STRUCTURE OF CHEMICAL COMPOUNDS, METHODS OF ANALYSIS AND PROCESS CONTROL

NATURAL COUMARINS: METHODS OF ISOLATION AND ANALYSIS

A. V. Lozhkin[1] and E. I. Sakanyan[1]

Translated from Khimiko-Farmatsevticheskii Zhurnal, Vol. 40, No. 6, pp. 47 – 56, June, 2006.

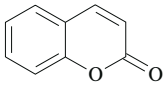
Original article submitted November 22, 2004.

With a view to optimizing of the quality control of sweetclover herbs and related preparations containing coumarins among the active substances, methods for the isolation and analysis of compounds of this class — in particular, coumarin and furocoumarin — are reviewed. The group of possible analytical methods includes gravimetric, titrimetric, photocolorimetric, and polarographic analysis, and the more recently developed spectroscopic (UV and IR), fluorimetric, and chromatographic (GC and HPLC) techniques.

Coumarins constitute a class of compounds representing 2H-1-benzopyran-2-one derivatives. These compounds are most frequently encountered in plants of the *Apiaceae* (Lindl.), *Rutaceae* (Juss.), *Fabaceae* (Lindl.), and *Hyppocastanaceae* (DC) families [1, 2]. Coumarins can significantly vary both in the site of concentration (fruits, underground organs, crust, leaves, stems) and in quantitative content (usually from 0.5 to 2 %, sometimes up to 5 – 6%) [3, 4].

Coumarin derivatives are can be conditionally subdivided into several groups.

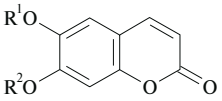
1. Unsubstituted coumarins:



coumarin

2. Hydroxy-, methoxy(alkoxy)-, and methylenedihydroxycoumarins and their glycosides, including

2.1. Compounds with hydroxy or alkoxy groups in the benzene ring:



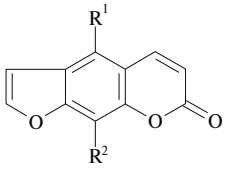
Umbelliferone: $R^1 = H$, $R^2 = OH$;
Esculetin: $R^1 = R^2 = OH$;
Scopoletin: $R^1 = OCH_3$, $R^2 = OH$;

2.2. Compounds with hydroxy or alkoxy groups in the pyrone ring (galfordin).

2.3. Hydroxy- and methoxycoumarins alkylated in the benzene or pyrone ring.

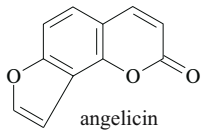
3. Furocoumarins, including

3.1. Psoralen derivatives:



Psoralen: $R^1 = H$, $R^2 = H$;
Xanthotoxin: $R^1 = H$, $R^2 = OCH_3$;
Bergapten: $R^1 = OCH_3$, $R^2 = H$;
Isopimpinellin: $R^1 = OCH_3$, $R^2 = OCH_3$.

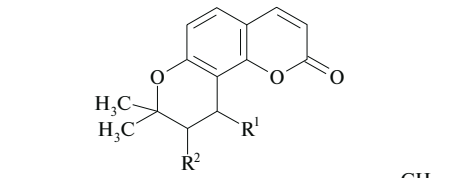
3.2. Angelicin derivatives:



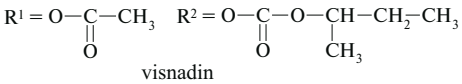
angelicin

4. Pyranocoumarins

[1] St. Petersburg State Chemico-Pharmaceutical Academy, St. Petersburg, Russia.

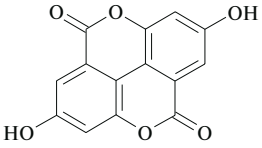


dihydrosamidin



visnadin

5. 3,4-Benzocoumarins



ellagic acid

6. Coumestans (coumestrol, etc.)

7. Compounds with more complicated structures involving the coumarin system (novobiocin, aflatoxin, etc.)

The physiological role of compounds belonging to this class is still not completely clear. It is known that coumarins are (i) involved in plant growth regulation (being antagonists of auxins), (ii) absorb UV radiation (thus protecting young plants from excess sunlight) [5], and (iii) protect plants against viral disorders [6].

One of the characteristic pharmacological properties of coumarin derivatives is anticoagulant action [7 – 10]; it is also known that coumarins produce coronary-dilative, β-blocking, and cholagogic action [11]. Many furocoumarins possess photosensitizing [3, 7] and spasmolytic activity. Some coumarins and furocoumarins were reported to produce bacteriostatic and antimytotic effects [1, 3, 7]. Coumestrol and related compounds show pronounced estrogen properties. Some synthetic and natural coumarin derivatives exhibit anti-HIV activity [12].

Coumarin proper (*cis*-*o*-hydroxycinnamic acid lactone) is widely used in the perfumery industry. As to pharmaceutical properties, this compound was reported to inhibit the development of lymphidema [13], renal carcinoma [14], and melanoma [15]. On the other hand, coumarin exhibited hepatotoxicity in experiments with rat liver and produced carcinogenic action upon chronic administration in high doses [16].

Coumarin derivatives can produce rather significant side effects. In high doses, they induce headache, nausea, womiting, sleepiness, and, in extreme cases, serious liver damage with hemorrhage as a result of hypoprothrombinemia [9, 17]. It is or recommended to administer drugs containing coumarin derivatives in patients whose professional activities are related to attention concentration. This circumstance implies the necessary standardization of plants containing coumarins with respect to the quantitative content of these biologically active substances. In accordance with the recommendations of WHO [18] and ICH [19, 20], it is necessary to validate methods used for the characterization of identity, purity, and quantitative content of active substances, which imply the availability of the corresponding reference samples.

The group of plants containing considerable amounts of coumarin includes yellow sweet clover (YSC) (*Melilotus officinalis* L. Pall.). Investigations performed at the St. Petersburg State Chemico-Pharmaceutical Academy showed that this plant possesses antihypoxant, antiischemic, and other kinds of cardiotropic activity [21 – 23], which stimulated the development of technology and standardization of the dry plant extract, tablets [24 – 26], ointments and suppositories [27] based on this extract, as well of the liquid extract and related preparations (flocramel) [28]. However, all methods proposed for the quality control of YSC preparations (UV spectrophotometry, HPLC, TLC) stipulate the use of a reference sample representing the class of coumarin derivatives [29, 30].

An analysis of the available normative documentation for the reference samples used in Russia for the quality control of the initial plant materials and phytopreparations showed the absence of direct reference samples for coumarin derivatives. The existing standards for furocoumarins such as psoralen, xanthotoxin, and phloverin (representing a combination of pyranocumarins dihydrosamidin and visnadin) are uacceptable for the evaluation of plant material containing *cis*-*o*-hydroxycinnamic acid lactones and substituted analogs.

