-speed counter-current liquid chromatography and superheated solvent extraction. Liquid – liquid and solid-phase extractions are used most widely for purification and preconcentration of polyphenols.[111 – 120]

For correct quantitation of phenol derivatives, it is necessary to take into account their possible transformations. This is especially important for analysis of food and beverages. During their production, phenols present in the raw materials undergo various transformations.

The influence of different wine manufacture processes on the composition of phenols and the antioxidant properties of the red wine Spatburgurder (Pinot Noir) and Cabernet Franc have been studied.[121] The processes included the grape peel fermentation, mash heating and a combination of these processes. The highest concentrations of anthocyanins, flavan-3-ols, flavonols and stilbenes were found in the red wine prepared by process combination. Lower concentrations of biologically active compounds were present in the wine prepared by mash heating; the wine produced by peel fermentation contained the lowest amount of anthocyanins. The process of fermentation during production of wine from blackberry and cherry berries caused $\sim$25% decrease in the content of the total polyphenol fraction.[122]

Table 1. Eluents used for fractionation and separation of extracts.

Phenols	Thin layer, preparative and column chromatography
Procyanidins	MeOH, acetone – water, ethyl acetate – HOAc – H_2O
Anthocyanins	Bu^nOH – HOAc – H_2O (4 : 5 : 1); Bu^nOH – 2 N HCl (1 : 1), 1% HCl, HOAc – HCl – H_2O (15 : 3 : 82)
Anthocyanidins	HOAc – HCl – H_2O (30 : 3 : 10); HCO_2H – HCl – H_2O (5 : 2 : 3)
Phenolic acids	Bu^nOH – HOAc – H_2O (4 : 5 : 1); EtAc – HOAc – H_2O (3 : 1 : 1); EtOH – H_2O – NH_4OH (16 : 3 : 1); toluene – HOAc – H_2O (40 : 10 : 5)

Note. The following mixture are used as eluents in high performance liquid chromatography: organic solvent (methanol, acetonitrile, ethanol) – water – acid (HCl, acetic, trifluoroacetic, formic, phosphoric) or organic solvent – acid.

In the extraction of phenols from grapefruit and orange juices [123] and dry leaves of green tea,[124] high yields were attained by repeated liquid – liquid extraction with aqueous methanol and acidified methanol. However, solid-phase extraction [125] ensures higher yield of phenols and polyphenols from various samples than liquid – liquid extraction. The main eluents for fractionation and separation of extracts are presented in Table 1.

IV. Chromatographic determination of phenols and polyphenols in various objects

1. Determination in plant materials

Determination of phenols in objects with a large number of components (complex mixtures) is performed most often by chromatography and capillary electrophoresis. Due to the thermal and chemical lability of most compounds to be determined, thin layer chromatography, its high performance version, capillary electrophoresis and high performance liquid chromatography are used. Thin layer and flash chromatography were the first methods used to separate many biologically active compounds of the plant origin. These methods are distinguished by low cost and simplicity. High-performance and two-dimensional thin layer chromatography can be used for analysis of complex natural sources of phenols.

Plant extracts do not require thorough purification by thin layer chromatography. Examples of such analyses are qualitative and quantitative determination of flavonoids in the passion flower (*Passiflora coerulea*) [126] and beech extracts.[127] Flavonoids were detected by exposure to UV light,[128] while procyanidins and anthocyanins were visualised by spraying with a 5% solution of vanillin in an ethanol – HCl (4 : 1) mixture.[129] Aglycons and glycosides were detected in the UV light and then visualised by spraying with a solution of H_2SO_4 and heating.[130] Scanning densitometry was used to analyse plant pigments.[131] Simultaneous separation and detection of compounds with antioxidant properties was carried out on plates with 1,1-diphenylpicrylhydrazide [132] and silver nanoparticles [133] applied thereon as indicators.

