CURRENT STATE OF THE LIQUID COLUMN CHROMATOGRAPHY OF COUMARINS

I. CHARACTERISTICS OF THE METHOD, AND THE DETECTION AND CHROMATOGRAPHIC BEHAVIOR OF THE COUMARINS

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An analysis is given of literature information on the liquid column chromatography of coumarins for 1983-1990. Questions of the classification of the methods used, the choice of the optimum detection regime, and the conditions of chromatographic separation are discussed. The relationship between the structures of coumarins and their chromatographic behavior is considered. Conditions for the separation, isolation, and analysis of coumarins in various materials are given.

Coumarins (CMs) – derivatives of benzo-α-pyrone (I) – form an important group of biologically active compounds. According to Murray et al. [1], in 1982 representatives of this class of phenolic compounds numbered more than 800. At the present time, as Asenov and Gevrenova consider, the number of known CMs has probably reached 1000 [2]. The list of new CMs is continuously being supplemented. Great advances have been achieved in the study of bicoumarins [3], isocoumarin derivatives [4], aflatoxins [5], etc. To a large degree this has been favored by the use of the method of liquid column chromatography (LCC) and, especially, high-performance LCC (HPLC).

On the other hand, the use of new variants of LCC based on special chemically modified adsorbents is considerably expanding the possibilities of the phytochemical investigations of CMs, of the pharmaceutical and toxicological analysis of coumarin-containing drugs, and of biochemical transformations of the CMs [6].

Together with other chromatographic methods, LCC of the coumarins has been considered, in part, in reviews by Bhandori and Rastogi [7] and by Grigor'ev et al. [8]. A critical review of publications during the period 1976-1982 on the HPLC of mycotoxins, including those with an isocoumarin structure, has been made by Fremy et al. [9], and those during the last five years are partially represented in publications by Betina [10] and Scott [11].

The present review has the aim of filling gaps in this area by generalizing and systemizing literature information over the last eight years with emphasis on the use of different variants of LCC in methods of investigating CMs, including those of previously unknown structure.

METHOD OF LIQUID COLUMN CHROMATOGRAPHY FOR COUMARINS

Starting from analytical or preparative aspects of preparation, the systematization of LCC methods for coumarins is based on the generally accepted classification of LCC according to the size of the load on the column [22]. The level of pressure used can be varied within wide limits in all cases, but as one of the factors determining the time and efficacy of the separation procedure it permits some expansion of the traditional classification frameworks (Table 1). Here it is also possible to take into account new approaches in the area of nontraditional methods of LCC for coumarins, such as the affinity chromatography of stereoisomers of CM derivatives, chromatography on adsorbents of the affinity type, etc.

PREPARATIVE AND MACROPREPARATIVE (LARGE-SCALE) LIQUID COLUMN CHROMATOGRAPHY OF COUMARINS

At the present time, preparative LCC (PLCC) is encountered in almost any publication on the isolation of CMs. Like other LCC methods, with the exception of analytical methods,

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TABLE 1. Characteristics of the Method of Liquid Column Chromatography for Coumarins

Type of liquid column chromatography of coumarins	Time of separation, h	Load, g	Column dimension, mm	Pressure, MPa	Literature
I. Macropreparative (large scale)	>100	>100	800×150	0,1	[19]
II. Preparative					
a) atmospheric pressure	-	0,01—100,0	600×15	-	[89]
b) low pressure	-	300,0—350,0	440×37	0,16	[26]
c) medium pressure	10	350	735×26	1,0—1,2	[27]
			310×25		[39]
III. Micropreparative medium pressure	1,0—2,0	0,008—0,04	240×10	0,3—0,6	[46]
IV. High performance (HPLC)					
a) preparative	—	—	250×16	20—30	[43]
b) semipreparative	1,0	0,03	250×16	30	[27]
c) analytical	0,4—0,5	—	250×4	-	[27, 43, 81]

PLCC is used in two main directions – a) to obtain purified total CM fractions [12-16], and b) to isolate or separate individual CMs [17-19] – and it continues to make an enormous contribution to the search for new coumarins.