In this context, it is of interest to review the possible methods of isolation, synthesis, and analysis of this class of natural substances with a view to the development of an adequate reference sample and its use for the quality control of drugs, parent substances, and preparations based on the YSC extract.

Coumarins typically appear as colorless or yellow crystalline substances, well soluble in organic solvents (chloroform, diethyl ether, ethyl alcohol), as well as in fats and fatty oils. Coumarin and its derivatives exhibit sublimation on heating to 100°C.

METHODS FOR ISOLATION OF COUMARINS

Coumarins are usually isolated from plants by extraction with solvents such as ethanol, methanol, benzene, chloroform, diethyl and petroleum ethers, or their combinations [31 – 33]. The most exhaustive extraction of coumarins (in the free form and as glycosides) is achieved with ethanol and its aqueous solutions, either in the cold or on heating. The total dense extract obtained after the evaporation of extractant is purified by treatment with chloroform and diethyl or petroleum ethers. Extraction with petroleum ether provides a good yield of furocoumarins, which can be isolated in crystalline form [3]. In some cases, the extracts were additionally purified using activated charcoal [34], boiling water (followed by

the condensation and separation of hydroxylated and methylated coumarins with chloroform [35], ethyl acetate and butanol [36]), or a chloroform – ethanol 97 : 3 mixture (for the separation of analogous derivatives [37]. Sometimes, the ethanol extract is used without additional treatments [32, 38 – 41].

For the isolation of the total 7-hydroxylated coumarins from the roots of *Helianthus annus* (L.), it was suggested to use sequential extraction with acetone and acetone – methanol (1 : 1) mixture, followed by the separation of pigments in a separatory funnel with a hexane – ether (6 : 4) mixture [42]. In some cases, the raw plant material is initially treated with petroleum ether and then exhaustively extracted with chloroform or methanol [3]. Peucedanin was successfully isolated by extraction with methanol in a Sohxlet apparatus [43]. An analogous procedure using successive extraction with *n*-hexane, dichloromethane, and methanol followed by preparative TLC was used for the isolation of six furocoumarin derivatives from *Angelica archangelica* (L.) [44]. The same extractants were used to obtain the total coumarins and furocoumarins from *Metrodorea flavida* [45]. Hydroxy- and alkoxycoumarins and their glycosides were isolated from *Aesculus hippocastanum* (L.) seeds by extraction with 80% aqueous ethanol followed by treatment with hot water, filtration, and multiple re-extraction with chloroform, ethyl acetate, and butanol [46]. Esculin and fraxin were isolated from chestnut crust by extraction with methanol [33]. Sequential hexane and methanol extraction of *Kielmeyera reticulata* (Saad.) sprouts yielded 4-phenylcoumarins and 4-*n*-propylcoumarins [47].

Isolated coumarins can be purified from accompanying substances by means of saponification, which was described in detail in [3, 48]. This method is based on the ability of the lactone (α-pyrone) ring to open under the action of alkalis with the formation of coumarinates (*o*-coumaric acid salts) and to close again upon subsequent acidification. A significant disadvantage of this method is the possible formation of secondary products and the dehydration and isomerization of some hydroxycoumarins.

Subsequent operations are usually aimed at the separation of the total coumarins and the isolation of individual compounds. Early investigations employed crystallization, fractional distillation, and sublimation in high vacuum [3]. However, since many coumarins possess close solubilities in organic solvents, even multiple recrystallization from solution did not provide reliable results. For this reason, subsequent progress in the chemistry of coumarins led to the development of various chromatographic techniques, which are free of disadvantages inherent in the early methods.

The first stage of partition is provided by column chromatography using various sorbents and solvent systems. In particular, the separation of hydroxy- and alkoxycoumarins substituted at the benzene cycle can be performed on a silica gel column sequentially eluted with hexane, hexane – chloroform mixture (with chloroform gradient), and chloroform – methanol (9 : 1, 8 : 2, 7 : 3) [35] or chloroform – ethanol (97 : 3) mixture [37]. The same task can be solved using an aluminum oxide column and ethyl acetate – benzene system (2 : 1) [36] or benzene [40] or a silica gel column and the following solvents: chloroform – benzene (1 : 1), chloroform, chloroform – ethanol (99 : 1, 98 : 2 and so on to 90 : 10), benzene – butanol (4 : 1, 3 : 1) [46]. The separation of 4-phenyl-, 4-*n*-propyl-, and 4-*n*-propylpyranocoumarins can be separated using a silica gel column eluted sequentially with hexane – ethyl acetate (gradient), methanol – water – dichloroethane, ad hexane – acetone systems [47].

Furocoumarins can be fractionated on an aluminum oxide column (activity degree III) eluted with petroleum ether, petroleum ether – chloroform (2 : 1), chloroform, and chloroform – ethanol (9 : 1, 4 : 1, 2 : 1) mixtures or on a silica gel column eluted sequentially with hexane – chloroform and chloroform – ethanol systems with increased proportion of a more hydrophilic component [38].

The presence of coumarins is indicated by the characteristic fluorescence of the corresponding sorbent bands under UV irradiation or by the positive response of characteristic chemical reactions of eluates.

The general laws governing the chromatographic behavior of coumarins are quite well established [3, 31, 49, 50]. According to one general rule, compounds with weak affinity to a sorbent should be isolated using active sorbents and weakly polar solvents, while strongly affine compounds should be isolated using low-activity sorbent layers and high-polarity solvents. In particular, coumarins containing phenolic or alcohol hydroxy groups are better adsorbed on aluminum oxide and eluted with large volumes of polar solvents (e.g., ethanol), sometimes with 0.5% acetic or hydrochloric acid. Methylated derivatives and pyranocoumarins are more weakly retained on sorbents. In the course of elution, it is expedient to gradually replace the hydrophobic solvent by a hydrophilic one [3, 51].

It was suggested to separate coumarins (including their mixtures with other low-molecular-weight biologically active substances) and to purify and analyze the components using column chromatography with affinity-type sorbents involving phenolic and polyphenolic ligands. In particular, it is possible to use epoxy-activated sorbents based on the HW-35 matrix containing resorcinol and catechol ligands. The possible mobile phases include water, aqueous ethanol solutions, and aqueous solutions of mineral acids, neutral salts, and their combinations. It was demonstrated that the results obtained with such chromatographic systems significantly exceed those provided by the classical silica gel, polyamide, and dextran sorbents [52].

