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Protective properties of wine products and the role of high performance liquid chromatography in the study of these properties

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Abstract. Data on the biologically active substances present in wines and wine products, the methods of their determination, and changes under chemical, radiation and other types of action are generalized. The role of high performance liquid chromatography in the studies of the protective properties of wines is demonstrated. Particular attention is devoted to problems of counterfeiting of wine products and the possibility to reveal it by using amperometric determination of the antioxidant activity. The bibliography includes 117 references.

I. Introduction

The history of wine making has lasted for more than 6000 years. Wine making process and the properties of wine are the subjects of a multitude of publications. Recent years have witnessed enhanced interest in the composition and properties of wines, which was aroused due to so-called 'French paradox'. It was noticed that France where people drink a lot of young red wines have low rates of heart diseases and cancer. This is attributed to the presence of large amounts of antioxidants and other beneficial compounds in wines. Therefore, studies of the composition of wines and their ability to regulate the redox processes in living systems that are induced by exposure to light, radiation and other factors attract considerable attention. Several reviews are devoted to these issues.[1 – 6]

The antioxidant and radioprotective properties of wines are caused by the presence of biologically active phenol derivatives, including flavonoids, the composition of these compounds being determined by both the wine making procedure and the time and conditions of storage of the finished product. The composition of wines is studied most often by HPLC, and the mechanism of change in the wine properties under external impacts is elucidated using radiation chemical simulation, in particular, pulse radiolysis.

However, before a work by Ulyanova,[7] these methods had not been combined to study the chemical changes in the wine composition on exposure to radiation. Determination of the products formed upon this treatment plays an important role in the studies of the mechanism of protective properties of wines. The calculations of the degrees of conversion are also necessary for evaluating the radiation stability of wines, as γ-irradiation is used more and more often for biological stabilization of wines in wineries.

This review integrates the data on flavonoids contained in wines. The antioxidant activity assays, first of all, chromatography, are considered in detail. The data on the effect of ionizing radiation on polyphenols present in wine are described systematically and analyzed. The purpose of this work was to demonstrate the enormous role of high performance liquid chromatography in the investigation of radioprotective and antioxidant properties of wines, which is caused by the unique potential of this method used to solve the following problems:

— separation of the mixture of antioxidants, including flavonoids followed by online determination of antioxidant activities of single compounds by various methods;

— determination of the total phenolic content;

— prediction of biological activities of wine antioxidants from their chromatographic characteristics;

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— chromatographic determination of the products of radiation-induced chemical transformations in wines under conditions of simulated oxidative stress.

The review also pays attention to the problem of counterfeiting of wine products and the possibility to reveal it by using amperometric determination of the antioxidant activity.

II. Flavonoids as antioxidants

Antioxidant can be defined as any compound that, being present in a low concentration with respect to the oxidized substrate, substantially retards or prevents the substrate oxidation.[8] The antioxidant and pro-oxidant are analogous to the terms 'reductant' and 'oxidant' in the context of biological systems. However, it should be borne in mind that, although any antioxidant is a reducing agent, not any reducing agent is an antioxidant and protector. Depending on the type and concentration of free radical initiators, polyphenols can behave as either antioxidants or pro-oxidants.

The most important natural antioxidants are vitamins E and C, polyphenols, flavonoids and aromatic hydroxy acids. Among all food products with a multitude of natural antioxidants, of special note is grape wine, which exceeds in activity vitamins C and E and β-carotene. More than 400 natural compounds are present in wines. These include carbohydrates and their derivatives, about 20 organic aliphatic acids and salts, dozens of aromatic acids and esters, amino acids, phenols and mineral substances, some valuable enzymes, vitamins and micro nutrients.[9] The presence of flavonoids, anthocyanins and other phenolic derivatives is considered to be responsible for the antioxidant properties of wine.[10–16] A great advantage of wine over vegetable food is that the phenolic compounds present are soluble in water–organic media and, hence, occur in biologically available forms, whereas in fruits and vegetables they occur as polymeric, insoluble or highly bound forms.[17]

Cognac spirits also contain phenolic compounds that have passed there during maturation in oak casks; the antioxidant activities of cognacs are still much lower than those of wines.

Flavonoids represent an extensive family of polyphenolic compounds the structures of which contain a common C_6-C_3-C_6 skeleton consisting of two benzene rings (**A** and **B**) connected by heterocyclic pyran ring **C**. Flavonoids are especially important antioxidants due to their low redox potential, which allows them to act as reducing agents, hydrogen donors and free radical traps. It is known that flavonoids, like other phenolic compounds, can retard peroxidation of cell membrane lipids, thus preventing the damaging action of free radicals and premature ageing of cells in living bodies.[18] The diversity of flavonoids in nature is caused by the fact that they usually contain several hydroxy or methoxy groups in the aglycone, the substituents being mainly in positions 5, 7, 4′, 3′ and 5′.

The flavonoids present in grapes include anthocyanins (anthocyanidin glycosides), flavanols, flavonols and flavanonols (Fig. 1). It is noteworthy that compounds of the first two groups are especially abundant in grapes and wine, and they significantly influence the quality of wine; moreover, anthocyanins are responsible for the colour of red wines, while flavanols affect the taste (astringency and bitterness). The flavonoids of grapes can be hydroxylated at all positions characteristic of these natural polyphenolic compounds and glycosylated, for example, at the C(3) atom.

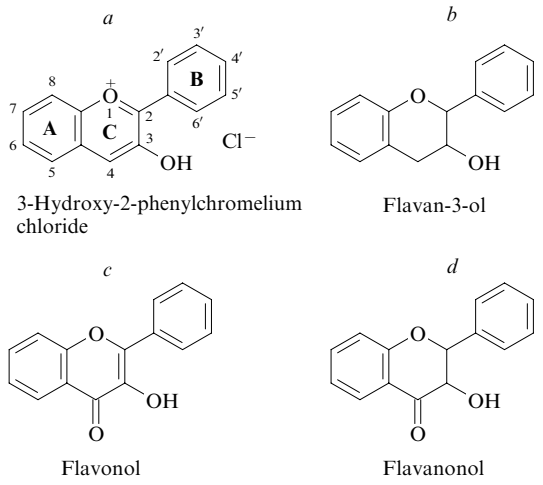


Figure 1. Parent structures of various flavonoids present in grapes: anthocyanins (*a*), flavanols (catechins) (*b*), flavonols (*c*), flavanonols (*d*).

The flavanols of grapes, which are more exactly called flavan-3-ols, have a hydroxyl in position 3 and are known both as monomers and as oligomers and polymers.[19] The principal monomeric flavanols of grapes are (+)-catechin and its isomer, (−)-epicatechin, and also gallic acid ester, (−)-epicatechin-3 gallate, which is present in a much lower amount (Fig. 2). Gallocatechin, catechin 3-gallate and gallocatechin 3-gallate have been found in some table grape varieties. Flavanol oligomers and polymers are also called condensed tannins or procyanidins. Tannins are able to interact with proteins and precipitate them. The name 'procyanidins' is related to the fact that on heating in acid medium, these compounds are converted to red anthocyanidin pigments. The structural units in type B procyanidins are connected by C(4)−C(8) bonds and/or C(4)−C(6) bonds. In type A procyanidins, C(2)−O−C(7) or C(2)−O−C(5) bridges are additionally formed. Figure 2 presents the structural formulae of the major flavanols present in grapes and wine.

The major grape flavonols are quercetin 3-glycosides. Each grape variety has its own specific flavonol profile, and this can be used for taxonomic characterization. Before 2006, it was considered that grape cultures used to make white wine contain no flavonols, until Rodrigues Montealigre detected a minor amount of the glycoside isorhamnetin in the grape skins.[19] Meanwhile, myricetin, laricitrin and syringetin derivatives seem to be specific to red grapes. It was shown that the content of flavonols, especially quercetin, in the skins of Pinot Noir grapes considerably depends on exposure to sunlight. Figure 3 shows the formulae of some wine flavonols.

III. Methods for determination of polyphenols in wines

Currently, the polyphenolic composition of wines arouses enormous interest in wine making specialists, because polyphenolic compounds are responsible for the quality (taste and colour) of wines and for the beneficial properties of

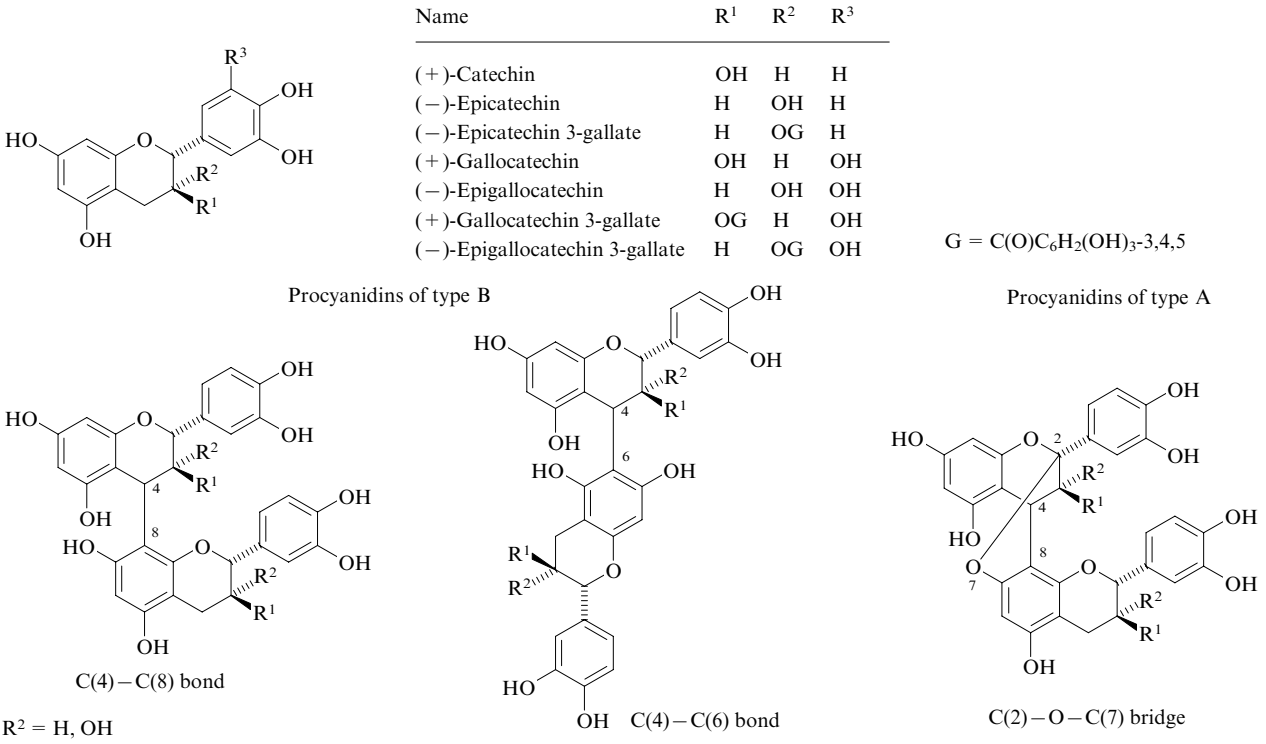


Figure 2. Major flavanols of grapes and wine.[19]