Gas chromatography is of limited utility for the analysis of natural biologically active compounds due to the necessity of derivatisation of phenols into methyl or trimethylsilyl derivatives. Nevertheless, such analysis is practised. Gas chromatography with mass spectrometric detection was used to determine phenolic acids in a coriander extract,[134] the main flavonoids and their glycosides in the willow wood [135] and phenols in the wormwood oil.[136] Fiamegos *et al.*[137] have developed a procedure for simultaneous extraction and derivatisation of flavonoids and phenolic acids for their determination in plant extracts by gas chromatography with mass spectrometric detection.

High performance liquid chromatography is used most often for determination of phenols. This is due to the following advantages of the method: high selectivity of the sorbents, sensitivity and selectivity of the diode array, ultraviolet, fluorescence and mass spectrometric detectors and mild conditions of analysis under which the analytes are not decomposed. High performance liquid chromatography on reversed phase columns with binary solvent systems and diode array detector is used both for routine analyses and to study plant extracts with complex composition. The detection limit of flavonoids can be reduced by using electrochemical or fluorescence [138] detectors. Examples of solvent systems and columns for this type of chromatography of plant extracts are summarised in Table 2.

Chromatography with amperometric detection is used to estimate the antioxidant activities of foodstuffs, beverages and drugs.[149, 150] Most natural biologically active phenols can be oxidised on an electrode; therefore, the amperometric detector is convenient for their determination. This allows one to establish the presence of unknown compounds in complex natural sources of phenols, whereas classical chemical methods [151] are only

Table 2. Results of analyses of the extracts of some flavonoids in plant samples by high performance liquid chromatography.

Sample	Identified compounds	Extractant	Column	Mobile phase	Detection	Ref.
Red clover	11 flavonoids	ethanol, methanol; acid hydrolysis	HP ODS Hypersil	H_2O/AcOH – MeOH	UV-MS	139
Passion flower (*Passiflora incarnata*)	10 flavonoids	'dry' extract	LiChrospher RP-18	H_2O – THF – MeCN – Pr^iOH	UV-MS	140
Cistus (*Cistus ladanifer*)	8 flavonoids	chloroform, methanol	LiChrosorb RP-18	0.1 M NH_4OAc in H_2O – THF – MeCN – Pr^iOH	UV-MS	141
Goatweed herb (*Hypericum perforatum*, St John's wort)	16 phenolic compounds	methanol	TP 544 RP-18	H_2O/H_3PO_4 – MeCN – MeOH	UV-MS	142
Oregano, caraway, fennel	flavonoids, methoxyflavonoids	methanol – water (62.5%)	Phenomenex RP C_{18}	H_2O/HCO_2H – MeOH	UV, MS-MS	143
Siberian larch and Gmelin larch bark	21 flavonoids	ethyl acetate	TLC: silica gel, HPLC: Separon C_{18}	H_2O/AcOH – MeCN	UV, IR, NMR	144
Gnidia (*Gnidia socotrana*) herb	flavone and coumarin derivatives	ethanol, heptane, ethyl acetate, n-butanol	RP 18	MeOH; H_2O/AcOH – MeCN	UV, UV-MS	145
Cyclopia	8 flavonoids, two isomers separated	methanol	Phenomenex Synergy MAX-RP C_{12}	H_2O/AcOH – MeCN – THF	electrochemical detector	146
Veronica (*Veronica thymoides*)	acylated flavonoids and glycosides	methanol, light petroleum	polyamide column for flash chromatography, TLC: silica gel	MeOH – $CHCl_3$, MeOH – H_2O	fluorescence detector	147
Brazilian pine tree	6 flavonoids	methanol – water, hexane	Phenomenex Synergy C18	MeOH – $CHCl_3/HCO_2H$	DAD, MS	148

Notes. The following designations are used: UV-MS a combination of UV and mass spectrometric detectors, MS-MS is tandem mass spectrometry, TLC is thin layer chromatography, DAD is diode array detector.

suitable for estimating the overall antioxidant activity of the sample or the activity of a known compound.