The efficiency of separation provided by column chromatography is checked by thin-layer chromatography (TLC) and, less frequently, by paper chromatography. These methods show homogeneity of the isolated substances and reveal even trace amounts of coumarins. TLC is typically performed on Silufol or Sorbfil plates [35, 53] and sometimes on aluminum oxide (activity degree II) [41] and silica gel [46, 55 – 57] layers, which are eluted with the following sol-

vent systems: benzene – acetone (1 : 2); benzene – methanol – acetone (8 : 2 : 10); hexane – chloroform, toluene, and *n*-butanol (for hydroxy- and methoxycoumarins) [35, 46], ethanol – chloroform (5.5 : 4.5); chloroform – formamide (for scopoletin) [36, 54]; ethyl acetate – benzene (1 : 2); diethyl and petroleum ethers (for furocoumarins) [54], and others.

Aluminum oxide is commonly considered the best sorbent for TLC separation of coumarins, the best eluents being petroleum ether – ethyl acetate (2 : 1), petroleum ether – chloroform, cyclohexane – ethyl acetate (3 : 1), benzene – ethyl acetate (in various proportions), and pure benzene.

Paper chromatography can be performed on various available media [24, 38, 58, 59]. It should be noted that, in view of the selective solubility of coumarins in aqueous and nonpolar solvents, the paper is usually impregnated with a 20% aqueous solution of ethylene glycol or propylene glycol, formamide (of DMF) in methanol or acetone, or sodium borate and phosphate solutions. If the hydroxy-containing coumarins stay at the start, the paper is impregnated with some other solvent [3] or chromatographed on a nonimpregnated paper [60].

The spots of coumarins on thin-layer and paper chromatograms are usually revealed by UV fluorescence at certain characteristic wavelengths, before or after the treatment with an aqueous-ethanol solutions of potassium hydroxide or with ammonia vapor, or using some other color reactions. The fluorescent color does not provide accurate identification of the structure of coumarins; nevertheless, sometimes it is possible to determine the type of functional groups [3, 57, 59].

Individual compounds can be separated using preparative TLC techniques. The identity of isolated compounds is established using melting points and IR, NMR [44, 61], and mass spectra [51, 62]. The IR spectra of coumarins exhibit characteristic absorption bands at 1750 – 1700 cm^{-1} (–C=O groups) and 1620 – 1470 cm^{-1} (–C=C– groups of aromatic rings) [3, 62 – 64]. The crystalline structure of synthetic compounds can be determined by conventional x-ray analysis (e.g., with the aid of SHELXL97 software [65]). Coumarins with side chains containing asymmetric carbon atoms [47, 51], chiral carbons in the furan cycle of a substituted dihydroangelicin, and the pyran cycle of substituted khellactone can be identified using specific optical rotary power [44].

The structures of coumarins isolated from plants can be confirmed by methods of direct synthesis, such as Perkin condensation (from salicylaldehyde and acetic anhydride), Pechmann reaction (from phenol and maleic acid or β-ketoester), Knoevenagel reaction (condensation of *o*-hydroxybenzaldehydes with esters of malonic and other acids), and other techniques described in [3].

METHODS OF ANALYSIS OF COUMARINS

The titrimetric method of quantitative determination of coumarin derivatives, which is now rarely used, is based on the ability of a lactone (α-pyrone) ring to open under the action of alkalis [66]. The excess alkali is titrated with hydrochloric or sulfuric acid. This procedure gave understated results, and a modified variant was developed in which mercury oxide prevented premature closure of the lactone ring [67]. An important advantage of the titrimetric method is that it does not require the use of reference samples of coumarins, whereas disadvantages are the low specificity of this procedure and the toxicity of mercury compounds. Nevertheless, titrimetry was included into the normative documentation for psoralen and related preparations [60].

The gravimetric method described in [34, 66] represents a variant of the lactone probe. Despite the advantage of a high accuracy of determination (comparable with that of spectrophotometric analysis) [24], this technique did not find use due to the rather laborious and time-consuming procedure.

It was also reported on the possible use of permanganometric titration for the quantitative determination of coumarin [66], but this technique does not provide sufficient accuracy of results.

The acid properties of some natural coumarins were studied by potentiometric titration in aqueous solutions and in some nonaqueous solvents. The known influence of the coumarin structure on the character of potentiometric curves can be used to establish the structure of unknown compounds, in addition to the information provided by the absorption spectra [68].

Calorimetric methods of the quantitative determination of coumarins are based on their ability to form stable colored (from a light brown to cherry tint) compounds with diazonium salts [3, 32, 60]. This method was successfully used for the determination of coumarins isolated from *Heracleum sp*. bergapten in *Pastinaca sativa* (L.) fruits, psoralen in parent substance and preparations [60], atamantin in *Peucedanum oreoselinum* (L. Moench.) roots, and xanthotoxin and bergapten in *Ammi majus* (L.) fruits and ammifurin preparation [3]. It was reported that a photocolorimetric technique was used for the determination of a synthetic anticoagulant sincoumar [69]. However, it should be noted that azoaddition is not a specific reaction for coumarins and their derivatives (analogous effects are inherent in phenols, aromatic amines, and flavonoids). Therefore, thorough purification from interfering substances must precede the photocolorimetric measurements. It is also necessary to take into account that the optical absorption spectra of diazo derivatives of coumarins exhibit differences related to features of the structure of the particular initial compound [70].

The spectrophotometric method is based on the ability of coumarins to absorb in the UV spectral range. It was established that the main absorption bands in the spectra of

coumarins and furocoumarins are related to the π electron transitions from bonding to antibonding molecular orbitals [48]. The presence of several characteristic high-intensity bands in the 220 – 350 nm interval makes possible the use of UV spectrophotometry for the quantitative determination of coumarins. The characteristic absorption peak positions were reported, in particular, in [3, 62 – 64, 69, 71].

The UV spectrophotometry is the most promising method for the group analysis of coumarins, since the analytical procedure is simple and the components can be determined using differences in their absorption spectra (without preliminary separation). However, this method requires the use of reference samples in order to provide more accurate determination of the target components as compared to the case of using calibration plots and specific absorption coefficients.

The results of UV spectrophotometric measurements showed that the absorption spectra of synthetic anticoagulants (dicoumarin, phenpromaron, and sincoumar) exhibit two characteristic bands, at 280 and 306 nm. The former band has a vibrational nature, while the latter corresponds to *p*-π conjugation of the benzene and pyrone cycles [72].

A special method based on direct spectrophotometry at 352 nm with the use of a working reference sample (WRS) of xanthotoxin was developed for the quality control of ammifurin. The accuracy of determination was increased by the joint use of TLC and spectrophotometry. The TLC can be performed on KSK_{254} 5/40 silica gel eluted with a petroleum ether – ethyl acetate (1 : 1) system. Then, the bands are cut out, the compounds are eluted with ethyl alcohol, and the optical density of eluate is determined at 352 nm [32, 73]. An analogous procedure can employ chromatography on a paper impregnated with a methanol solution of formamide and eluted with a heptane – benzene (4 : 1) system.