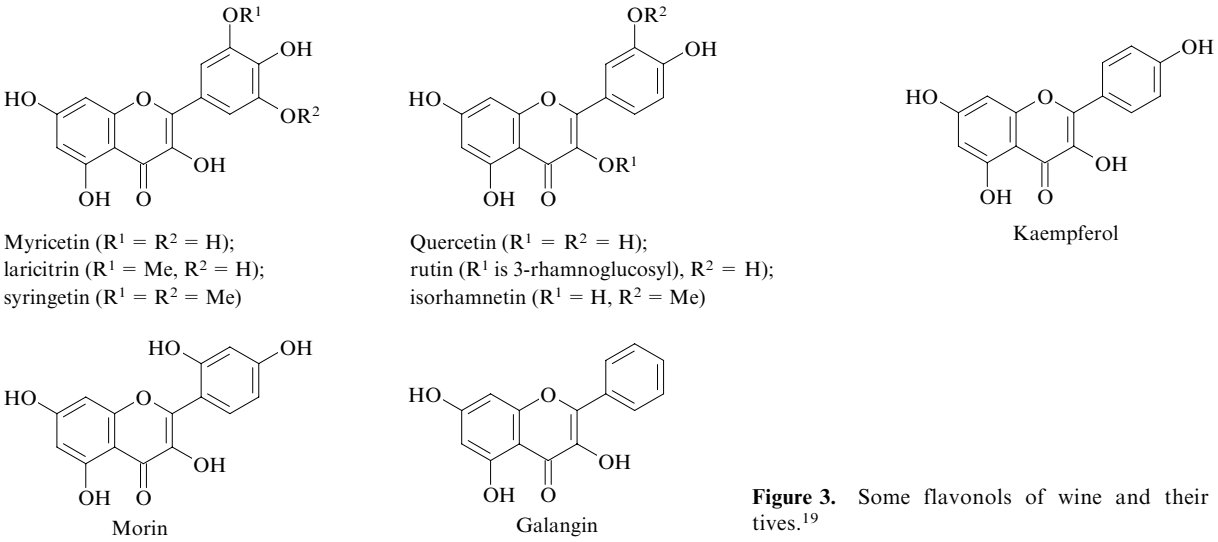


Figure 3. Some flavonols of wine and their derivatives.[19]

wines that follow from their antioxidant activity (the ability to prevent cardiovascular diseases and cancer). From the polyphenolic composition of wine, it is possible to determine its geographic origin and the variety of grape used to produce this wine.[20] The importance of this problem has initiated the use of a broad range of methods including thin layer,[21] gas[22] and high performance liquid chromatography[23–26] and capillary electrophoresis[27] to analyze these natural objects.

Reversed-phase HPLC is among the methods used most frequently to determine polyphenols in wines. This is due to the following advantages of the method: mild temperature conditions of analysis under which the test compounds are not destroyed; the sensitivity and selectivity of detectors used, and long service life of the reversed-phase C18 columns, which provide high separation selectivity and good reproducibility of the results. The difficulties associated with the determination of flavonoids in wines are mainly related to the lack of necessary standard specimens.

Consider the possibilities of standardless identification of these compounds. In hydroxylated flavan derivatives, *e.g.*, naringenin (spectrum *1* in Fig. 4), which absorb only in the UV region at 275–280 nm, the C(2)−C(3) single bond (Fig. 5) effectively separates the two benzene rings and electron transitions occur only in isolated chromophores. In flavonols and flavones, *e.g.*, apigenin (spectrum *2* in

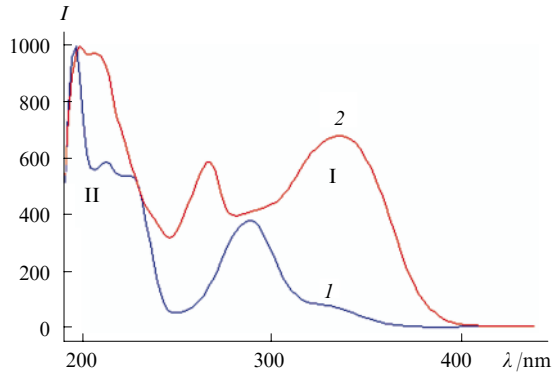
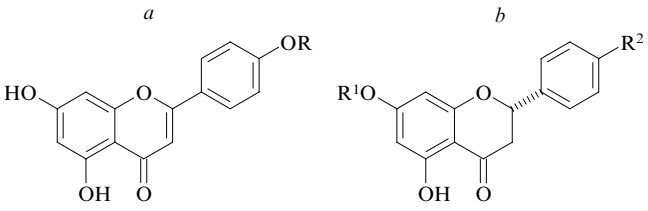


Figure 4. Optical absorption spectra of naringenin (*1*) and apigenin (*2*).[7]
Here and below *I* is intensity in arb.u.



Apigenin (R = H), acacetin (R = Me)

Pinocembrin ($R^1 = R^2 = H$), naringenin ($R^1 = H$, $R^2 = OH$), naringin ($R^2 = OH$, R^1 is 3-rhamnoglucosyl)

Figure 5. Some flavones (*a*) and flavonones (*b*) of wine and their derivatives.

Fig. 4), the carbonyl group in position 4 is conjugated with ring **B** (see Fig. 5); therefore, the spectra exhibit two absorption bands: the intense band I in the long-wavelength region (320–370 nm) is related to ring **B** and short-wavelength band II (200–240 nm) corresponds to ring **A**.

The pattern of the absorption spectra of polyphenols depends on the number and positions of hydroxy groups. The introduction of every next hydroxyl into ring **A** or **B** results in a long-wavelength shift of the corresponding bands, which is demonstrated in Fig. 6 in relation to the flavone acacetin and the flavonol galangin (see Figs 3 and 5): the spectrum of the latter exhibits a strong band at 320–370 nm.

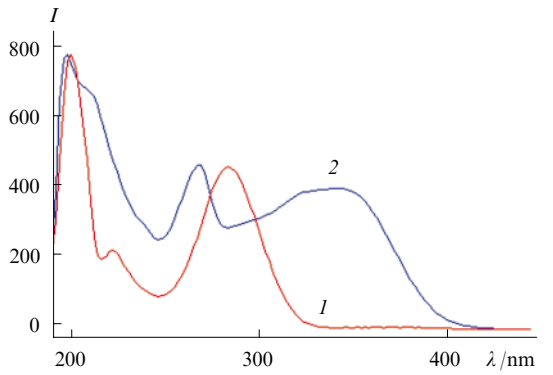


Figure 6. Optical absorption spectra of acacetin (*1*) and galangin (*2*).[7]

The differences between the optical absorption spectra can be used to draw tentative conclusions about the class to which an unknown natural flavonoid belongs. Further identification of the pigments is based on their chromatographic parameters, *i.e.*, their retention times are measured. Apart from the general trend of increasing retention times with the increase in the molecule polarizability and molecular mass, retention is also affected by factors of specific interaction with molecules of the mobile phase. Glycosylation enhances intermolecular interaction of flavonoids with the polar eluent, which results in shorter retention times in the reversed phase. For example, as can be seen from the chromatogram of a model mixture (Fig. 7), rutin and naringin are eluted on the 31st and 33rd minutes, respectively, while their aglycones quercetin and naringenin are eluted on the 49th and 51st minutes.

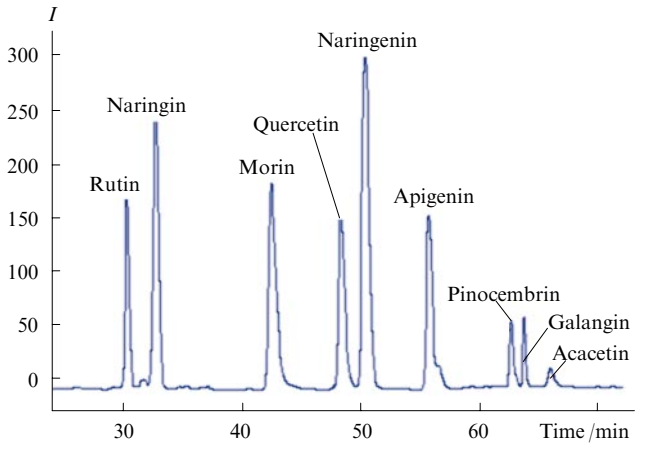


Figure 7. Chromatogram of a model mixture of flavonoids.[7]
For the structures of compounds, see Figs 3 and 5.