The basic feature of PLCC is the possibility of isolating gram amounts of substance, but in view of the factors determining the chromatographic behavior of CMs in PLCC (column dimensions, load of sample), preparative columns have a low separating power. Only by selecting suitable fractions of partially overlapping peaks of CMs is it possible to exclude a large amount of impurities. For example, in the method of obtaining furocoumarins (FCMs) – xanthotoxol (II) and xanthotoxin (III), sphondin (IV), isopimpinellin (V), and bergapten (VI) from drug raw material patented by Sledzinska et al. [19] the purity of the compounds isolated by macropreparative LCC reaches 99% [19]. The control process is usually monitored by the TLC method [20, 21] or by refractometric detection.

As practice shows, less lengthy procedures based on rough purification or fractionation of the sample, followed by rechromatography of individual fractions in the same or a different chromatographic system (CS) are more effective and more economical. Therefore, in view of the possibility that appears in this case of selecting a variant of LCC and of a combination of CSs, and also of isolating minor components, it is just such a scheme that has come into predominant use in phytochemical practice, for example [12, 13].

A negative aspect of the PLCC method is its low reproducibility, since under the conditions of PLCC an adsorption mechanism of separation is realized most frequently and the complete preliminary saturation of the adsorbent with the mobile phase (MP) is rarely achieved. Literature sources very rarely give detailed characteristics of the CSs: the adsorbent–MP system and the dimensions of the column used in PLCC, and also the volumes of the eluents. This fact complicates the drawing up of special reviews and the systematization of information on the PLCC of coumarins. However, where necessary, the conditions of preparative separation can easily be selected by scaling up analytical LCC by means of the well-known formula

$$m_1 = m_2\left(\frac{d_1}{d_2}\right)^2 \times \frac{l_1}{l_2},$$

where m_1 and m_2 are the loads, d_1 and d_2 the internal diameters, and ℓ_1 and ℓ_2 the lengths of the preparative and analytical columns, respectively. In order to increase the efficiency of the CS, Bidlingmeyer's other recommendations [22] must also be followed.

In addition to the new chromatographic materials that have come into use in the PLCC of the coumarins (reversed-phase analogues of silica gel (RF-SG) [19] and of Sephadex [17], SG impregnated with Ag^+ ions [23, 25] etc.), a number of changes in the technical side of the method connected with the use of high pressure, greatly accelerating the separation process, must also be mentioned [26, 27]. In the low-pressure LCC regime in small columns, the pressure can be achieved by means of a peristaltic pump as, for example, in the gel permeation chromatography of coumarin rodenticides [28, 29]. For large columns, piston pumps are more

frequently used. Thus, the amount of certain separated CMs – sibiricin (VII) and murrayone – reached 162 and 125 mg, respectively, in the work of Kumar et al. [26]. By this method, Di Paolo has isolated new chromonocoumarins: frutinone A (VIII) and frutinone B (IX) from Polygala fruticosa [30].

Medium-pressure preparative LCC is being used ever more widely in the phytochemical investigations of CMs. Its use for the intermediate purification of hydroxycoumarins (HCMs) has been described by Zdero et al. [31, 32], Van Wagenen et al. [33], Bittner et al. [34], Marco [35], and Giammona et al. [32a]. Dubois et al. have separated glycosides: esculin, fraxin, and palustroside [32b]. Thus, special investigations of this method carried out by Zogg et al. taking as an example the separation of FCMs (III-VI), angelicin (X), isobergapten (XI), pimpinellin (XII), and psoralen (XIII) have shown that medium-pressure preparative LCC is not only not inferior to preparative HPLC but is economically superior to it [27] (Table 3). The most effective separation is achieved on column filled with the use of vacuum or under nitrogen pressure, and the mean size of the SG particles (LiChrosorb Si60) must be 15 μm (range of the fractions, 5-25 μm) [36]. It has been reported that the isolation of individual FCMs with a purity of not less than 95% can be achieved even at a load of up to 10 mg/g of adsorbent [37]. The use of the method is considerably simplified by the procedure of optimizing the conditions of medium-pressure LCC with the aid of TLC proposed by Nyiredy et al. and demonstrated in the separation of the FCMs (IV-VI, XI, and XII) [38]. A similar method was used by Glowniak et al. in the development of a procedure for separating unbelliprennin, imperatorin (XX), (III), (V), and (VI), and (XII) from Archangelica fruits [39].