Quantitative determination of peucedanin in plant materials and preparations is possible using TLC on a plate with nonbonded silica gel eluted with a 1 : 2 mixture of petroleum and diethyl ethers [43]. Then, the bands are eluted with ethanol, the optical densities of these solutions are measured at 298 nm, and the specific absorption coefficient is determined and used to calculate the drug content. In this case, a significant advantage of the UV spectrophotometry is the possibility to determine the presence of oreozoline (impurity), which absorbs at 345 nm [74]. The quantitative determination of pterixin in *Libanotis densiflora* and in the related preparation (libaverin) can be performed using the following procedure: TLC on KSK_{254} silica gel in *n*-hexane – benzene – methanol (5 : 4 : 1) system; band elution with ethanol; optical density measurements at 322 nm. The drug content is calculated using the specific absorption coefficient of pterixin.

Analogous chromatospectrophotometric methods were developed for the quantitative determination of psoralen and total furocoumarins in *Psoralea drupacea* (Bge.) with the use of WRSs of psoralen and angelicin. Measurements of the specific absorption coefficient of xanthotoxin can be used for the spectrophotometric evaluation of the quality of *Pastinaca sativa* (L.) fruits [32].

The content of psoralen and bergapten in the drug furalen can be quantitatively determined without preliminary separation of the total coumarins, using the optical density measurements at 297.5 nm [43] or at two wavelengths [75]. The latter principle was used for the analysis of fig tree *Ficus carica* (L.) leaves and parent substance of psoberan and related preparations [76]. This method is based on a modified chromatospectrophotometric procedure, whereby the total furocoumarins are determined from the optical density at 298 nm, the content of psoralen is determined from the measurements at 246 and 268 nm corresponding to the maximum difference in the absorption intensity, and the content of bergapten is calculated as the difference. This procedure employs the WRS of psoralen.

The UV spectrophotometric measurements can be used for determining the molecular weights of coumarins and furocoumarins [75, 77]. Using the UV spectra, it is possible to identify coumarins isolated from plants [44, 48] and to study their physiological functions [78]. The UV absorption measurements were also used for evaluation of the bioavailability of dicoumaron [79].

The polarographic method was used for the analysis of ready-to-use preparations containing furocoumarins. This technique is based on the reduction of the α-pyrone ring (at the double bond in position 3,4) on the mercury dropping electrode [80]. The results of investigations of the physicochemical properties of natural coumarins were used for the development of methods of qualitative, quantitative, and functional analysis included in pharmacopoeial articles for the parent substances of khellin, psoralen, visdnadin, pastinacin, some raw plant materials, and ready-to-use preparations. In particular, the quality of *Pastinaca sativa* (L.) fruits can be monitored using polarographic and chromatopolarographic methods employing the WRS of xanthotoxin [32]. A combination of polarography and chromatography was also used to study the chemical composition of the coumarin fraction in some plants and natural products [81]. A polarographic method was used for the quantitative determination of coumarin and cinnamic acid used as stabilizers in a PBS injection solution [82]. An advantage of polarographic methods is the possibility of rapid and reliable determination of total coumarins without their preliminary isolation. However, the high sensitivity of this technique to various electrochemically active impurities makes necessary thorough preparation of sample solutions. Unfortunately, at present, polarographic methods are practically not used for the determination of coumarins.

Fluorescent analysis can be used for the determination of coumarins and furocoumarins because many of them exhibit characteristic (yellow, green, blue, or violet) fluorescence in the visible and UV spectral regions in neutral alcohol solutions, in alkali solutions, and in concentrated sulfuric acid under UV excitation. The fluorescence is less pronounced in acid medium but is enhanced due to the formation of a

quinoid structure in the alkali medium. It was suggested to identify coumarins in phytochemical preparations using the Stokes shift and the position of the fluorescence peak [83]. Fedorin [84, 85] measured the fluorescence spectra of coumarins and furocoumarins and established a linear relation between the peak intensity and concentration, which made possible the use of fluorodensitometry for the quantitative determination of furocoumarins (psoralen and angelicin) in ready-to-use preparations of psoralen. The formation of fluorescent compounds by components of the ammifurin, beroxan, and pastinacin preparations in a bromine-saturated alkali solution was employed for their identification using fluorescence excitation (380 nm) and emission (480 nm) peaks [86].

A fluorimetric method was developed for the quantitative analysis of furocoumarins in *Pastinaca sativa* (L.) fruits using the excitation at 350 nm and the emission at 470 nm. An analogous method was proposed for determining khellin, a structurally close coumarin analog, in *Ammi visnaga* (L. Lam.) fruits [32].

A selective fluorimetric procedure was proposed for the quantitative determination of neodicoumarin via sequential treatment of the sample solution in DMF by ammonia solution and saturated magnesium chloride solution in the same solvent [87].

The fluorescence of coumarin derivatives depends on the concentration of hydrogen ions. For this reason, knowledge of changes in the color and intensity of fluorescence at various pH for coumarins with different structures is necessary for reliable use of the fluorimetric techniques. Data on the character of fluorescence for 98 coumarin derivatives were reported by Goodwin and Cavanaugh [88]. However, quantitative fluorimetric analysis did not find application because the linear dependence of the fluorescence intensity on the concentration of coumarins is not always clearly manifested.

It was reported that 7-(2-bromoethoxy)coumarin can be used as a fluorescent label for the quantitative fluorimetric analysis of drugs with molecules containing tertiary nitrogen atom [89]. Fluorescent microscopy can be used for the localization and quantitative determination of hydroxylated coumarin derivatives playing the role of photoprotectors in *Dasycladis vermicularis* (Scopoli) Krasser algae [5]. Another interesting fluorimetric method proposed for the rapid analysis of coumarin in fuels is based on the reaction of alkaline hydrolysis with formation of *o*-coumarinate ions and their subsequent conversion into *o*-coumarate ions exhibiting intense fluorescence [90].

Paper chromatography is most frequently used in combination with other physicochemical analytical techniques. Fore example, separation of total coumarins by paper chromatography (petroleum ether; DMF in acetone) followed by polarographic determination was used for the analysis of coumarins in *Pastinaca sativa* (L.) fruits and the drug beroxan [32]. A combination of paper chromatography with photocolorimetric measurements based on the reaction with dinitrided sulfanilic acid was used for the analysis of furocoumarins in *Psoralea drupacea* (Bge.) [91], psoralen and bergapten in *Ficus carica* (L.) leaves [92], and psoralen in some umbellate species. A significant disadvantage of paper chromatography is the time-consuming procedure and the need for concentrating extracts because of the low sorption capacity of this chromatographic medium [60].