The difficulties arising in the determination of phenols by chromatographic methods are mainly related (apart from the necessity of preliminary purification of the extracts before the introduction into the column) to the lack of standards due to the great diversity of natural forms of these compounds. Therefore, the so-called fingerprint method is used in some cases for analysis of complex plant extracts.[152, 153] These fingerprints are specific for each plant species and can be used to identify the source of a plant raw material. Secondary metabolites, including phenolic acids and flavonoids, are used as chemical markers in the fingerprints.[154] The plants species are differentiated based on the variation of the composition and content of these compounds.[155] A combination of the fingerprint method and chromatographic analysis of biological fluids is used to identify fake drugs.[156]

A number of methods have been described for quantitative and qualitative determination of proanthocyanidins and their derivatives,[157] chromatography and spectrometry being used most often.

The spectrometric methods are based on reactions giving chromophores (the Folin – Ciocalteu reagent, the use of HCl – BuOH, vanillin, and so on). However, these methods do not provide information on the amounts and structures of oligomers with different degrees of polymerisation. Spectrophotometry is used to determine the total amount of procyanidins in complex samples,[93] however lately it has been replaced by mass spectrometry. Gel chromatography,[158] thin layer and column chromatography are used to isolate anthocyanin and procyanidin fractions and to separate them according to the degree of polymerisation.[92–94] A mixture of individual procyanidins (from monomers to trimers) can be separated by reversed phase high performance liquid chromatography.[99, 159] The method proposed by Svedstrom *et al.*[91] made it possible to separate epicatechin oligomers (from monomers to hexamers) present in the extract of hawthorn leaves and to isolate previously unknown trimers and pentamers using a polyamide column and an acetonitrile-based eluent.

Capillary electrophoresis is gaining increasing acceptance for determination of pharmacologically important compounds in plants.[160–162] *trans*-Resveratrol was determined using a borate buffer (pH 8 – 11), as borates form complexes with the hydroxy groups of flavonoids, thus facilitating the separation.[163] The buckthorn flavonoids were separated in the presence of a modifying agent, dimethyl-β-cyclodextrin.[164] Anthocyanins have been separated by capillary electrophoresis in an acidic medium.[165]

Neutral molecules can be separated in an electric field by micellar electrokinetic capillary chromatography. By varying the concentration of the micelle-forming agent (most often, sodium dodecyl sulfate), the system selectivity can be changed. Various modifying agents are used: methanol for determination of flavonoids, phenolic acids and aldehydes,[166] butan-1-ol for flavonoids and iridoids[167] and acetonitrile for coumarins.[168] Drawbacks of the method include lower sensitivity and reproducibility compared to high performance liquid chromatography.

2. Determination in foodstuffs and beverages

Most often, phenols are detected by spectrophotometry using a number of wavelengths: 230, 280, 360, 503 nm, *etc*. In some studies dealing with particular groups of flavonoids, only one

wavelength was used, namely 280 nm for catechins [169–173] and 520 nm for anthocyanins.[174–178] Other studies made use of wavelengths of 280, 306 and 360 nm for phenol detection.[179–184] Since many phenols have several absorption maxima, they are often determined using simultaneous scanning at several wavelengths called diode array detection.[185–190] High performance liquid chromatography with diode array detection is often used to determine phenols present in foodstuffs and beverages. The key advantage of this method is the low detection limits. For example, the detection limit for catechins in the green tea extract was reported to be 0.05 mg ml^{-1} (with detection at 205 nm).[191] Examples of using the diode array detection in the determination of phenols and polyphenols in fruit and vegetable extracts and in beverages by high performance liquid chromatography are summarised in Table 3.

Determination of phenols in foodstuffs and beverages is often carried out by reversed phase chromatography on modified silica gels, because reversed phase columns ensure high separation selectivity and better reproducibility of the results and have longer service life. Some types of modified silica gels used most often in the analysis of phenols in various objects are summarised in Table 4.