Nevertheless, one of the main positions among the methods for the preparative LCC of the coumarins continues to be occupied by preparative HPLC. In spite of the smaller load and smaller column dimensions (see Table 1), the high rate of feed of eluent leads to a rapid occurrence of the separation process, which when necessary, permits the cycle to be repeated several times with the achievement of a satisfactory separation or the preparative processing of CMs. The wide use of preparative HLPC has been reported in all stages of the isolation of CMs from plant extracts: purification [16], and the separation or isolation of individual CMs [21, 32, 40-44]. It must be borne in mind that the specific nature of the apparatus for HPLC sets high demands on the purity of the solvents, the sensitivity of the detector, the quality of the column filling, etc. [6].

MICROPREPARATIVE LIQUID COLUMN CHROMATOGRAPHY OF THE COUMARINS

In the LCC of the coumarins, the area of preparative separation began to be singled out comparatively recently. Thus, after known methods of semipreparative HPLC, for example [27], had received their development, this scale of separation of the coumarins was also found in other variants of LCC [46].

On the other hand, the domain with a load on the column of 4-20 mg is interpreted by some authors as "... separation in milligram amounts ..." [39]. But the micropreparative separation that they give, in spite of using columns of the same dimensions as those in preparative HPLC (Table 3, No. 4), must, from the "pressure" characteristic, be assigned to medium-pressure LCC, since its interval (0.3-0.6 MPa) is close to the medium-pressure preparative LCC of [27] and agrees with the figures in Speckenbach's review [45]. As Wawrzynowicz et al. have shown, with an increase in the amount of CMs to be analyzed to 40 mg rechromatography is already necessary [46]. Therefore the specific load in the medium-pressure micropreparative LCC of the coumarins must not exceed 1-2 mg/g of adsorbent.

Thus, even on the micropreparative scale of separation the analytical aspect of LCC appears, and in the case of a poor separation of the peaks this must be successfully realized through TLC monitoring [39, 46]. Furthermore, in the low- and medium-pressure regimes an intermediate area exists between the preparative and analytical LCC methods with a load on the column in the range of 0.05-0.5 mg/g of adsorbent in which selective, well filtering, hydrophilic polymeric adsorbents, including modified ones, are used: affinity adsorbents [47-49], adsorbents of the affinity type (AAFT) [50-52], etc. The main feature of the latter, apart from their original structure, is the possibility of combining analytical (in scale) separation with the isolation of sufficient amounts of CMs for spectral identification.

ANALYTICAL LIQUID COLUMN CHROMATOGRAPHY OF THE COUMARINS

With respect to the totality of characteristics satisfying the demands set for analytical methods, HPLC has no equals among other methods of LCC. Appearing as the concluding stage

of qualitative and quantitative analysis, it greatly simplifies methods of investigating CMs in the area of the taxonomy [21, 53, 54] and physiology of plants [55-58] and fungi [59, 60], and also the study of the metabolism of the CMs in microorganisms and in the animal and human organism [61-67], their determination in blood plasma [68-75], and the analysis of coumarin-containing drugs [76-80]. This is favored by the high rate of separation (up to 30 coumarins in 25-30 min [81]) and by some methods of concentrating components of a sample by the method of solid-phase extraction on minicolumns of the Sep-Pak type [71, 82, 84], on phenyl-silica gel [85, 86], etc., that are characteristic for HPLC. In some investigations, methods have been used which lower the limit of detection of the coumarins by increasing their adsorption in the working range of the detector with the aid of so-called "post-column" "reagents" [28, 87].

Thanks to the possibility of realizing, under HPLC conditions, all known separation mechanisms, including affinity chromatography, and also processes of optimizing and modeling procedures for separating complex mixtures of CMs [88], analytical HPLC serves as methodological basis for PLCC. Therefore, below, in considering concrete conditions for separating CMs and the relationship of the structures of CMs with their chromatographic behavior, it is just to analytical HPLC that our main attention will be devoted.

DETECTION OF COUMARINS IN LIQUID COLUMN CHROMATOGRAPHY

The UV detector has the widest linear dynamic range among those used in the LCC of coumarins [90].