All compounds comprised in the model mixture have a hydroxy group at C(5). This group can form an intramolecular hydrogen bond with the keto group at C(4).[28] As a result, polarity decreases and the retention time increases, *i.e.*, these compounds should be retained more strongly than the corresponding isomers with a hydroxyl at any carbon atom other than C(5) in the flavonoid molecule. Pinocembrin is retained more strongly than naringenin, despite the presence of an additional OH group. Presumably, the polarity of pinocembrin decreases because the intramolecular hydrogen bond formed by the OH group at C(5) and the keto group at C(4) is stabilized by the electron-donating OH group at C(7). It can also be seen in the chromatogram of the model mixture that, despite the larger number of polar groups in the molecule, acacetin and galangin, which are flavone derivatives, are retained on the reversed phase longer than pinocembrin (a flavan derivative). This may be attributable to the fact that flavone molecules are planar due to the presence of a double bond between C(2) and C(3). It remains unclear why apigenin, which differs from acacetin only by the 7-hydroxy group being present in the molecule instead of the 7-methoxy

group, is retained less strongly than pinocembrin. Considering the pair of isomers, quercetin and morin, which differ by the position of one OH group in ring **B**, suggests that the former is longer retained than the latter due to the presence of an intramolecular hydrogen bond between C(3)′ and C(4)′ atoms. (+)-Catechin (see Fig. 2), which is not contained in the model mixture, is retained much weaker than quercetin, because the OH group at C(5) in its molecule is hydrogen-bonded to the neighbouring keto group and, moreover, the molecule is planar.

Note that various HPLC procedures are quite efficient for determining phenolic monomers and oligomers in wine; however, only gel chromatography is suitable to determine polymeric compounds. This is caused by the large number of possible low-molecular-mass isomers and low contents of these substances in wine. Procyanidin polymers can be determined by HPLC procedures that include preliminary acid-catalyzed purification in the presence of a nucleophilic agent.

1. Wine sample preparation for the assay

Sample preparation is used in almost all works dealing with determination of the polyphenolic composition of wine. The key sample preparation techniques are liquid extraction into various organic solvents[29,30] and solid phase extraction.[31,32] Note that the use of liquid extraction for the wine sample preparation is mainly encountered in the publications of the 1980–1990s, while currently the solid phase extraction is more abundant, *i.e.*, the former method is obsolete. The drawbacks of liquid extraction are the long duration of the sample preparation procedure, which may lead to partial loss of extracted polyphenols upon oxidation.[33] Decomposition of polyphenolic compounds is facilitated by enzymes that are present in wine, by light and by atmospheric oxygen, which cannot be avoided during liquid extraction. In addition, the liquid extraction procedure is difficult to automate, which may be important for wine making.

As organic solvents, diethyl ether, ethyl acetate, alcohols (ethanol, methanol) and acetone are used most often. For example, Vanhoenacker *et al.*[34] described a method for determination of 24 phenolic compounds including phenolic acids, flavonoids (kaempferol, quercetin, myricetin and so on) and stilbenes in the diethyl ether extracts of red wines by HPLC and capillary electrophoresis in combination with mass spectrometry.

Octadecylsilyl-grafted silica gel is the sorbent of choice for solid phase extraction of wines. De Villiers *et al.*[35] used the solid phase extraction on styrene divinylbenzene cartridges Strata SDB-L to prepare samples for the simultaneous determination of polyphenols, organic acids and sugars in wines. Wine was adjusted to pH 2.5 and passed through these cartridges. Organic acids and sugars were eluted with 20 mM sulfuric acid, while monomeric polyphenols were eluted with ethyl acetate. The phenolic fraction (18 compounds) was analyzed by reversed-phase liquid chromatography with diode array detection.

In 1990, solid phase microextraction (SPME) was proposed,[36] which is now widely used in chemical assays of various specimens including wines. The key advantage of the SPME method is that no solvent is used; the sampling, extraction, preconcentration and sample injection are performed in one stage; a very small sample volume is needed and the sample preparation time and cost are reduced.

Turbulent chromatography is a modern sample preparation method. This method is best suited for the samples containing low-molecular-mass impurities that interfere with determination of macromolecules. A turbulent chromatography column may be a part of a LC/MS system for online determination of flavonoids and reservatrol in wines.[37]

The group polyphenol determination requiring no sample preparation may be quite sufficient for the study of the relationship between the general antioxidant activity and the contents of particular classes of polyphenolic compounds in wine and the changes in these characteristics during storage.[38] Since the absence of sample preparation was among the requirements to the procedure of wine analysis after radiolysis in our works, of particular interest are publications devoted particularly to the direct sample injection into the chromatograph.[39–42]

2. Detectors for determination of flavonoids in wines

Flavonoids and other polyphenolic compounds in wines can be detected by means of UV detectors[43] and also by diode array,[44] fluorimetric[45] and electrochemical detectors.[40]

Zhang *et al.*[46] reported a simple, selective and sensitive method for determination of 20 phenolic compounds including quercetin, kaempferol and isorhamnetin in wines by HPLC with chemiluminescence detection. The method is based on acceleration of chemiluminecence reaction between cerium(IV) compounds and rhodamine 6G in the presence of phenolic compounds.

Carando *et al.*[42] determined procyanidin dimers (B1, B2, B3 and B4) and trimers (C1 and T2) in white wines by HPLC with a fluorimetric detector also without preliminary sample preparation.† It was reported that UV detection is suitable for the determination of these compounds only in red wines, whereas for analysis of white wines containing a lower amount of procyanidins it is insufficiently sensitive. However, the joint use of fluorimetric and spectrophotometric detectors[45] can increase the selectivity and sensitivity of determination of catechin, epicatechin, *trans*-reservatrol and vanillic and syringic acids in wine samples.

Tinttunen *et al.*[40] used reversed-phase HPLC with diode array and coulometric detectors without the sample preparation stage to determine a number of phenolic acids, catechin, epicatechin, *trans*-reservatrol, rutin, quercetin and cyanidin in wine samples. The diode array detector ensures a wider range of linearity for the compounds, it is more reliable and provides more reproducible results. The detection limits for almost all compounds to be quantified, except for rutin, are lower in the case of coulometric detection. To increase the sensitivity of a diode array detector, the samples can be concentrated *in vacuo* at a temperature below 40 °C to decrease their volume 2–2.5-fold.[47]

Alcalde-Eon *et al.*[48] identified for the first time the *cis*-isomers of delphinidin-, cyanidin- and petunidin-3-*p*-coumarylglycosides in red wine by HPLC-MS using a diode array detector and a electrospray ionization mass spectrometer with an ion trap type analyzer.

† Procyanidins B1–B4 are diastereomers with the C(4)–C(8) bond incorporating catechin and epicatechin residues; in types C and T trimers, flavanol moieties are also connected by the C(4)–C(8) bonds.

3. Sorbents for flavonoid determination in wines

Currently, there is a rather broad selection of C18 columns that provide appropriate separation of the compound mixture (for details, see Section III.5).

Recent years have seen enhanced interest in the use of monolithic stationary phases for the solution of various analytical problems by HPLC. The Chromolith HPLC columns provide good separation over much shorter periods of time than usual packed columns. They are made of porous monolithic quartz rods with bimodal pore structure (a combination of macropores and mesopores). Macropores ensure high flow rate of the mobile phase even at low pressure. Mesopores form a large homogeneous surface for absorption and high performance chromatographic separation. Repollés *et al.* [49] employed a monolithic-type reversed-phase C18 column (Chromolith Performance) to separate several flavonoids of wine, which required only 15 min owing to the enhanced hydrodynamic properties of the column. Meanwhile, it takes 30–60 min to achieve a similar result on a conventional packed column. A chromatographic system including two columns with parallel gradients was employed to separate natural antioxidants in beer and wine.[43] The separation was first on a C18 sorbent, phenyl silica gel or polyethylene glycol-modified silica gel and then on a monolithic-type C18 column. The selectivity of phenyl silica gel columns is based on π–π-electron interactions, whereas the selectivity of polyethylene glycol-modified silica gel is related to hydrogen bonding with polar adsorbate molecules and hydrophobic interactions, which are also involved in the former case. It was shown [50] that phenyl silica gel in combination with a Chromolith RP-18e monolithic column ensures better separation of flavonoids than polyethylene glycol-modified silica gel.

4. Mobile phases for determination of flavonoids in wines

Optimization of the mobile phase composition is an important stage in the development of a chromatographic procedure. Acetonitrile–water or methanol–water mixtures with the addition of a weak acid are used most often as solvents to determine phenolic compounds in beverages and food products, in particular, in wines.

If diode array detection is used, the initial concentration of the acid (phosphoric or acetic) should be 2% to 10% (pH in the range of 2–4). The final acid concentration may be lower. The use of phosphates or other additives as mobile phase components should be eliminated due to peak broadening and co-elution of some components. The test compounds often have a broad range of polarity; therefore, a gradient mode of elution or isocratic elution with several mobile phases is needed to determine them. If the mobile phase contains methanol, the methanol concentration is 5 vol.%–10 vol.% in the beginning of the gradient and 40 vol.%–100 vol.% at the end of the gradient.[51] In the case of acetonitrile, the elution is started at 0%–10% and is completed at 30%–90%. Czyzowska and Pogorzelski [52] used the isocratic mode to determine anthocyanins, flavan-3-ols, flavonols and phenolic acids in three brands of Bulgarian wine, while stilbene determination in the same brands of wine was performed using gradient elution.

In the case of the direct injection of a wine sample into a liquid chromatograph, enhanced requirements are imposed on the mobile phase composition. For solvents that may contain salts, the injection system becomes more complicated; in this case, even a three-dimensional gradient can be used. Revilla and Ryan [41] succeeded in separating 15 phenolic compounds present in wine, in particular, flavan-3-ols, anthocyanins and flavonol derivatives by means of reversed-phase chromatography with a diode array detector and using a binary concentration gradient of salt-free solvents and direct sample injection into the chromatograph.

If mass spectrometric detection and an acetonitrile–water mobile phase are used, formic acid is preferred over acetic or trifluoroacetic acid. The optimal concentration of formic acid is 0.1% for the negative ion mode and 0.5% for the positive ion mode. If methanol is a component of the eluent, 1% acetic acid provides better sensitivity.

5. Examples of determination of flavonoids in wines

Table 1 presents examples of HPLC determination of flavonoids in grapes and wine. It can be seen from the Table that most analyses take more than 1.5 h except for the

Table 1. Examples of determination of flavonoids in grapes and wine by gradient elution high performance liquid chromatography.