Thin-layer chromatography is free of many disadvantages inherent in paper chromatography. TLC provides a quite rapid separation of components in a sample mixture and, similar to paper chromatography, can be used for the identification of coumarins in various samples. Joint TLC – colorimetric methods based on the azo-addition reaction with TLC separation on an aluminum oxide layer eluted in the hexane – benzene – methanol (5 : 4 : 1) system were developed for the quantitative determination of peucedanin in *Peucedanum morrissonii* (Bess.) [93] and for the analysis of beroxan, pastinacin, and psoralen preparations [31]. Colorimetric determination of xanthotoxin, imperatorin, and bergapten in *Ammi majus* (L.) fruits can be performed after TLC separation on silica gel impregnated with formamide and eluted in dibutyl ether. In order to determine psoralen alone and together with bergapten in *Ficus carica* (L.) leaves, the extract was purified from ballast substances and chromatographed in a thin layer of aluminum oxide in diethyl ether, after which the bands were analyzed by UV spectrophotometry [60]. Two-dimensional TLC on silica gel eluted in hexane – carbon tetrachloride – *tert*-butylamine (180 : 12 : 9) and hexane – toluene – ethyl acetate – acetic acid (100 : 10 : 10 : 0.5) in combination with spectrophotometry can be used for the determination of bergapten.

In the recent decade, tasks related to the isolation of coumarins and the quality control of related preparations were most frequently solved using GC and HPLC techniques.

Gas chromatography (GC) was predominantly used for the identification and quantitative analysis of furocoumarins in preparations and raw plant materials. Investigations of the chromatographic behavior (retention times) of substituted furocoumarins revealed the following general laws: (i) on passage from hydroxy- to methoxycoumarins, the retention time decreases (because of reduced adsorption via hydrogen bonds); (ii) furocoumarins with O-alkyl substituents at C_5 are eluted after 8-hydroxy isomers; (iii) the logarithm of the relative retention time is a linear function of the molecular weight [73]. These GC data can be used for determining the structure and estimating the retention time of analogous coumarins [94]. GC was successfully used for determining psoberan in parent substances [95].

A procedure developed for the analysis of coumarins in *Phlojodicarpus sibiricus* roots and the drug phloverin employs extraction with chloroform and GC measurements using an absolute calibration technique in comparison to the WRS of phloverin. It was found that dihydrosamidin and visnadin are eluted in a common symmetric peak. Their separate determination is possible upon the conversion of salts (formed in the course of alkaline hydrolysis) into free acids,

which are extracted with ether and measured by GC [32]. An analogous method was used for the analysis of *Ammi majus* (L.) fruits and ammifurin preparation, according to which the samples are extracted with ethyl alcohol and measured by GC in comparison to the WRS of xanthotoxin [73]. GC was also successfully used for the detection of coumarin and its metabolites in the liver of experimental animals as well as in the human liver [96].

High-performance liquid chromatography (HPLC) is now most widely used for the analysis of coumarin and furocoumarin derivatives. Opletal et al. [97] described the simultaneous determination of coumarins (scopoletin, daphnoretin, umbelliferon) and cardiotonic glycosides in *Coronilla varia* (L.) seeds. An analogous HPLC procedure was used to study the effect of methoxsalen (xanthotoxin) on the metabolism of nicotine [98].

Biavatti et al. [41] studied the dependence of the content of coumarins in *Mikania laevigata* on the conditions of collection, method of extraction, and the type of extractant. HPLC in the isocratic mode was used for the separation (without preliminary purification) and simultaneous qualitative and quantitative determination of coumarins in the crust of *Aesculus hippocastanum* (L.) [33] and for the quantitative determination of coumarin in the leaves of *Mikania glomerata* (Spreng) [57]. HPLC in the gradient mode with a fluorescent detector was used for the separation of coumarins in the study of their biochemical functions in the roots of sunflower (*Helianthus annuus* L.) [42].

A very promising method is offered by the combination of HPLC with mass spectrometry (MS). The HPLC/MS techniques were developed for determining the indirect anticoagulants [99] and rhodenticides [100] of the coumarin series (4-hydroxycoumarin derivatives) in blood plasma in the course of toxicological analysis. Another interesting example is the use of chromatomass spectrometry for detecting coumarins in tobacco products [101].

METHODS FOR THE ANALYSIS OF COUMARIN DERIVATIVES STIPULATED BY FOREIGN PHARMACOPOEIAS

A review of several foreign pharmacopoeias, including the U.S. Pharmacopoeia (23rd Edition), European Pharmacopoeia (3rd Edition, Suppl. 2001), and British Pharmacopoeia (16th Edition, 1998) showed that the number of coumarin-based preparations is not large.

USP-XXIII includes articles on two furocoumarins (parent substances of methoxsalen and trioxsalen), warfarin sodium (4-hydroxycoumarin derivative), related ready-to-use medicinal forms, and novobiocin (an antibiotic standardized by biological methods). All parent substances are represented by reference samples.

The identity of preparations is verified using IR and UV spectroscopy, HPLC, TLC (with reference samples), melting points, and characteristic sodium ion reaction. The quantitative determination is performed by HPLC. The dissolution test for capsules and methoxalen solution is performed using UV spectrophotometry at 252 nm [102].

BP-98 [56] includes articles on fig (*Ficus carica*) fruits, which contain various substituted furocoumarins, and on three parent substances of synthetic coumarin derivatives (acenocoumarol (nicoumalone), warfarin sodium, and warfarin sodium clathrate). The identification of acenocoumarol (parent substance and tablets) is performed using IR spectroscopy in comparison to the reference sample. Tablets are quantitatively analyzed using UV spectrophotometry in combination with dinitriding and azo-addition reactions. The tests for related compounds is performed by TLC. The parent substance is determined using an alkalimetric technique (alternatively, spectrophotometry at 306 nm). The quality of *Ficus carica* fruits is determined by the content of water-soluble extractable substances.

The quality of warfarin sodium according to BP-98 is checked using the same method as that stipulated by the European Pharmacopoeia [103], which also contains a particular article on coumarin as a reactant (Coumarin R).

METHODS FOR COUMARIN DETERMINATION IN YELLOW SWEET CLOVER

We suggest isolating the total coumarins from YSC grass (*Melilotus officinalis* L. Pall.) by extraction with aqueous ethanol solutions of various concentrations, followed by purification with chloroform. The qualitative composition of coumarins was determined using various chromatographic techniques (paper, TLC, HPLC), which showed that YSC grass contains no less that six compounds belonging to the class of coumarins, including 2H-1-benzopyran-2-one, umbelliferon, and scopoletin. The analysis of a dry extract revealed four coumarins. Unsubstituted coumarin predominates both in medicinal forms and in the dry YSC extract [29, 104].