It is known that the absorption maximum of coumarin in the UV region is located at 275 nm. Its substituted analogues that are most widely distributed in nature have pronounced absorption maxima in the 250-260 and 310-345 nm regions [91]. This factor determines the choice of the wavelengths of the UV detector in the analysis of a mixture of CMs, which is usually carried out at 254 nm [41, 81, 91] or at 310 or 335 nm [93]. For the detection of FCMs, 313 nm [27, 44, 94] and 320 nm [95-98] are used, in addition to 254 nm. In spite of contours of their UV spectra close to those of coumarin, it has been proposed to use for drugs derived from 4-hydroxycoumarin (4-HCM) and their metabolites the interval of a less pronounced absorption maximum – 303-313 nm [65, 67, 68, 75, 77, 99, 102]. Only for some CMs is the long-wave region of the UV spectrum used; for example, esculetin (XIV) is determined at 350 nm [58], and aflatoxins at 365 nm [103].

The conditions for detecting CMs on analysis in the presence of other phenolic compounds requires a correspondence of the selected absorption maximum wavelength and the phenolic compounds of interest to the researcher. Therefore it may represent one magnitude common for the given components or two optimum ones (two-wavelength detection). Most frequently, to determine CMs with derivatives of hydroxybenzoic acid and other phenolic compounds use is made of 275 nm [104], 280 nm [61, 105, 106], and 254 nm [107], or the latter two simultaneously [108].

Mixtures of HCMs and flavonoids are analyzed at 337 nm [109] or 340 nm [110, 111], and those of coumestrols and phenolic compounds at 351 nm [112].

Investigations of metabolic processes of CMs and pharmacokinetics are always characterized by low concentrations of CMs in the materials for analysis. For example, the number of metabolites of xanthotoxin [62] and warfarin (WFR) [63] reaches 10-12, which complicates their differentiation. The resolution of such problems is favored by the use of instruments which are less common but possess the greatest sensitivity (10^{-9} g) among optical detectors – fluorimeters, and also UV detectors with photodiode arrays. Similar analytical problems in the determination of CMs labeled with radioactive isotopes are solved with the aid of scintillation detectors [47, 62] which, for known reasons, have limited use (Table 2).

In view of the fact that all CMs, with rare exceptions, are capable of fluorescing, the use of a fluorimeter for detection in the LCC of coumarins predominates over UV detection, particularly in methods for quantitative determination, in spite of its comparatively narrow dynamic range. So far as concerns nonfluorescing CMs, UV detectors with photodiode arrays are used more frequently [68, 100, 113], and these are also used in the analysis of FCMs [114] and chromonocoumarins [30]. However, the limit of detection when a fluorimetric detector is used not only depends on the concrete compound but is also determined by the variant of LCC. Thus, the limit of detection of WFR in the free state (RF-HPLC) is twice that achieved when it is in the form of ion pairs with surface-active substances – ion-pair (IP)

TABLE 2. Limits of Detection for: a) Warfarin; b) Methoxy Derivatives of Psoralen by the HPLC Method with Various Detectors

Detector	Limit of detection, g/ml	Literature	Limit of detection, g/ml	Literature
	a)		b)	
1. Ultraviolet	5×10^{-7}	[99]	6×10^{-8} —10^{-9}	[72]
2. Fluorescent	10^{-9}	[63]	10^{-9}	[64]
3. UV with photodiode array	10^{-10}	[40]	—	—
4. Mass spectrometric	2 ng in the sample	[71]	—	—
5. Scintillation	—	—	5×10^{-11}	[62]

HPLC [28, 29]. On passing to luminescent detection, the limit of detection of some CM derivatives decreases hundredsfold, and in the regime of normal-phase (NP) HPLC the sensitivity of the method is six times greater than that of RF-HPLC [115].

If the components of the MP have strong absorption in the UV region or fluoresce, a differential refractometer is used [16, 101, 116], and this is particularly effective in the case of high concentrations of CMs. The advantages of this include the possibility of monitoring the saturation of the adsorbent with the MP.

The use of an electrochemical detector has been described by Sontag et al. in a method for the quantitative determination of bergaptol [117]. Furthermore, in many publications devoted to the analysis of isolated CMs mass spectrometry is used to identify the peaks immediately after separation [16, 22, 54, 96, 113, 118]. Spink et al. have proposed to link a UV detector with a device for the thermospray ionization of a mass spectrometer, which ensures the reliable and selective recording of hydroxy derivatives of WFR with a detection limit of 2-10 ng [71].