To increase the sensitivity and selectivity of determination of phenolic acids and aldehydes, simultaneous diode array and fluorescence detection is used. In order to ensure the highest sensitivity of the analysis, a fluorescence detector should be time

Table 3. Results of determination of phenol and polyphenol antioxidants in fruits, vegetables and beverages by high performance liquid chromatography.

Object	Identified compounds	Detector	Quantifiable concentrations	Ref.
Apples, grapes, beans	(+)-catechin, (−)-epicatechin, 3 catechin derivatives	DAD	0.1–3.9 mg per kg – –	192
Grapefruit juice	naringin	DAD 280 nm	2.5 mg $litre^{-1}$	193
Grape berries	*trans*-resveratrol, rutin, quercetin, quercitrin	DAD 306 nm	4.5–10.1 mg per 100 g – 0.16–0.45 mg per 100 g –	194
Bulgarian wine, 11 brands	quercetin, myricetin, morin, kaempferol	DAD 360 nm	the highest total content of flavonols in the brand Melnic: 12.2–16.9 mg $litre^{-1}$	195
Green and black tea	16 phenols including (−)-epigallocatechin	DAD 205 nm	2.84 mg g^{-1} (black tea) (23.46–36.53) mg g^{-1} (green tea)	196–199
Juices and beverages	21 phenols including: quercetin (−)-quininic acid	DAD 215 nm	 0.03 mg $litre^{-1}$ 12.5 mg $litre^{-1}$	200

Table 4. Characteristics of some reversed phase columns used in analysis of phenol derivatives in various objects by high performance liquid chromatography.

Object	Column			Elution time and mode	Identified compounds	Ref.
	sorbent type	sorbent grain diameter /μm	column size /mm			
Juices (apple and multifruit)	Shim-Pack VP-ODS	5	250 × 4.6	80 min, gradient	31 phenols	201
Cranberry	XDR C_{18}	5	150 × 4.6	47 min, gradient	13 phenols	202
Apples, pears	Nucleosil 120 C_{18}	5	250 × 4.6	25 min, gradient	5 phenols	203
Green and black tea, 8 brands	PartiSphere 5 C_{18}	3	110 × 4.6	27 min, gradient	15 phenols	191
Chocolate, 11 brands	Hypersil ODS	5	250 × 4.6	30 min, isocratic	9 phenols	173
Grapefruit, orange	μ-Bondapak C_{18}	5	250 × 4.6	20 min, isocratic	3 phenols	204
White wine, 11 brands	Alltima	3	100 × 4.6	20 min, isocratic	4 phenols	205
Grape berries	Hypersil ODS	5	150 × 4.6	25 min, gradient	4 phenols	206
Red and white wine, 5 brands	Nova-Pak C_{18}	4	150 × 3.9	34 min, gradient	17 phenols	207
Onion, soy	Hypersil 120	3	125 × 4.6	30 min, gradient	5 phenols	208
Green tea	YMC-Pack ODS-AQ	5	150 × 4.6	50 min, isocratic	6 phenols	209
Orange juice	Luna	3	150 × 2.0	15 min, gradient	10 flavonoids	181
Lemon peel	Nova Pak C_{18}	5	150 × 4.6	60 min, gradient	7 flavonoids	210
Wine [a]	Spherisorb ODS 2	3	250 × 4.6	55 min, gradient	17 phenols	211
Orange and grapefruit juice concentrates	Alltech C_{18}	5	250 × 4.6	30 min, isocratic	3 flavonoid glycosides	123
French wine [b]	Nucleosil 100 C_{18}	5	250 × 4.0	75 min, gradient	1 phenol	212
Linseed	Sperisorb S-ODS	5	250 × 4.0	50 min, isocratic	6 phenols	213
Lentil, beans	Nucleosil 120 C_{18}	5	250 × 4.6	20 min, gradient	5 phenols	214