The qualitative analysis of coumarin derivatives contained in the dry YSC extract and related preparations (melilotin tablets, ointments, flokramel) can be performed using both chemical techniques and physicochemical methods.

Chemical methods for the identification of coumarins in YSC grass include the conventional azo-addition reaction with diazo component, lactone probe [4], and some other color reactions. These reactions can be used either jointly with paper chromatography and TLC, or separately — by direct interaction with the extract.

The qualitative determination of total coumarins is expediently performed by TLC on Sorbfil plates eluted in a benzene – ethyl acetate (1 : 2) system, followed by the treatment of the plate with an ethanolic alkali solution, and the bands are identified using mobilities (R_f) and the characteristic yellow color. However, as was noted above, the reaction with diazo compounds is not specific of coumarins and the R_f val-

ues are not always reproduced. For this reason, it is expedient to perform TLC in comparison to the WRSs of predominating compounds.

In the case of UV spectrophotometric analysis, coumarins are identified by the presence of absorption bands at certain specified wavelengths. HPLC analysis is based on the determination of retention parameters. Using these techniques, methods have been developed for the simultaneous qualitative and quantitative analysis of dry YSC extracts and melilotin tablets. The quantitative determination of the total coumarins in YSC grass can be performed by gravimetric, titrimetric, and photocolorimetric analysis, UV spectrophotometry, and chromatography (HPLC) [104]. The total content of coumarins in YSC grass can be also estimated using UV spectrophotometry. The extraction is performed by ethyl alcohol without preliminary purification. The total content of coumarins is determined with recalculation for the specific absorption coefficient at 275 nm. The totalf coumarins in YSC extracts can also be determined either directly, using spectrophotometric measurements at 305 nm and the specific absorption coefficient of scopoletin [24], or upon reactions with an alkali solution and freshly prepared solution of dinitrided sulfanilic acid [105].

For dry YSC extracts and melilotin tablets, the test for identity can be combined with the quantitative analysis of coumarins by means of reverse-phase HPLC with UV detector and coumarin (WRS) as the internal standard. The components are identified using retention chromatograms and the relative optical densities *A* (254/220), *A* (317/254), and *A* (317/220) [106].

REFERENCES

1. A. Z. Abyshev, É. M. Agaev, and Yu. B. Kerimov, *Chemistry and Pharmacology of Natural Coumarins* [in Russian], Baku (2003), pp. 5 – 8.
2. S. K. Cherepanov, *Vascular Plants of Russia and CIS Countries* [in Russian], Mir i Sem'ya-95, St. Petersburg (1995).
3. G. A. Kuznetsova, *Natural Coumarins and Furocoumarins* [in Russian], Nauka, Leningrad (1967).
4. M. I. Gabyshev, *Materials for the Investigation of Medicinal Plants of Yakutia* [in Russian], Yakutsk State University, Yakutsk (1977), pp. 140 – 152.
5. E. Pérez-Rodriguez, J. Aguilera, and F. L. Figueroa, *J. Exp. Bot.*, **54**(384), 1093 – 1100 (2003).
6. R. H. Goodwin and C. Taves, *Am. J. Bot.*, No. 37, 3 (1950).
7. Ya. I. Khadzhai, Terpenoids and Coumarins, *Trudy Bot. Inst. im. V. L. Komarova*, Ser. V, Rast. Syr., No. 12, 25 – 30 (1965).
8. K. M. Lakin, T. V. Smirnova, G. M. Vishnyakova, et al., *Khim.-Farm. Zh.*, **23**(10), 1212 – 1213 (1989).
9. N. M. Vavilova, *Homeopathic Pharmacodynamics* [in Russian], Medical Center "Everest", Moscow (1994), Vol. 1, pp. 318 – 321.
10. *Register of Drugs in Russia* [in Russian], Yu. F. Krylov (ed.), InPharmChem, Moscow (2004).
11. *Pharmaceutical Substances: Syntheses*, *Patents*, *Applications (4th Edition)*, Vol. 1, Thieme, Stuttgart – New York (2001), pp. 280, 346, 530, 1035.
12. W. Ch. Evans, *Trease and Evans' Pharmacognosy (14th Edition)*, Saunders, London – Philadelphia (1996), p. 455.
13. S. Jamal and J. R. Casley-Smith, *Ann. Trop. Med. Parasitol.*, **83**, 287 – 290 (1989).
14. M. E. Marshall, J. L. Mohler, K. Edmonds, et al., *Cancer Res. Clin. Oncol.*, No. 120, 39 – 42 (1994).
15. M. E. Marshall, K. Butler, J. Cantrell, et al., *J. Cancer Chemother. Pharmacol.*, No. 24, 65 – 66 (1989).
16. B. D. Carlton, J.-C. Aubrun, and G. S. Simon, *Fund. Appl. Toxicol.*, No. 30, 145 – 151 (1996).
17. L. V. Pastushenkov and E. E. Lesiovskaya, *Plants-Antihypoxanths (Phytotherapy)* [in Russian], St. Petersburg State Chem.-Pharm. Inst., St. Petersburg (1991), pp. 26 – 27.
18. *WHO Expert Committee on Specifications for Pharmaceutical Preparations (34th report)*, Annex 3 (WHO Technical Report Series, No. 885), WHO, Geneva (1999).
19. *ICH Harmonized Tripartite Guideline*, Text on Validation of Analytical Procedures (Q2A), (1994).
20. *ICH Harmonized Tripartite Guideline*, Validation of Analytical Procedures: Methodology (Q2B), (1996).
21. E. E. Lesiovskaya, O. M. Lokteva, N. Yu. Frolova, and B. K. Kotovskii, *Abstracts of Papers*, *All-Russia Sci. Conf. "Current Problerms in R&D of New Drugs"* [in Russian], St. Petersburg (1996), pp. 143 – 144.
22. O. M. Lokteva, *Abstracts of Papers*, *Int. Conf. "Pharmacy XXI: Innovations and Traditions"* [in Russian], St. Petersburg (1999), pp. 174 – 176.
23. O. M. Lokteva, *Author's Abstract of Cand. Sci. (Biol.) Thesis* [in Russian], St. Petersburg (1999).
24. I. I. Chemesova, S. L. Chubarova, E. I. Sakanyan, et al., *Rast. Resur.*, **36**(1), 86 – 91 (2000).
25. N. A. Gromova, B. K. Kotovskii, and S. A. Minina, *Abstracts of Papers*, *All-Russia Sci. Conf. "Chemistry and Technology of Drugs"* [in Russian], St. Petersburg (1994), pp. 22 – 23.
26. V. M. Kosman, I. I. Chemesova, B. K. Kotovskii, and S. L. Chubarova, *Abstracts of Papers*, *Int. Conf. "Farmacy XXI: Innovations and Traditions"* [in Russian], St. Petersburg (1999), pp. 236 – 237.
27. L. M. Markova, B. K. Kotovskii, N. K. Borovskaya, et al., *Abstracts of Papers*, *Int. Conf. "Farmacy XXI: Innovations and Traditions"* [in Russian], St. Petersburg (1999), pp. 60 – 61.
28. N. V. Marchenko, *Author's Abstract of Cand. Sci. (Pharm.) Thesis* [in Russian], St. Petersburg (2002).
29. A. V. Lozhkin, B. K. Kotovskii, E. I. Sakanyan, and S. L. Petrova, *Abstracts of Papers*, *Int. Conf. "85 Years of St. Petersburg State Chem.-Pharm. Academy"* [in Russian], St. Petersburg (2004), pp. 286 – 287.
30. A. V. Lozhkin, E. I. Sakanyan, L. F. Strelkova, et al., *Proc. 9th Int. Congr. "PhytoPharm 2005" and Int. Conf. Young Scientists of Eur. Phytochem. Soc. "Plants & Public Health*," St. Petersburg (2005), pp. 264 – 268.
31. V. P. Georgievskii and G. V. Fedorin, *Rast. Resur.*, **7**(2), 275 – 279 (1972).
32. V. P. Georgievskii, N. F. Komissarenko, and S. E. Dmitruk, *Biologically Active substances of Medicinal Plants* [in Russian], Nauka, Novosibirsk (1990), pp. 9 – 11, 14 – 34, 152 – 191.
33. G. Stanić, B. Jurišić, and D. Brkić, Croat. *Chem. Acta*, **72**(4), 827 – 834 (1999).
34. G. K. Nikonov, *Author's Abstract of Cand. Sci. (Chem.) Thesis* [in Russian], Moscow (1964).