Sample	Column			Mobile phase		Number of identified compounds	Ref.
	type of sorbent	column size /mm	sorbent grain diameter /mm	eluent	elution time /min		
Red grapes of three varieties, white grapes of three varieties	LiChrospher 100 RP-18	250 × 3	5	$MeOH-AcOH-H_2O$ A: 10–2–88%, B: 90–2–8%	100	11	18
Red wines at different maturation stages	Kromasil 100-C18	200 × 4.6	5	A: HCO_2H-H_2O (2 : 98 v/v) B: $HCO_2H-MeCN-H_2O$ (2 : 80 : 18 v/v)	45	27	53
Muscat wines of Portugal	LiChrospher C18	250 × 4	5	A: 0.5% HCO_2H B: $HCO_2H-MeCN-H_2O$ (5 : 400 : 595 v/v)	90	25	54
Red wine of Portugal	C18 ODS	250 × 4.6	–	A: HCO_2H-H_2O (1 : 9 v/v) B: $HCO_2H-MeCN-H_2O$ (1 : 8 : 1 v/v)	100	8 oligomeric pyranoanthocyanins	55

Table 2. Content [a] of single phenolic compounds in red and white wine samples after a 1- and 3-year storage.[7]

Sample	Phenolic content /mg dm^{-3}							
	quercetin		(+)-catechin		caffeic acid		gallic acid	
	1 year	3 years	1 year	3 years	1 year	3 years	1 year	3 years
Cabernet Sauvignon No. 1, France	24.6	4.3	49.6	24.9	–	–	61.6	56.6
Cabernet Sauvignon No. 3, Chile	26.0	–	89.7	21.5	–	–	26.9	54.4
Merlot No. 1, France	38.3	7.7	–	73.8	–	–	47.9	40.7
Merlot No. 2, Chile	50.7	6.1	–	–	–	–	58.6	49.6
Chardonnay No. 1, France	–	–	4.0	29.7	8.0	19.3	–	–
Chardonnay No. 2, Chile	–	–	6.5	24.6	9.6	27.0	–	–

[a] A dash means 'below the detection limit'.

example reported by Guadalupe *et al.*,[53] who separated 27 compounds in only 45 min.

Thus, reversed-phase HPLC equipped with various types of detectors (most often, diode array and mass spectrometric) is suitable for determination of flavonoids in wines. The success of analysis mainly depends on the optimal selection of separation and detection conditions.

We performed chromatographic analysis of red wines Cabernet Sauvignon and Merlot and white wines Chardonnay produced in France and in Chile for the contents of single phenolic compounds before and after a three-year storage.[7] It can be seen from Table 2 that the sample of French Cabernet Sauvignon showed a decrease in the contents of identified phenolic compounds, especially quercetin and (+)-catechin, after the storage for three years. More pronounced changes were noted for the Chile Cabernet Sauvignon, namely, no quercetin was detected, the (+)-catechin content decreased by 76%; simultaneously the concentration of gallic acid doubled. In the Merlot samples, the content of gallic acid decreased to the same extent, namely, by 15%. As regards white wines, the concentrations of (+)-catechin and caffeic acid considerably increased in both samples.

IV. Assays of the antioxidant activity of wine

Currently, study of the antioxidant properties of wine, including the influence of particular components on the antioxidant activity, as well as the development of an objective, reliable and reproducible rapid assays of the overall antioxidant activities of wine-making products is a topical problem of the theory and practice of wine making. The topicality of the problem is also due to the fact that standardization of this characteristics for wine certification and the use of this characteristics as both an objective criterion of the beneficial effect of wine on the human health and an indicator of high quality of finished wine products is being extensively debated in the top wine manufacturing countries.

All existing assays of the antioxidant activities of wine-making products can be classified into two large groups: direct and indirect ones. Direct methods are those based on the detection of trapping of some free radicals generated into the medium; this detection can be accomplished by various methods (spectrophotometric, fluorimetric, electrochemical and other procedures are known). The most popular among these procedures are TEAC, ORAC, TRAP, FRAP (see below and Table 3). Indirect methods include the measurement of various physicochemical char-

Table 3. Signal-forming reactions and methods of detection in the determination of the antioxidant activity by the TEAC, FRAP, ORAC and TRAP assays.

Assay	Signal-forming reactions	Method of detection	Ref.
TEAC	Methmyoglobin + H_2O_2 ⟶ ferryl myoglobin + ABTS ⟶ $ABTS^{+\cdot}$ $\xrightarrow{\mathbf{AO}}$	spectrophotometric (decrease in the absorption of $ABTS^{+\cdot}$ at λ = 600 nm)	56, 57
FRAP	Fe^{III} – tripyridyltriazine $\xrightarrow{\mathbf{AO}}$ Fe^{II} – tripyridyltriazine	spectrophotometric (increase in the absorption at λ = 593 nm)	58
ORAC	R $\xrightarrow{O_2,\ 37\ ^\circ C}$ $RO_2^{\cdot}$ $\xrightarrow{\mathrm{OAC}+\mathbf{AO}}$	fluorimetric (decrease in the OAS fluorescence)	59
	or Fe^{2+} (Cu^{2+}) + H_2O_2 ⟶ $HO^{\cdot}$ $\xrightarrow{\mathrm{OAC}+\mathbf{AO}}$		
TRAP	R $\xrightarrow{O_2,\ 37\ ^\circ C}$ $RO_2^{\cdot}$ $\xrightarrow{\mathbf{AO}}$	electrochemical	60

Note. Examples of studies that used these methods are given; **AO** is antioxidant; R = AAPH (see text); OAC is the optically active substrate.

acteristics of wine related to the antioxidant activity. However, these characteristics are not always directly related to the antioxidant activity. For example, oxygen, being involved in the wine maturation process, affects both the content and the qualitative composition of phenolic compounds. Nevertheless, no direct correlation was revealed between the concentration of oxygen and the antioxidant activity of wine. Sulfur dioxide is widely used in wine making to stabilize fermentation, and subsequently it serves as an antimicrobial agent and antioxidant. In model solutions, its antioxidant activity is rather high; however, analysis of wine-making products did not reveal a correlation between the contents of free or total sulfur dioxide and antioxidant activity of wine.

1. Direct methods

When considering the direct methods for determining the antioxidant activity, it is noteworthy that currently only a method based on suppression of the formation of the $ABTS^{+\bullet}$ radical cation [2,2′-azinobis(3-ethylbenzothiazoline 6-sulfonate)] in the presence of antioxidants has been commercialized by the Randox company. This is the so-called Trolox equivalent antioxidant capacity (TEAC) assay. Note that this method has a high percentage of error and also takes a lot of time. By using their assay, the Randox company, together with the Environmental Testing Service of California (USA), investigated the correlation between the general antioxidant activity and the phenolic content of California and New York wines.[61] Alonso *et al.*[62] presented a new electrochemical assay of antioxidant activity, which is a modified Randox assay that rapidly and easily provides reliable results. It is based on measurement of the current quantity needed to generate the $ABTS^{+\bullet}$ radical cation in the presence and in the absence of the compound or sample with supposed antioxidant activity. With the electrochemical generation of the radical, there is no need to use other reagents, the reaction is more easily controlled, and no temperature control is required.

The ferric reducing/antioxidant power (FRAP) assay is based on the reduction of the Fe^{III} complex with tripyridyltriazine to the Fe^{II} complex, which is bright-blue coloured and shows an absorption band at $\lambda = 593$ nm. The amount of antioxidants is, in this case, proportional to the amount of the coloured compound formed. The range of suitable antioxidants is very narrow (ascorbic and uric acid, *etc.*).

The oxygen radical absorption capacity (ORAC) assay utilizes the ability of antioxidants to react with the peroxyl radical formed upon the thermal decomposition and reaction with molecular oxygen of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH). The reactive oxygen species can also be generated using the $Cu^{2+} - H_2O_2$ or $Fe^{2+} - H_2O_2$ system. For determination of the ability to trap oxygen radicals, fluorescence of the the substrate (B-phycoerythrin or fluorescein) arising upon its interaction with reactive oxygen species is recorded. If the test sample contains antioxidants, fluorescence declines with respect to the reference. The use of peroxyl or hydroxyl radicals as pro-oxidants and proteins as the substrates to be oxidized favourably distinguishes the ORAC assay from other methods, because the formation of exactly these radicals in a living body may cause various diseases.

The total radical trapping antioxidant parameter (TRAP) assay exploits the ability of antioxidants to react with the same peroxyl radical AAPH as is used in the ORAC assay. The reaction is monitored electrochemically using an oxygen electrode, which is a considerable drawback of the method.

A water-soluble derivative of vitamin E called Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is used as the reference in the TEAC, ORAC and TRAP methods.

The Roginsky assay based on suppression of lipid peroxidation by antioxidants is sensitive to the polyphenolic qualitative composition and, when dealing with phenolic dimers and polymers, it can give incorrect results that do not take into account the activities of all hydroxy groups.

2. Indirect methods

The total phenolic content is often estimated by the Folin–Ciocalteu method[63–65] after oxidation of the phenolic compounds with the Folin–Ciocalteu reagent consisting of a mixture of phosphotungstic and phosphomolybdic acids, which are thus reduced to a light-blue-coloured mixture of tungsten and molybdenum oxides. The absorbance of the solution at 755 nm after the reaction is proportional to the phenolic content.

The coulometric determination of the antioxidant activity of a wine material is based on sample titration with electrochemically generated bromine, resulting in oxidation of phenolic compounds.[66]

Höner *et al.*[67] reported an antioxidant activity assay based on the ability of antioxidants to trap free radicals in the Briggs–Rauscher oscillating reaction, which occurs with hydrogen peroxide, iodic acid, manganese(II) sulfate and starch. The pH of the reaction mixture is close to the pH of the human gastric juice (pH ~2), whereas the Randox assay and most other methods measure the antioxidant activity at the blood pH (pH 7.4). Thus the method based on the Briggs–Rauscher reaction, unlike other methods, is suitable for determining the antioxidant activity of wine in the stomach. In the same publication, it is reported that the wine antioxidant activity data determined from the Folin–Ciocalteu index, the Randox assay and the Briggs–Rauscher reaction assay are fairly well correlated with one another. This confirms the great role of phenolic compounds in free radical trapping.