[a] The wine brand was not specified. [b] Red and white wine, 5 brands each.

programmed for the optimal excitation and fluorescence wavelengths for the group of phenols with similar spectral characteristics. The use of statistic multivariant methods is of interest for composing an optimal program for detection of a complex sample.[188] After optimisation of all individual parameters of the analysis, the detection limit for *trans*-resveratrol using fluorescence detector was 0.003 mg litre^{-1}, and using diode array detector, this value was an order of magnitude higher, 0.02 mg litre^{-1} (Ref. 187).

The sensitivities of various versions of detection in the determination of flavonoids in orange juice by high performance liquid chromatography have been compared.[215] The detection limits and the concentrations of some flavonoids found in orange juice are presented in Table 5. It can be seen that all detection methods ensured high reproducibility and allowed quantitative determination of low levels of flavonoids.

Table 5. Detection limits and results of quantitative determination of flavonoids in orange juice by high performance liquid chromatography with different detectors.[186]

Compound	Detector		
	UV	mass spectrometer	electro-chemical
Detection limits (μg in 1 ml) for some flavonoids			
Quercetin	0.15	0.2	0.3
Narirutin	0.2	0.1	1.0
Eriocitrin	0.2	0.08	0.5
Hesperidin	0.3	0.1	0.5
Naringin	0.25	0.15	1.0
Content (mg per 100 g) of flavonoids detected in juice			
Quercetin	1.34 ± 0.15	0.90 ± 0.03	1.18 ± 0.11
Narirutin	5.10 ± 0.17	5.4 ± 0.2	5.0 ± 0.6
Eriocitrin	1.0 ± 0.2	0.9 ± 0.3	–
Hesperidin	73 ± 7	78.9 ± 0.4	79.3 ± 0.5
Naringin	2.6 ± 0.3	0.14 ± 0.0	–

The compounds to be determined may have a broad range of polarity; therefore, their analysis requires either a gradient elution mode or several mobile phases with isocratic elution. Thus the isocratic mode was used to determine anthocyanins, flavan-3-ols, flavonols and phenolic acids in three brands of Bulgarian wine and gradient elution was employed to determine stilbenes in the same brands of wine.[122] In some cases, virtually no sample preparation was required. For analysis of *trans*-resveratrol isomers in red wine, the sample was directly applied (without the sample preparation stage) on a chromatographic column and the fractions containing each of the target stilbenes were isolated by reversed phase high performance liquid chromatography.[216] Then these fractions were analysed by the gas chromatography – mass spectrometry technique.‡

A popular method for detection of phenols is coulometric detection. Unlike amperometric analysis, in this case the analyte is completely oxidised. The coulometric detector has been used for qualitative and quantitative determination of phenols in vegetable oils.[195] The advantages of this detector are the simple design and the possibility of simultaneous determination of eluates with different oxidation potentials. This detector ensures a lower detection limit and a higher selectivity than the UV detector when used for quantitative determination of biologically active phenols in plant extracts. However, it suffers from a number of drawbacks, namely, contamination of the electrode by oxidation products and, as a consequence, a change in the electrode characteristics, dependence of the signal on the eluent flow rate and limited applicability in the gradient elution mode.[218] It has been found[219] that phenolic acids and tocopherol show the maximum coulometric detector response at low potentials (100 – 450 mV), while flavonoids show the maximum responses in two ranges: 0 – 300 and 600 – 900 mV.