CHROMATOGRAPHIC BEHAVIOR OF COUMARINS IN VARIOUS CHROMATOGRAPHIC SYSTEMS

The traditional CSs, representing the most widely used group, in the LCC of coumarins is represented by silica adsorbents (SG of various brands), so-called normal phases (MPs), and their chemically modified analogues, the reversed phases (RPs). At the same time, some properties of RP sorbents are possessed by certain unmodified sorbents — polyamide [117, 119, 120], Sephadex [18, 20], Toyopearl [50, 51], and Spheron. In this case, the factor determining the selectivity of the CS is, as a rule, the composition of the MP.

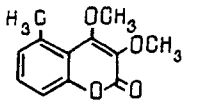
Substantial progress in methods for obtaining modified adsorbents [121] has been brought about by the appearance of CSs of a complex type in the LCC of coumarins. Their efficiency is due not only to the structure of the ligand but also to the contribution of all the component parts of the adsorbent (matrix—insert—ligand) in the process of interacting with the substance being analyzed.

NORMAL-PHASE AND REVERSED-PHASE CHROMATOGRAPHIC SYSTEMS

The chromatographic behavior of coumarins in the system is determined predominantly by the mechanisms of hydrophobic interaction with the adsorbent. That ratio of polarities of the MP and the adsorbent at which their half-sum is equal to the polarity of the sample is considered to be the optimum for LCC [88]. This is most frequently achieved by the empirical choice of the composition of the MP.

The main eluents in the RP—HPLC of the coumarins — binary mixtures of MeOH or MeCN with water — are widely used in the preparative separation of the HCMs [15, 31, 32, 42, 54, 125] and FCMs [19, 46, 122-124]. The elution of the CMs in such CSs takes place in the order of increase of their lipophilic properties, which are determined by the structure and positions of the constituents. In analytical methods, the above mentioned MPs readily separate mixtures of HCMs [59, 126], FCMs [42, 127, 128], pyranocoumarins (PCMs) [12], aflatoxins [103], and coumarin and phenolic compounds [129]. The order of elution or order of increasing retention time on the adsorbent for a number of CMs has the following form [46, 127, 130]: 4-HCM < 4-CH_3(XIV) < (XXI) < (II) < coumarin < (III) < (V) < (VI) < (XIII) < (XX) < (XVII).

It follows from the Scheme that the influence of polar radicals in the structure of a CM in the direction of a decrease in retention under the conditions of RP chromatography is enhanced if they are present in the immediate vicinity of the lactone group. The existence of such a relationship permits a hypothesis to be put forward according to which the differentiation of the polar properties of the coumarins with the same number of substituents but different distributions of them takes place through a mechanism of the formation of "hydration" centers (Fig. 1). A clear confirmation of this is the order of elution of derivatives of 3-methoxypereflorin (XXXI), the retention times of which increase with a decrease in the polarity of the substituents at C_8 and also with their removal to the C_7 and C_8 atoms [32]:



XXXI

8-OH<8-OCH_3<7-OH<8-CH_3<6-OCH_3

An analogous situation is observed for other HCMs [93]: 6,7-diOH < 7,8-diOH < 7-OH < 7-OH, 8-OCH_3 < 7-OH, 6,8-diOCH_3.

Coumarins having a 4',5'-dihydropyran ring with a hydroxyl-containing radical (rutaretin, Fig. 1b) are eluted not only earlier than other hydroxyl-containing FCMs (II, XXV) but also than some HCMs, such as leptodactylone [93] (Fig. 1c).

The steric hindrance caused by hydrophobic 8- or 3-alkyl, alkoxy or aryl substituents prevents the hydration of the polar lactam group and also of 4-OH and 7-OH groups and, thus, is responsible for the extremely lipophilic nature of such CM derivatives as imperatorin, osthole (XVII), WFR, and pheprocumon (PPN).