The interest in using electrochemical methods for estimation of the antioxidant status of biologically active compounds has increased in recent years. This is due to high sensitivity and high speed of this group of methods and the lack of influence of the sample colour on the assay results. Furthermore, these methods are easily commercialized and used for mass rapid assays in industry. Among the electrochemical methods and devices, note voltammetry (constant-current voltammetry or cathodic voltammetry) with a mercury film working electrode implemented in a TA-2 voltammetric analyzer (Tekhnomet, Tomsk). Our works on antioxidant activity determination were based on a reported method.[68] The instrument Tsvet Yauza 01-AA (OJSC Research and Production Association Khimavtomatika, Moscow) is also suitable for implementation of the voltammetric technique based on the detection of the electric current arising upon oxidation of the test molecules on the working electrode surface at a definite potential. The following reaction takes place on the working electrode:

$$Fl-OH \longrightarrow Fl-O^{\bullet} + e^{-} + H^{+}$$

Fl is the flavonoid residue

The arising currents are very low, 10^{-6}–10^{-9} A. The signals are amplified and then converted by means of an analogue-to-digital converter into digital signals, which are displayed on the computer monitor. The working electrode is made of glassy carbon, which is most versatile for determination of polyphenolic compounds. The potential can vary from 0 to 2 V, and the ionization potentials of phenolic compounds are in the range of 100–1000 mV. The instrument is suitable for direct quantitative assays of the antioxidant activities of samples containing biologically active compounds. The amperometric detector can operate in three modes: constant-potential and pulse-potential modes and with potential sweep over the whole range. By varying the polarity and the magnitudes of applied potentials, it is possible to determine not only the overall antioxidant activity but also the activities of particular classes of biological compounds.

Mannino *et al.*[69] proposed an enzymatic assay of antioxidant activity of wine using an electrochemical detector. The assay is based on the ability of some enzymes, for example, laccase and tyrosinase, to catalyze the oxidation of a broad range of organic and inorganic compounds, in particular phenolic compounds. Enzymes provide quantitative oxidation of all or virtually all of the hydroxy groups of antioxidant wine components. The assay is characterized by the absence of enzyme inactivation during the reaction and higher stability in the presence of sample components.

V. High performance liquid chromatography in the investigation of the antioxidant and radioprotective activities of wine

As has already been noted in the Introduction, the role of HPLC in the study of the antioxidant activity of wines is determined by the following possibilities of the method:

— separation of a mixture of antioxidants including flavonoids followed by the on-line determination of the antioxidant activities of single compounds;[70, 71]

— determination of the total phenolic content;[38, 72]

— prediction of biological activities of wine antioxidants from their chromatographic characteristics;[73]

— chromatographic determination of the products of radiation-induced chemical transformations in wines under conditions of simulated oxidative stress.

Since HPLC is suitable for determination of the total phenolic content of wine, it can be regarded as an indirect method for determination of the antioxidant activity and an alternative to determination of the Folin–Ciocalteu indices. It should be borne in mind that the chromatographic technique has an obvious advantage over the Folin–Ciocalteu method, as it allows one to identify and quantify single phenolic compounds.

The instrumentation for liquid chromatography with two detectors, diode array spectrophotometric and chemiluminescence detectors, was utilized[70] to determine the antioxidant activity of rutin, quercetin and other phenolic compounds present in wine. The HPLC determination of the antioxidant activities of phenolic compounds (hydroxy acids, flavonoids, tocopherols) using a coulometric detector was reported.[64] It was shown that the lower the oxidation potential of the phenolic compound, the higher the antioxidant activity.

Tsao and Yang[72] presented a method for separation and quantitative determination of five main groups of natural phenolic compounds: simple phenolic acids (hydroxybenzoic and hydroxycinnamic acids), flavan-3-ols, flavonols, anthocyanins and dihydrochalcones. It is very important that the antioxidant activities found in this work are in good agreement with the Folin–Ciocalteu indices.

Mulero *et al.*[38] determined the overall content of several compounds in wine samples by HPLC with a diode array detector. The reference compounds were cyanidin-3-rutinoside (λ = 510 nm) for anthocyanins, rutin (λ = 360 nm) for flavonols, chlorogenic acid (λ = 320 nm) for hydroxycinnamic acid derivatives and *trans*-reservatrol (λ = 320 nm) for stilbenes. The following anthocyanins were detected: delphinidin-3-glycoside, cyanidin-3-glycoside, petunidin-3-glycoside, peonidin-3-glycoside, malvidin-3-glycoside, cyanidin-3-*p*-coumarylglycoside, petunidin-3-*p*-coumarylglycoside, malvidin-3-*p*-coumarylglycoside. The total phenolic content was calculated as the sum of the amounts of anthocyanins, flavonols and hydroxycinnamic acid derivatives. It was shown that the content of anthocyanins decreases during storage, whereas the contents of hydroxycinnamic acid derivatives, flavonols and stilbenes do not change significantly. It was found that stilbenes and flavonols are mainly responsible for the antioxidant properties of wines, as the best correlation between the compound contents and the antioxidant activity of wine samples was observed for these classes of compounds.

The antioxidant activity is a particular case of biological activity. Currently, a topical issue is predicting the biological activity from the known molecular structure.[73] One of the best methods for establishing the structure–property relationships for the compounds in question is chromatography, because chromatographic characteristics are rather easily determined in experiment and are well correlated with many physicochemical and structural parameters. By using various chromatographic procedures, detectors and columns, it is possible to reveal the types of intermolecular interactions determined by the differences in the structure of compounds and simulate biochromatographic processes that occur upon administration of physiologically active substances into a living body. In addition, the use of chromatographic parameters for the estimation of the biological activities of organic compounds is justified by the fact that in a chromatographic column, as in biological systems, the substance is partitioned between phases with different physicochemical properties. A sorption mechanism of interaction of a pharmaceutical agent or any biologically active compound is often implemented *in vivo*; therefore, it appears pertinent to study the sorption characteristics of these compounds by chromatography. The chromatographic retention values can serve to predict the biological activity of organic compounds. There are models that enable rather accurate and reliable calculation of toxicity parameters of compounds from their chromatographic characteristics.[73]

1. Radiation-induced chemical transformations of wine components

Since consumers have a negative attitude towards chemical additives (preservatives, stabilizers, *etc.*) the use of which in food industry is also undesirable from the hygienic standpoints, wineries use more and more often physical treatment for biological stabilization of wines and juices. These methods include heat and cooling (cryo) treatment,[74] irradiation (γ-, UV and IR), sonication and microwave treat-

ment. Electrodialysis was proposed for biological stabilization of unstable grape wines with residual sugar and low alcohol content and juices.[75] In addition, physicochemical treatment of grapes was performed in order to increase the phenolic, particularly flavonoid, content. For example, it was demonstrated[76] that electromagnetic field treatment of Cabernet Sauvignon grapes changes the content of phenolic compounds in the wine material. In experiments of Cantos *et al.*,[77] the Monastrell grape was UV-irradiated after being harvested in order to increase the contents of reservatrol and 3,4,3′,5′-tetrahydroxy-*trans*-stilbene, which have properties beneficial for humans. Both compounds refer to so-called phytoalexins[78] — special compounds synthesized by a plant in response to infection or stress, UV irradiation functioning as the latter. The reservatrol concentration in the grape was found to reach a maximum on the fourth day of irradiation; its content increased twofold with respect to the initial level. The contents of non-stilbene phenolic compounds did not change. Upon irradiation of other grape varieties, the reservatrol concentration may increase 10-fold or even 2000-fold. Reservatrol can exist in the *cis*- and *trans*-forms. It was reported[79] that on exposure to UV radiation, *trans*-reservatrol is converted to *cis*-reservatrol; irradiation conditions (duration of exposure, distance to the UV lamp and UV wavelength) should be thoroughly controlled, otherwise, by-products may be formed in concentrations depending on the initial concentration of *trans*-reservatrol.

Pulse radiolysis in combination with kinetic spectroscopy can be used to study the interaction of single biologically active compounds or classes of compounds (condensed or hydrolyzed tannins) contained in wines with free radicals ($HO^{\bullet}$, $N_3^{\bullet}$, $O_2^{-\bullet}$).[80] For both condensed and hydrolyzed tannins, a correlation was found between the rates of reactions of these compounds with the $HO^{\bullet}$ radical, which is here a measure of the antioxidant activity, and the number of neighbouring OH groups in their molecules.

Phenolic compounds in young wines exist as unstable conformations and on exposure to ionizing radiation, which causes oxidative destruction, they are converted to other, more reactive species — semiquinones, which can either condense to give polymeric compounds with a different antioxidant activity or regenerate to the initial compounds.[81,82] Apparently, among compounds with equal or similar degrees of conversion, those present in lower concentrations are more radiation-labile, because in this case, the probability of interaction with the reactive species (radical) is lower. During the maturation, the conformations of the phenolic compounds present in first-year wines pass to stable forms which are more resistant to radiolysis.

The major component of wine is water. Water radiolysis[83] yields equal amounts of hydroxyl radicals $HO^{\bullet}$ and hydrated electrons (e_{aq}^-), and also hydrogen peroxide (H_2O_2), hydrogen atoms ($H^{\bullet}$) and hydrogen molecules.

$$H_2O \rightsquigarrow HO^{\bullet}, H^{\bullet}, e_{aq}^-, H_2O_2, H_2$$

Radiolysis of ethanol gives rise to e_{aq}^-, $H^{\bullet}$ and carbon- and oxygen-centred radicals $CH_3{}^{\bullet}CHOH$ and $CH_3CH_2O^{\bullet}$.

These reactive species can react with dissolved molecular oxygen to give the superoxide radical anion ($O_2^{-\bullet}$) and peroxide radicals $HOO^{\bullet}$ and $CH_3CH(OH)OO^{\bullet}$.

$$e_{aq}^- + O_2 \longrightarrow O_2^{-\bullet}$$

$$H^{\bullet} + O_2 \longrightarrow HOO^{\bullet}$$

$$CH_3{}^{\bullet}CHOH + O_2 \longrightarrow CH_3CH(OH)OO^{\bullet}$$

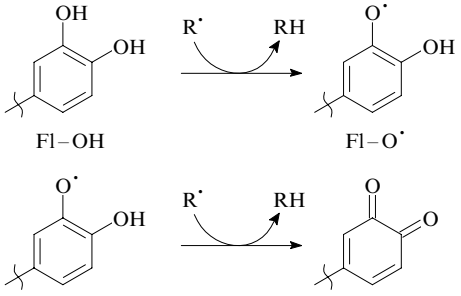
The polyphenol compounds (flavonoids) can reversibly react with molecular oxygen to give labile oxocharge transfer complexes. Hence, they can change the local concentration of oxygen in the cell and either protect readily oxidizable areas of the cell or, in the case of oxygen shortage, supply O_2. Oxidative destruction of flavonoids at room temperature is also possible. For example, oxidation of a water–ethanol solution of quercetin in a weakly basic medium gives 3,4-dihydroxybenzoic (protocatechuic) and 2,4,6-trihydroxybenzoic acids and the decarboxylation product of the latter — 1,3,5-trihydroxybenzene (phloroglucinol). The oxidative destruction with air oxygen is likely to be a typical process for all flavonoids but it is fully suppressed when the hydroxyl hydrogen at C(3) is replaced by the glycoside moiety, as in the rutin molecule.[84] The ease of oxidation of flavonoids is important for the formation of various conformations.