In the studies of complex natural objects by any chromatographic method, identification of the components is an important stage. This task is solved by comparing the retention times, spectroscopic data and/or retention indices of the analytes with the same parameters of standard (reference) compounds. In analysis of natural objects, the most informative data can be obtained by combined use of all characteristics.[220] The phenol derivatives determined by high performance liquid chromatography are identified by comparison of spectroscopic characteristics (mass spectra)[221] and retention indices[222,223] with the respective characteristics of the standard compounds. In the absence of standard compounds,[224] the impurities in dihydroquercetin can be identified by combined consideration of the retention indices and spectral ratios at different wavelengths. It is possible to use retention indices in micellar electrokinetic chromatography.[225]

In recent years, high performance liquid chromatography with mass spectrometric detection[226–230] or combinations of diode array and mass spectrometric detection with various ionisation sources[231–234] have been used most often to identify phenols present in plant raw materials and foodstuffs. The latter technique is especially valuable in the study of acylated flavonoid glycosides present in plants, vegetables or fruits in small amounts.

Using high performance liquid chromatography with mass spectrometric detection and with chemical ionisation under atmospheric pressure, extracts of about 40 fruit and vegetable species have been characterised qualitatively and quantitatively.[235] Seven flavonoids were isolated and identified. For example, apples contained up to 2 mg of quercetin per 100 g of the sample weight and parsley contained up to 185 mg of apigenin per 100 g of the sample weight.

Studies of flavonoids in many spice plants carried out by high performance liquid chromatography with electrospray ionisation mass spectrometry detection have shown[143] the possibility of determining molecular masses of aglycons and glycosides.

Capillary electrophoresis starts to be actively used to determine phenols in juices, tea, vegetable oils, wines and berries.[236–241] Advantages of this method include the possibility of introducing the sample into a capillary in a dilute form under pressure,[242] *i.e.*, the sample preparation stage is avoided, and performing the experiments over a broader pH range[243,244] than in the case of high performance liquid chromatography.

A review[245] cites an interesting example of using capillary electrophoresis for the separation of six diastereomeric flavanone-7-*O*-glycosides. The separation was carried out at pH 10 in a phosphate buffer with addition of chiral compounds, *viz*., cyclodextrins and their derivatives, to the supporting electrolyte. Under chosen conditions, lemon juice was analysed and the mixture of four flavanone-7-*O*-glycosides present was separated into isomers.

Currently, capillary electrophoresis is used as a technique supplementary to high performance liquid chromatography for the separation and determination of phenols.

3. Determination in biological fluids

Biological fluids (blood, urine) are analysed for the presence of antioxidants and to estimate the bioavailability of these compounds (after consuming various kinds of food), study their metabolism and influence on the human health. In virtually all studies of this type, high performance liquid chromatography and capillary electrophoresis techniques are used. The applications of

‡ The original procedure for the analysis of hydrophobic samples including direct injection of plant or animal fats into a column has been reported.[217] The procedure is applicable to determination of biologically active compounds in plant oils.

capillary electrophoresis, in particular, that with mass spectrometric detection, for analysis of antioxidants, metabolites and toxins have been surveyed.[245] In the present review, we will consider the use of high performance liquid chromatography in more detail.

The extraction of flavonoids from biological fluids is rather simple. A sample is centrifuged to remove proteins and other polymeric components and is subjected to solid-phase extraction with elution by methanol, ethanol and ethyl acetate. If necessary, enzymatic or acid hydrolysis is carried out. Sample preparation methods for blood samples have been compared.[246] The addition of anticoagulants does not influence noticeably the accuracy of analysis, 1% ascorbic acid mixed with orthophosphoric acid (10 μl ml^{-1}) being the best stabiliser. The samples should be stored at -20 °C.

Isoflavones were the first flavonoids to be determined quantitatively in human urine. Routine methods for analysis of human blood and urine for the presence of daidzein and equol (120 ng ml^{-1})[247] using reversed phase high performance liquid chromatography with UV detection have been developed. The high performance liquid chromatography technique with electrochemical detector is more sensitive, permitting determination of daidzein, genistein and equol at a level of 1–2 ng ml^{-1} (Ref. 248). By gas chromatography–mass spectrometry, isoflavone metabolites were identified in blood serum and rat internal tissues at a minimum content of 10–20 pg mg^{-1} (Ref. 249). A fast method for determination of isoflavone in urine by immunofluorometric assay was proposed by Uehara *et al*.[250] The method is sensitive (0.1 ng ml^{-1}) and reproducible (the root-mean-square deviation is 2%–10% between the series of analyses); however, like gas chromatography with the same detection method, this method is expensive and labour-consuming.