35. S. V. Terent'eva and E. A. Krasnov, *Rast. Resur.*, **39**(1), 55 – 63 (2003).
36. L. V. Ligai, A. A. Akopov,c and S. V. Men'kov, Proc. 56th Reg. Conf. on Pharmacy, *Pharmacology*, *and Staff Training (A Collection of Papers)* [in Russian], Pyatigorsk (2001), cc. 30 – 31.
37. A. I. Dergach, A. G. Kotov, S. N. Komissarenko, et al., *Rast. Resur.*, **35**(3), 81 – 85 (1999).
38. A. L. Shavarda, T. Yu. Danchul, L. I. Shagova, et al., *Rast. Resur.*, **37**(4), 62 – 64 (2001).
39. N. F. Komissarenko, *Khim. Prir. Soedin.*, No. 3, 141 – 144 (1969).
40. N. F. Komissarenko, *Khim. Prir. Soedin.*, No. 3, 624 – 628 (1970).
41. M. W. Biavatti, C. A. Koerich, C. H. Henck, et al., *Z. Naturforsch*, No. 59c, 197 – 200 (2004).
42. K. Serghini, A. Pérez de Luque, M. Castejón-Muñoz, et al., *J. Exp. Bot.*, **52**(364), 2227 – 2234 (2001).
43. B. A. Krivut, *Author's Abstract of Cand. Sci. (Biol.) Thesis* [in Russian], Tashkent (1973).
44. M. Muller, M. Byres, and M. Jaspars, et al., *Acta Pharm.*, No. 54, 277 – 285 (2004).
45. A. C. S. Baetas, M. S. P. Arruda, A. H. Müller, and A. C. Arruda, *J. Braz. Chem. Soc.*, **10**(3), 181 – 183 (1999).
46. N. F. Komissarenko, A. I. Dergach, A. N. Komissarenko, et al., *Rast. Resur.*, **29**(3), 53 – 59 (1994).
47. F. G. Cruz, L. de M. Moreira, N. A. S. Santos, and M. L. S. Guedes, *J. Braz. Chem. Soc.*, **13**(5), 704 – 707 (2002).
48. G. K. Nikonov and M. E. Perel'son, *Lek. Rast.*, No. 15, 87 – 125 (1969).
49. A. A. Akhrem and A. M. Kuznetsova, *Thin-Layer Chromatography* [in Russian], Nauka, Moscow (1965), pp. 83 – 84.
50. M. Sharshunova, V. Shvarts, and Ch. Mikhalets, *Thin-Layer Chromatography in Pharmacy and Clinical Biochemistry* [in Russian], Mir, Moscow (1980), pp. 301 – 621, 430 – 434.
51. J. Kitajima, Ch. Ocamura, T. Ishikawa, and Y. Tanako, *J. Pharm. Sci.*, **88**(12), 1939 – 1940 (1998).
52. V. V. Shkarenda, *Author's Abstract of Cand. Sci. (Pharm.) Thesis* [in Russian], St. Petersburg (1992).
53. N. A. Tyukavkina, I. A. Rulenko, N. N. Kolesnikova, and Yu. A. Kolesnik, *Farmatsiya*, No. 3, 22 – 23 (1997).
54. G. A. Kuznetsova and L. V. Kuz'mina, *Rast. Resur.*, **1**(1), 149 – 151 (1965).
55. V. I. Dikhtyarev, V. N. Kovalev, and N. F. Komissarenko, *Khim. Prir. Soedin*, No. 2, 258 – 259 (1982).
56. British Pharmacopoeia, CD 1998, Version 2.1, Crown Copyright (1998).
57. R. M. S. Celeghini, J. H. Y. Vilegas, and F. M. Lansas, *J. Braz. Chem. Soc.*, **12**(6), 706 – 709 (2001).
58. G. A. Denisova and S. Sh. Kerimov, *Rast. Resur.*, **2**(2), 182 (1966).
59. L. I. Kosheleva, G. K. Nikonov, and M. G. Pimenov, *Lek. Rast.*, No. 15, 140 – 148 (1969).
60. G. L. Genkina, *Khim.-Farm. Zh.*, **15**(11), 108 – 115 (1981).
61. V. de F. F. Monteiro, L. Mathias, J. C. Vieira Ivo, et al., *J. Braz. Chem. Soc.*, **13**(2), 281 – 287 (2002).
62. V. M. Malikov and A. I. Saidkhodzhaev, *Khim. Prir. Soedin*, No. 2, 250 – 281 (1998).
63. V. M. Malikov and A. I. Saidkhodzhaev, *Khim. Prir. Soedin*, No. 3, 384 – 432 (1998).
64. V. M. Malikov and A. I. Saidkhodzhaev, *Khim. Prir. Soedin*, No. 4, 560 – 593 (1998).
65. R. Krishna, D. Velmuragan, S. Shanmuga Sundara Raj, et al., *Cryst. Res. Technol.*, **36**(11), 1289 – 1294 (2001).
66. V. V. Suvorov, *Cultured Flora of the USSR* [in Russian], Sel'khozgiz, Moscow, (1950), Vol. 13, pp. 345 – 502.
67. N. A. Valyashko and É. G. Berdichevskii, *Zh. Obshch. Khim.*, **27**(8), 2302 – 2303 (1957).
68. V. P. Georgievskii, N. A. Kazarinov, and M. O. Karryev, *Physicochemical Methods of Analysis of Biologically Active Substances of Plant Origin* [in Russian], Ylym, Ashkhabad (1976), p. 50.
69. P. P. Lutsko, V. V. Mikhno, and I. G. Postriga'n, *Abstracts of Papers*, *The 4th Congr. of Ukrainian Pharmacists* [in Russian], Zaporozhye (1984), p. 237.
70. M. G. Pimenov, *Register of Plants-Sources of Coumarins* [in Russian], Nauka, Leningrad (1971), p. 9.
71. M. E. Perel'son, Yu. N. Sheinker, and A. A. Savina, *Spectra and Structure of Coumarins*, *Chromones*, *and Xanthones* [in Russian], Meditsina, Moscow (1975), pp. 9 – 35.
72. V. V. Buryak and N. K. Starchevskaya, *Farmatsiya*, No. 5, 26 – 31 (1981).
73. A. I. Gromakova, *Author's Abstract of Cand. Sci. (Pharm.) Thesis* [in Russian], Moscow (1982).
74. B. A. Krivut and M. E. Perel'son, *Khim. Prir. Soedin*, No. 2, 183 – 185 (1970).
75. B. A. Krivut and M. E. Perel'son, *Khim. Prir. Soedin*, No. 1, 3 – 6 (1970).
76. Ya. I. Éidler, G. L. Genkina, and T. T. Shakirov, *Khim. Prir. Soedin*, No. 1, 86 – 87 (1974).
77. M. E. Perel'son, A. I. Ban'kovskii, A. A. Kir'yanov, *Khim. Prir. Soedin*, No. 6, 381 – 383 (1966).
78. V. A. Katz, O. U. Thulke, and U. Conrath., *Plant Physiol.*, No. 117, 1333 – 1339 (1998).
79. C. Thanos, Z. Liw, M. Goddard, et al., *J. Pharm. Sci.*, **92**(8), 1677 – 1689 (2003).
80. Yu. E. Orlov and R. V. Piskareva, *Khim. Prir. Soedin*, No. 1, 87 (1974).
81. Yu. E. Orlov, *Abstracts of Papers*, *The 4th Congr. of Ukrainian Pharmacists* [in Russian], Zaporozhye (1984), p. 230.
82. Yu. E. Orlov and L. Ya. Sirenko, *Khim. Prir. Soedin*, No. 5, 717 – 718 (1991).
83. V. P. Georgievskii, *Abstracts of Papers*, *The 3rd Congr. of Pharmacists* [in Russian], Kishinev (1980), p. 242.
84. G. F. Fedorin, *Abstracts of Papers*, *The 3rd Congr. of Pharmacists* [in Russian], Kishinev (1980), pp. 242 – 243.
85. G. F. Fedorin, *Abstracts of Papers*, *The 4th Congr. of Ukrainian Pharmacists* [in Russian], Zaporozhye (1984), p. 172.
86. A. A. Khabarov, N. P. Khabarova *Abstracts of Papers*, *The 3rd Congr. of Pharmacists* [in Russian], Kishinev (1980), p. 278.
87. A. A. Khabarov, O. A. Grigor'ev, andS. I. Kobelev, *Byull. Izobret.*, No. 46 (1985).
88. R. H. Goodwin and F. Kavanagh, *Arch. Biochem.*, **27**(1), 152 – 173 (1950).
89. A. Z. Abyshev, V. G. Klimov, S. S. Krylov, et al., *Khim.-Farm. Zh.*, **19**(6), 756 – 759 (1985).
90. S. Kurata, N. Aizawa, H. Hirano, and M. Nagai, *Bunseki Kagaku Abstr.*, **52**(3), 187 – 195 (2003).
91. B. Z. Usmanov and N. K. Abubakirov, *Khim.-Farm. Zh.*, **1**(5), 42 – 46 (1967).
92. B. Z. Usmanov and N. K. Abubakirov, *Khim.-Farm. Zh.*, **1**(5), 295 – 298 (1967).