Ethanol, glycerol and tartaric acid are wine components with the second largest concentrations (after water), which are 2.0, 0.2 and 0.02 mmol litre^{-1}, respectively. Therefore, the probability of reactions of these compounds with free radicals formed upon water radiolysis is maximum. The reaction of ethanol molecules with $H^{\bullet}$ or $HO^{\bullet}$ furnishes carbon-centred hydroxyethyl radicals $CH_3{}^{\bullet}CHOH$. Due to the low concentration of flavonoids, the radiation-induced chemical transformations of these components occur mainly in the reactions with the above-mentioned reactive intermediates of radiolysis formed from the solvent (ethanol and water). Owing to the low redox potentials ($0.23 < E < 0.75$ V), flavonoids are able to reduce free radicals such as superoxide, peroxyl, alkoxyl and hydroxyl radicals the redox potentials of which are in the range from 1.0 to 2.13 V.[85] In these reactions, flavonoids act as hydrogen donors:

$$Fl{-}OH + R^{\bullet} \longrightarrow Fl{-}O^{\bullet} + RH$$

The aroxyl radical ($Fl{-}O^{\bullet}$) can react with a second radical, being converted to a stable quinone structure (Scheme 1).

Scheme 1



$R^{\bullet} = O_2^{-\bullet}, HOO^{\bullet}, HO^{\bullet}, EtO^{\bullet}$

In addition, it is known from the literature that radiolysis of aqueous and water–ethanol solutions of flavonoids yields labile 'red form' compounds,[86] which resemble anthocyanin pigments as regards optical characteristics and radiation resistance. The appearance of 'red forms' is

considered to be important in radiation genetics as they are used as markers of the radiation damage of plants.[87] Moreover, biologists lay emphasis on the detection of these compounds, which probably act as protective factors upon stress impacts on the plant cells.[88–90]

We used HPLC to identify the products of radiolysis of wine components,[91] of flavonoid and coumarin series, and to calculate the degrees of conversion of the starting compounds. Changes in the contents of single wine components were noticed by either increase or decrease in the chromatographic peak areas at $\lambda = 280$ and 334 nm. The identity of peaks before and after radiolysis was ascertained based on the retention times and optical absorption spectra. Some peaks completely disappear after radiolysis, *i.e.*, the corresponding components of wine undergo complete radiation-induced chemical transformation to give radiolysis products; some new peaks corresponding to the radiolysis products appear instead. Upon the radiolysis of flavonoids, a product was detected only for pinocembrin, which is eluted ahead of the major component, as can be seen from Fig. 8. Figure 9 presents the degrees of conversion of flavonoids. The highest degrees of conversion are noted for quercetin, morin, acacetin and apigenin. This may be related to stabilization of radicals due to bond conjugation in the structures. In addition, the following trends can be observed: quercetin and naringenin are more radiation-sensitive than their glycosides, rutin and naringin.

Since wines contain trace amounts of metal ions, in particular iron(II), the following reaction is possible:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\bullet} + HO^-$$

resulting in the formation of reactive hydroxyl radicals. On the one hand, flavonoids, especially quercetin, have pronounced chelating ability with respect to Fe^{2+} (structure **1**) and, hence, they inhibit peroxidation.[92]

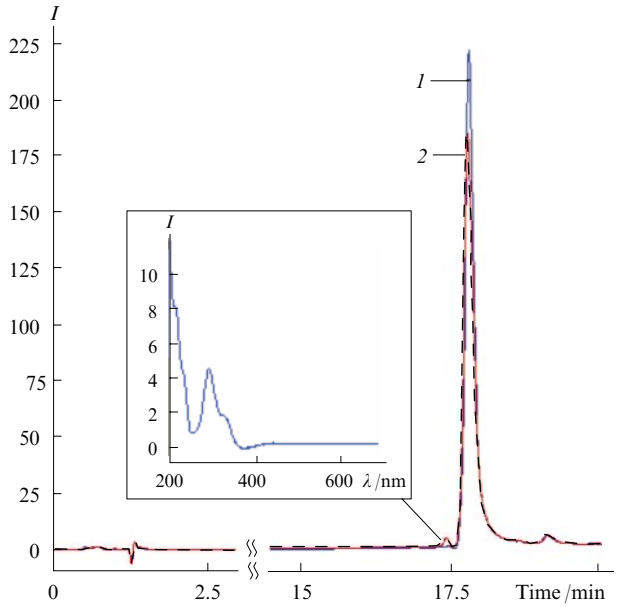


Figure 8. Chromatograms ($\lambda = 280$ nm) of a solution (10^{-3} mol litre^{-1}) of pinocembrin in 40% ethanol before (*1*) and after (*2*) irradiation.[7]
The inset shows the spectrum of the radiolysis product.

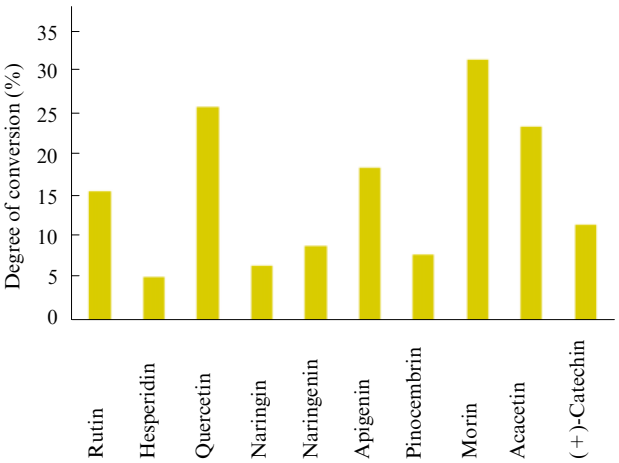
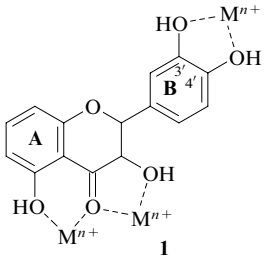


Figure 9. Degrees of conversion of flavonoids upon exposure to radiation in aerobic conditions.[7]
Irradiation dose 2.5 kGy.

Structure **1**

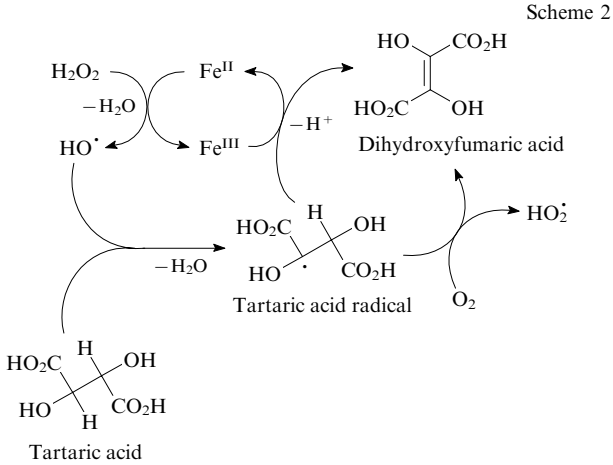


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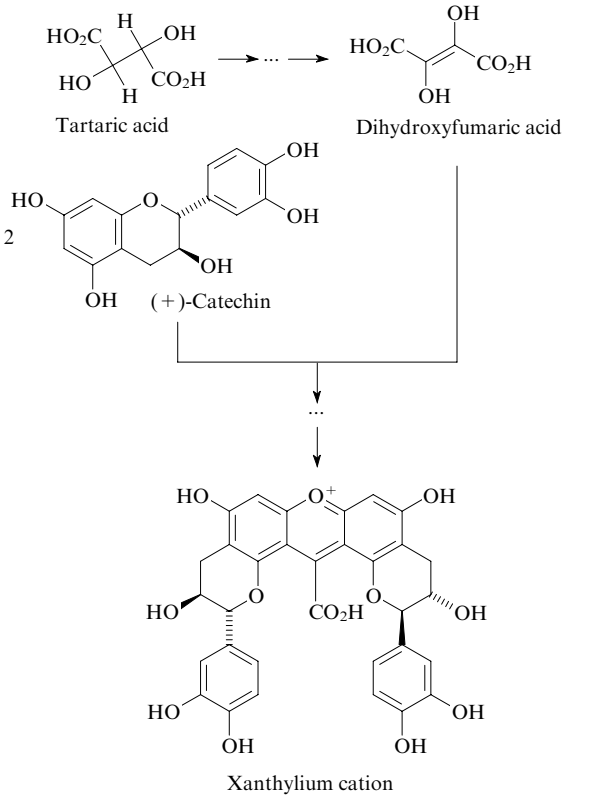
In addition, these compounds show reducing properties with respect to Fe^{3+} ions, thus accelerating peroxide decomposition and promoting the formation of hydroxyl radicals.[93]

γ-Irradiation of such a complex mixture as wine can induce not only the formation of new products from the starting compounds as they are attacked by free radicals but also reactions of the radiolysis products with wine components. It was demonstrated[94] that the reaction of tartaric acid with hydroxyl radicals yields dihydroxyfumaric acid,

Scheme 2



Scheme 3



which in turn reacts with the major flavonoid of wine — (+)-catechin (Schemes 2, 3). This produces the xanthylium cation — a yellow pigment, which was detected by liquid chromatography in combination with mass spectrometry.