The total amount of quercetin and its metabolites in biological samples is determined using liquid chromatography with mass spectrometric detection[251–254] and some other methods, for example, versions of high performance liquid chromatography with mass spectrometric,[252] UV,[255,256] affinity chromatographic,[257] fluorescence and electrochemical[258,259] detection. Gas chromatography with mass spectrometric detection has been proposed for determining quercetin and its conjugates in human urine and blood plasma in concentrations of down to 10 pg ml^{-1} (Ref. 260). The characteristics of some methods for determination of quercetin and other biologically active phenols are given in Table 6.

Thus, nowadays, reversed phase high performance liquid chromatography combined with various detection techniques (most often, diode array and mass spectrometric detection) is

Table 6. Characteristics of some methods for determination of biologically active phenols in various samples.

Sample	Identified compounds	Extractant	Hydrolysis	Method [a]	Detection limit /ng ml^{-1}	Ref.
Blood, human blood serum and plasma, rat urine	(+)-catechin, quercetin, *trans*-resveratrol	ethyl acetate	see [b]	GC-MS	0.01	261
Human blood and urine	quercetin and its glycosides	aqueous methanol	HCl–methanol, 5 h (blood), 8 h (urine)	HPLC, fluorescence detector	–	262
Rat blood plasma	quercetin	preliminary purification on a C_{18} column, extraction with a toluene–CH_2Cl_2 mixture	ascorbic acid + β-glucuronidase	HPLC, electrochemical detector	0.63	263
Rat urine and blood	flavonoids, phenylalkanoic acids	water, methanol, fractionation (SPE C_{18})	HCl–methanol, 45 min	RP HPLC-UV (C_8, C_{18})	–	264
Human blood plasma	naringin, naringenin	ethanol, fractionation (Sep-Pak, C_{18}, 80% methanol)	–	RP HPLC-UV (C_{18})	2	265
Human blood plasma	catechin, epicatechin, 3 derivatives	ethyl acetate	HCl–methanol, 30 min	RP HPLC-UV (C_{18})	–	266
Human urine	apigenin, acacetin	addition of 2 M NaOAc + 10% ascorbic acid	β-glucuronidase + arylsulfatase, 60 min	RP HPLC-UV with column switching (C_8, C_{18})	10–70	267
Human blood plasma	naringenin, hesperetin	SPE on a C_{18} cartridge	β-glucuronidase + arylsulfatase, 18 h	HPLC-UV, C_8	10	268
Rat blood, urine, tissues	a mixture of 8 catechins	methanol–ethyl acetate–sodium dithionite (2 : 1 : 3)	β-glucuronidase + arylsulfatase, 45 min	RP HPLC (C_{18}), electrochemical detector	5	269
Human blood serum	6 phenolic acids and aldehydes	formic acid–acetone–water (4 : 140 : 56)	–	HPLC-MS (C_{18})	8	270

Notes. The following designations are used: GC-MS is gas chromatography–mass spectrometry, RP HPLC-UV is reversed phase high performance liquid chromatography with UV detection, SPE is solid-phase extraction, HPLC-UV is high performance liquid chromatography with UV detection, HPLC-MS is high performance liquid chromatography with mass spectrometric detection.

[a] The hydrocarbon sorbent is indicated in parentheses. [b] Prior to the analysis, the sample was modified with bis(trimethylsilyl)trifluoroacetamide.

one of the most efficient methods for separation and identification of biologically active phenols and polyphenols in various objects.

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[a] — *Russ. Chem. Bull., Int. Ed. (Engl. Transl.)*
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