93. A. V. Ananichev and D. A. Pakaln, *Med. Prom-st SSSR*, No. 11, 51 – 53 (1966).
94. M. de Freitas Soares, F. Delle Monache, V. E. Fonseca Heinzena, and R. A. Yunes, *J. Braz. Chem. Soc.*, 10(3), 189 – 196 (1999).
95. Ya. I. Eidler, G. L. Genkina, and T. T. Shakirov, *Khim. Prir. Soedin*, No. 2, 155 – 156 (1974).
96. S. L. Born, D. Caudill, B. J. Smith, and L. D. Lehmann-McKeeman, *Tox. Sci.*, No. 58, 23 – 31 (2000).
97. L. Opletal, K. Vockac, V. Hanus, et al., *Folia Pharm. Univ. Carol.*, No. 21 – 22, 89 – 94 (1998).
98. W. Zhang, T. Kilicarslan, R. F. Tyndale, and E. M. Sellers, *Drug Metabol. Dispos.*, No. 26, 892 – 902 (2001).
99. M. Kollroser and C. Schober, *Clin. Chem.*, **48**(1), 84 – 91 (2002).
100. T. Grobosch, B. Angelow, and D. Lampe, T + K, **72**(1), 46 – 55 (2005).
101. M. A. Givel, *Tobacco Control*, No. 12, 401 – 405 (2003).
102. *The Pharmacopoeia of the United States of America*, XXIII Rev., The National Formulary, XVIII Rev., New York (1995).
103. *European Pharmacopoeia*, Third Edition, Supplement 2001, Council of Europe, Strasbourg (2000).
104. V. N. Bubenchikova and I. L. Drozdova, *Khim.-Farm. Zh.*, **38**(4), 24 – 25 (2004).
105. V. YU. Myasnikov, N. V. Marchenko, E. V. Ivanov, et al., *Proc. Int. Conf. "Current Problems in R&D of New Drugs of Plant Origin"* [in Russian], St. Petersburg (2001), pp. 336 – 340.
106. V. M. Kosman, I. G. Zenkevich, and N. F. Komissarenko, *Rast. Resur.*, **33**(3), 32 – 37 (1997).