2. Dependence of the antioxidant activity of wine on the composition

Considering published data and our results, one can state that the antioxidant activity of wine depends on the content of flavonoids, which have high antioxidant activities. The content of flavonoids depends on the grape variety, climatic conditions of grape vegetation, and the wine production process (maturation time and so on) and wine product storage conditions.[63] Currently, the relationship between the antioxidant activity and the chemical structure of phenolic compounds, including flavonoids, has not been ultimately clarified. However, it was found that the antioxidant activity increases in those cases where electron delocalization in the resulting aryloxyl radical and, hence, stabilization of the radical are possible. Thus, the antioxidant activity is enhanced due to the following factors:

— the presence of a double bond in positions 2, 3 of the heterocyclic moiety, because this enables electron delocalization between rings **A** and **B**;

— the presence of a hydroxy group in position 3 of the heterocycle;

— the presence of a hydroxy group in position 3′ of ring **B** and further increase in the number of hydroxy groups;

— transfer from glycosides to their aglycones (polyphenols).

According to these trends, the antioxidant activity of quercetin is higher than those of rutin or kaempferol but lower than that of myricetin. Flavanonols and flavanones are weak antioxidants in comparison with flavones or flavonols due to the lack of a double bond in position 2, 3.

Attempts to predict the antioxidant activity of wine based on its composition usually fail because single flavonoids present in the mixture can be in either antagonistic or synergistic relations.[95] In the former case, the antioxidant activity of a flavonoid mixture is lower than the sum of the antioxidant activities of single components, while in the latter case it is higher. The situation is complicated by the fact that synergistic or antagonistic behaviour depends also on the antioxidant activity assay used. Hidalgo *et al.*[95] determined the antioxidant activities of 11 single flavonoids (cyanidin-, malvidin-, delphinidin, peonidin- and pelargonidin-3-*O*-glycosides, catechin, epicatechin, kaempferol, myricetin, quercetin and quercetin-3-β-glycoside) and various flavonoid pairs using two methods. A study of the ability of these compounds to trap free radicals demonstrated that only kaempferol and myricetin form a synergistic pair. When the FRAP method was used, a mixture of epicatechin and quercetin-3-β-glycoside showed very pronounced synergistic effect, while a mixture of myricetin and quercetin showed an antagonistic effect. It was also reported[96] that the joint presence of quercetin, rutin and reservatrol, for example, in red wine enhances the antioxidant action of each of the components.

During storage of wine, the antioxidant activity with respect to free radicals gradually decreases, which is caused by oxidative destruction of polyphenolic compounds, depending on the material of the tank in which the wine was stored. It was shown by HPLC[16] that the loss of catechin, epicatechin, procyanidin B2, myricetin and monomeric anthocyanins is the lowest when this material is polyethylene terephthalate, which is able to absorb oxygen. The Folin–Ciocalteu index cannot serve as a measure of wine stability against oxidation, because this parameter changes slightly.

During ageing of red wines the contents of polymers formed by malvidin- and peonidin-3,5-diglycosides and their acyl derivatives increase. Noguer *et al.*[97] were able to isolate the polymeric fraction from wine matured for seven years by high-speed counter-current chromatography; they obtained the chromatographic profile of the fraction by HPLC and estimated the antioxidant activity by the FRAP and ORAC methods under conditions simulating the digestion in the stomach and intestines. It was found that this fraction makes the greatest contribution to the total antioxidant activity of the studied wines. Furthermore, the antioxidant activity of both polymers and low-molecular-mass phenols increases under digestion simulating conditions.

Netzel *et al.*[98] studied the effect of different wine production processes on the phenolic composition and on the antioxidant properties of red Spätburgunder (Pinot Noir) and Cabernet Franc: fermentation of grape skin, heating of the must or combination of these processes. The antioxidant activity was determined by the TEAC assay and the content of polyphenolic compounds was determined by reversed-phase HPLC using a C18 Prontosil-Eurobond column. Lower antioxidant concentrations were found in the wine prepared by must heating. The lowest content of anthocyanins was found for the wine prepared by fermentation of grape skin. The highest concentrations of anthocyanins, flavan-3-ols, flavonols and stilbenes were

determined in the red wine obtained by combining the two processes.

The phenolic composition of the Cabernet Sauvignon wines manufactured from one and the same variety of grapes but harvested in five different regions of China was determined by HPLC-MS.[99] The wines from grapes vegetating in the South-West of China were found to contain exceptionally high amounts of cyanidin and quercetin derivatives but a very low amount of epicatechin.

Fernández-Pachón *et al.*[100] determined the antioxidant activities of white, red and dessert wines using a number of methods including TEAC [the ability to suppress the formation of the ABTS radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD)] and ORAC. The antioxidant activities proved to be higher for red wines than for white or dessert wines at any determination method. The best correlations with respect to the total phenolic content were obtained for the ORAC methods. The authors also separated the phenolic compounds of red wines into three fractions (phenolic acids, flavan-3-ols and anthocyanins, flavanols) by solid phase extraction. The second fraction was most active in all cases. It is noteworthy that the ratios of the antioxidant activities *in vitro* and *in vivo* are also considerably different for red and white wines, the latter being inferior: only consumption of red wine results in a considerable increase in the antioxidant activity of blood plasma.[101]

3. Study of the radioprotective properties of wines and single polyphenols

We estimated the possibility of using *Saccharomyces cerevisiae* yeast cells (race Feodosiya 7) as a biological object for determining the radioprotective activity and the reactivating action ‡ of single phenolic compounds: rutin, hesperidin, quercetin, naringin, naringenin, apigenin, pinocembrin, morin, acacetin and (+)-catechin. The investigation results are summarized in Table 4. Under simulated oxidative stress conditions, all of these compounds showed radioprotective activity with respect to yeast cells, *i.e.*, they favoured preservation of the cell viability on exposure to γ-radiation. Conversely, no reactivating action was found for these compounds. The most pronounced radioprotective action was inherent in hesperidin, (+)-catechin and naringin, while naringenin, pinocembrin and acacetin were least active. As noted above, quercetin and naringenin are more radiation-labile than their corresponding glycosides, namely, rutin and naringin. One could expect that the radioprotective activity of the former two compounds would also be higher; however, the radioprotective activity of rutin is similar to that of quercetin, while the activity of naringin is much higher than the activity of naringenin.

Table 4. Radioprotective activity and reactivating effect of single flavonoids (%).

Compound	Radioprotective activity	Reactivating effect
Reference	100	100
Rutin	125	63
Hesperidin	182	65
Quercetin	127	78
Naringin	157	69
Naringenin	112	64
Apigenin	127	85
Pinocembrin	113	72
Morin	139	81
Acacetin	114	72
(+)-Catechin	169	88

‡ Reactivating ability is the influence of a compound on the pre-irradiated cell.

The radioprotective activity and the reactivating effect of coumarins were also studied.[102] Estimation of the radioprotective activities of coumarin and 4-hydroxycoumarin with respect to the *S. cerevisiae* yeast cells demonstrated the effect of the coumarin structure on the type of protective action in living systems: coumarin functions as a radioprotector, while 4-hydroxycoumarin does not show radioprotective activity but does have reactivating properties.

The obtained results convincingly demonstrate that the *S. cerevisiae* yeast cells can serve as a model system for estimation of the protective properties of flavonoids not belonging to yeast metabolites and allow the use of this method for establishing the radioprotective properties of wines.

Table 5 summarizes the results of determination of the antioxidant and radioprotective (with respect to the *S. cerevisiae* yeast) activities of wine and the total mass concentrations of phenolic compounds in these wines (determined from the Folin–Ciocalteu index).

It can be seen from Table 2 and 5 that of two wines of the same name, the wine with higher antioxidant activity has a higher content of quercetin, (+)-catechin and caffeic acid, *i.e.*, compounds that refer to the group of potent antioxidants. In the case of gallic acid, no such trend was identified. As regards the phenolic content, both red and white wines of the same name but produced in different regions slightly differed from one another. However, the phenolic content is on average 8 times higher in red wines than in white wines. For practically similar phenolic contents of wines of the same name, the antioxidant activities of the samples are different. Indeed, the antioxidant activity of

Table 5. Antioxidant (AOA) and radioprotective (RA) activity of wine samples and total phenolic contents of wines (ω).

Sample	ω /g litre^{-1}	AOA /mg litre^{-1}	RA (%)
Red dry table wines			
Cabernet Sauvignon No. 1, France	2.4	135.1	124
Cabernet Sauvignon No. 2, Chile	1.9	593.0	132
Cabernet Sauvignon No. 3, Chile	2.2	802.2	140
Merlot No. 1, France	1.8	111.1	101
Merlot No. 2, Chile	1.9	757.9	127
Merlot No. 3, Chile	2.0	517.7	135
White dry table wines			
Sauvignon Blanc No. 1, France	0.3	72.1	102
Sauvignon Blanc No. 2, Chile	0.2	162.6	103
Sauvignon Blanc No. 3, Chile	0.2	153.5	101
Chardonnay No. 1, France	0.3	87.9	105
Chardonnay No. 2, Chile	0.3	282.1	103
Chardonnay No. 3, Chile	0.2	137.6	102

red wines from Chile is five times higher and that of white wines is twice higher than those of French samples of the same varieties of wine. Thus, not only phenolic but also some non-phenolic compounds are responsible for the antioxidant behaviour of wines. Radioprotective properties were found for almost all samples of red wines. The sample Merlot No. 1 was an exception; the percentage of surviving yeast cells differed only slightly from the reference in this case. It should also be noted that the sample Cabernet Sauvignon No. 1, having low antioxidant activity, still showed high radioprotective activity on exposure to γ-radiation. The antioxidant activity of the Chilean white wine, Chardonnay No. 2, was more than twice as high as this value for the French Cabernet Sauvignon; however, like other white wines, this sample showed almost no radioprotective properties. It is known that storage of wines is accompanied by various oxidative condensation and polymerization reactions of phenolic compounds, which changes the biochemical composition of wine and affects their protective properties. According to a publication,[103] after three years of storage, red wines completely lose the radioprotective properties. Generally, the radioprotective activity was found only for red wine of the first year of storage. Although there is no clear-cut correlation between the radioprotective and antioxidant activities, wines with the lowest antioxidant activity have also the lowest radioprotective activity, whereas wines with the highest antioxidant activity have the highest radioprotective activity.

VI. The problem of detection of counterfeit wine products

Currently, the quality of cognac type products made in Russia and in the C.I.S countries or imported from other countries is being sharply deteriorated. Therefore, detection of counterfeiting is now a very topical issue.[104–113] The counterfeiting of cognacs is represented by a variety of frauds ranging from crude ones (the use of synthetic flavouring agents) to more subtle frauds such as mimicking the age and replacement of aged cognac spirits by younger ones.

The compounds present in the cognac spirits can be classified into compounds that got there during distillation and those formed during maturation in oak casks.[113] The latter group of compounds deserves particular attention. The oak wood, like other wood, contains lignin. It is contained in cell walls and intercellular space of plants and fastens together cellulose fibres. Together with hemicelluloses, lignin determines the mechanical strength of stems and stalks. In addition, lignin reduces cell wall permeability for water and nutrients. It is not an individual compound of a strictly definite chemical composition. The branched macromolecules of lignin are mainly composed of the substituted phenolic alcohol residues. The ethanolysis of lignin follows two main routes:

coniferyl alcohol → coniferaldehyde →
→ vanillin

sinapyl alcohol → sinapaldehyde →
→ syringaldehyde

The degree of hydrolysis along either of these routes is not always unambiguous.[114]

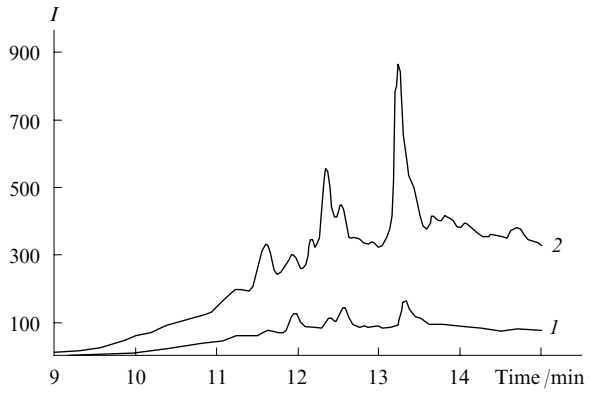


Figure 10. Typical chromatograms of counterfeit (*1*) and genuine (*2*) cognacs.[104]

The aromatic aldehydes thus formed are mainly responsible for the taste and the flavour of the finished product. In addition, dynamics of their accumulation during long maturation reflects the manufacturing process. According to available information, compounds formed along the former of the above ethanolysis routes predominate during the first years of maturation, while subsequently compounds of the latter route are mainly present. Hence, the ratio of compounds of these groups can to a certain extent reflect the age of some cognac spirits and cognacs. As the age of the cognac spirits increases, not only aromatic aldehydes are gradually accumulated but also aromatic acids and eugenol resulting from lignin destruction. Thus, method for discovery of counterfeiting should be based on integrated determination of aromatic aldehydes, the corresponding aromatic acids and precursors of aldehydes, *i.e.*, alcohols. The separation and quantitative determination of these phenolic compounds in cognacs is often done by chromatography.

Figure 10 presents typical chromatograms of counterfeit and genuine cognacs in the elution region of phenolic compounds contained in them.[115] It can be seen from the Figure that, first, the genuine cognac has a more diverse component composition and, second, it has higher contents of most components.

Table 6 presents the contents of vanillin and syringaldehyde determined in cognac samples by chromatography. It can be seen from the Table that cognacs Nos 1, 3 and 6 are crude frauds in which the syringaldehyde and vanillin molar ratio is 0.3–1.5, whereas in a genuine cognac, according to published data, it varies in the range of 2–4, *i.e.*, syringaldehyde considerably predominates. In other samples, this requirement is met; in the counterfeit cognac from French spirits (sample No. 8), this value reaches 2.9. In the genuine cognacs (samples Nos 11, 14, 15 and 17), the molar ratio of the syringaldehyde and vanillin concentrations is 2.2–3.4. In sample No. 5, these components were not detected at all, *i.e.*, the age is completely adulterated. It can also seen from the Table that except for the Russian counterfeit five-star cognac (sample No. 10) in which the syringaldehyde content was 3.19 mg litre^{-1}, the concentration of this phenolic compound in genuine cognacs is much higher ranging from 2.27 to 4.11 mg litre^{-1}. In high-quality samples, high contents of vanillin and coniferaldehyde are accompanied by a set of other components that are also formed upon

Table 6. Content of vanillin and syringaldehyde in cognac samples.[a]

No	Sample	Vanillin /mg litre^{-1}	Syring-aldehyde /mg litre^{-1}	R[b]
Adulterated cognacs				
1	Cognac A 5*	1.5	0.55	0.3
3	Cognac C 5*	1.30	0.79	0.5
6	Cognac E	0.50	0.88	1.5
8	Cognac from French spirits	0.43	1.46	2.9
9	Cognac D 5*	0.52	1.41	2.3
10	Cognac E 5*	1.27	3.19	2.1
Real cognacs				
11	Cognac F	0.86	2.27	2.2
14	Cognac (12 years)	0.66	2.62	3.4
15	Cognac (30 years)	1.31	4.11	2.7
17	Cognac I	0.78	2.53	2.8

[a] Altogether 17 samples were examined; in samples Nos 2, 4, 5, 6, 7, 12, 13 and 16, vanillin and syringaldehyde contents were below the detection limit. [b] R is the ratio of the molar concentrations of syringaldehyde and vanillin.

destruction of lignin and tannins. The presence of vanillin without the accompanying components may indicate that it was added to cognac or cognac spirit in a pure state or as a component of flavouring agents to impart better organoleptical properties to the product. In the framework of this study, it was impossible to draw conclusions about the origin of vanillin and syringaldehyde in the counterfeit samples where their contents and their ratio are high. In a genuine cognac, the increase in the age from 12 to 30 years was accompanied by a twofold increase in the vanillin content and a 1.6-fold increase in the syringaldehyde (samples Nos 14 and 15).

Apart from vanillin and syringaldehyde, all of the samples were found to contain gallic and syringic acids. Ellagic acid (a component of ellagotannins) was missing only in the counterfeit samples Nos 2, 4, 9 and 10. The balanced amount of this acid ensures full taste, while its excessive amount brings about bitterness and astringency. The sinapaldehyde and coniferaldehyde were not separated under the conditions used.

Counterfeiting can be revealed only by composing a data bank of cognac spirits from various manufacturers and for particular regions of manufacture and developing a particular qualimetric formula. The introduction of new regulatory documents (in addition to the existing ones), State Standards (GOSTs) that would regulate the quality control of the cognac products and cognac spirits is also necessary. They should be based on analytical procedures developed on the basis of modern techniques of instrumental quality evaluation and organoleptic examinations. In Russia, such integrated procedures that would be certified and implemented in laboratory practice are actually missing, only procedures for determining two or three polyphenolic components of the beverage by simple analytical methods being developed; these results are used to draw conclusions about the cognac age without taking into account some other very important characteristics.[116] Meanwhile, cognac is a multicomponent system in which more than 700 components have been identified to date,[117] and, hence, the quantitative determination of single compounds is quite complicated. Therefore, it would be of interest to develop a procedure suitable for rapid identification of the major part of fakes in a huge stream of test samples. A promising way to address this problem is to use amperometric measurements of the antioxidant activity, which characterizes the total content of phenolic compounds in the cognac being tested.[116] Apart from identification of the obvious counterfeiting cases, the antioxidant activity allows one to estimate to a certain degree the age of the genuine cognac. It should be admitted that implementation of such a procedure requires that a huge bulk of statistical data has been accumulated.

Table 7 presents the antioxidant activities of the same cognac samples. As can be seen, the antioxidant activities of genuine cognacs (samples Nos. 11, 13–17), except for sample No. 13 of three-year-old cognac, are much higher compared with counterfeit cognacs (samples Nos. 1–10). Apparently, this is caused by either dilution of cognacs by rectified ethanol or water–ethanol mixture or insufficient maturation period and, as a consequence, lower content of polyphenolic compounds. As the maturation period of a genuine cognac was increased from 3 to 12 years (samples Nos. 13, 14), *i.e.*, 4-fold, the antioxidant activity also increased 4-fold. On further increase in the maturation period of this cognac to 30 years (sample No. 15), the antioxidant activity increased by an additional 20%. The antioxidant activity of counterfeit five-star cognac (sam-

Table 7. Antioxidant activity (AOA) of cognac samples.

Sample	Adulterated cognacs	AOA /mg litre^{-1}	Sample	Real cognacs	AOA /mg litre^{-1}
1	Cognac A 5*	11.02	11	Cognac F	18.80
2	Cognac B	10.73	13	Cognac G (3 years)	10.95
3	Cognac C 3*	11.74	14	Cognac G (12 years)	44.04
4	Cognac D 3*	3.20	15	Cognac G (30 years)	53.32
5	Wine distillate	14.13	16	Cognac H	54.43
6	Cognac E	8.11	17	Cognac I	22.63
7	Cognac spirit	18.40			
9	Cognac D 5*	10.53			
10	Cognac E 5*	10.05			

ple No. 9) is three times higher than that of the three-star cognac (sample No. 4).

VII. Conclusion

The practical value of the natural polyphenolic compounds such as flavonoids and the like dictates the necessity to develop methods for their determination in complex mixtures of plant origin, in particular, in wines. This task is successfully solved by HPLC; however, the available information on the use of HPLC to analyze the products of radiation chemical transformations of wine flavonoids is scarce. It is necessary to study the stability of these phenolic components of wine upon stress impacts (γ-irradiation) in order to understand the mechanism of action of flavonoids in biological systems and estimate the antioxidant properties of wines.

The antioxidant properties of wines were estimated using the instrument Tsvet Yauza 01-AA (OJSC Research and Production Association Khimavtomatika, Moscow), which performs amperometric measurements. This instrument is suitable for determining the antioxidant activities not only for test samples but also for single phenolic compounds contained in them. The method is also promising for sample control and identification of counterfeit cognac products because it provides the total phenolic content of the tested cognac. The known value of antioxidant activity, apart from being used to reveal the clear counterfeits, can be used to estimate to some extent the age of the true cognac.

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