It must be mentioned that RP systems have proved more effective for the separation of such FCMs as isoimperatorin (XVIII), 8-geranyloxy-(XIII), 5-geranyloxy-7-methoxycoumarin, and mixtures of bergapten, phellopterin (XIX) and (XX), which are not separated on an NP system for which it is far more difficult to compose selective MPs. In some cases, satisfactory separation is achieved by lowering the concentration of MeOH in the MP (Table 4, No. 1). Thus, passing from 50% to 20% aqueous MeOH permits the separation of a mixture of unbelliferone (XXI), hydrangetin (XXII), and isofraxidin (XXIII) [93]. In this investigation, Thompson and Brown tested eluents for the analysis of multicomponent mixtures on 67 different CMs. The MPs proposed by the authors are used in differentiated fashion for CMs of phenolic (Table 4, No. 2) and neutral (Table 4, No. 3) natures and permit the separation of 10-11 substances under the conditions of isocratic and linear gradient elution [93]. An example of the analytical separation of a 25-component mixture of CMs has been demonstrated by Vande Castelle et al., who used a programmed gradient (Table 4, No. 4) [81].

For the analysis of multicomponent mixtures of HCMs, 70-80% aqueous MeOH containing 1% of acetic acid is recommended [93]. Eluents of the composition have been used in the identification of bergaptol (XXV) in essential oils [131] and of polar coumarin metabolites [61]. Nevertheless, the use of such a gradient (Table 4, No. 5) in the work of Peuch et al. [132] for the separation of esculin, (XXI), 4-CH_3-(XXI), and scopoletin (XXXV) requires improvement. This is due to the considerable latent period between the peak of the glycoside and the group of peaks of the aglycons, which increases the time of analysis to 45-50 min.

The addition of acid to the MP lowers the capacity factor (R') for (VI), (XX), and (XXXV) and a number of other alkyl- and alkoxycoumarins and FCMs by 10-15%, with the exception of 4-CH_3-(XIV) and (XV), the values of (R') for which increase [130]. Thijssen et al. [67] and Maupas et al. [73] have shown a sharp rise in R' in an acid MP (Table 4, Nos. 6 and 7) for compounds with the structure of WFR with an NH_2 group [67] or a chlorine atom in the $C_{4'}$ position [73] (at pH 4.0, the value of R' is 15-17), which is three times greater than for 4'-nitro-WFR, and this even if the amino group is acylated or is absent [67]. This effect appears particularly strongly for WFR, phepromaron, acenocoumarin (ACN), neodicoumarin (NCM), and dicoumarol (DCM) in LCC on the polymeric adsorbent Toyopearl and its modified analogs, and this not only at pH < 6 but also when neutral salts are present in the MP (pH 7) – for example, beginning from 0.05 M NaCl [52] (Table 4, No. 8). It is possible that this is connected with the formation of molecular complexes between the molecules of 4-HCM or lipophilic forms of its 3-aryl-substituted derivatives stabilized by hydrogen bonds (Fig. 2).

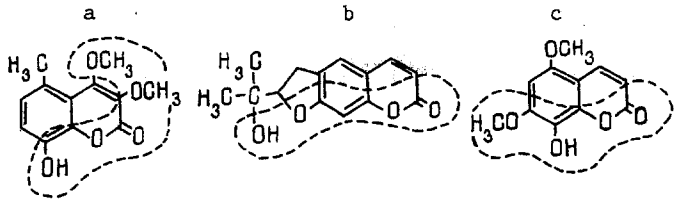


Fig. 1

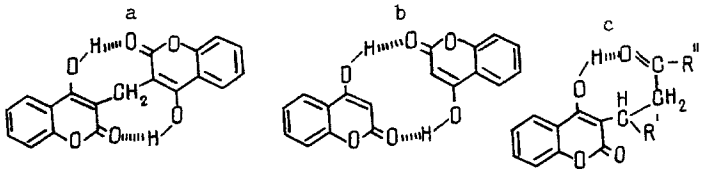


Fig. 2

Fig. 1. Hypothesis of "hydration" centers of coumarins: a) 8-hydroxy-(XXXI); b) rutaretin; c) leptodactylone.

Fig. 2. "Lipophilic" conformations of 4-HCM derivatives stabilized by intra- and intermolecular hydrogen bonds: a) dicoumarol [133]; b) 4-HCM; c) WFR, ACN.

TABLE 3. Chromatographic Systems for the Medium-Pressure Liquid Column Chromatography of Furocoumarins

Number	Adsorbent	Column dimensions, mm	Mobile phase	Literature
1	LiChrosorb Si60 (5 μm)	735×26 584×37	Ethyl acetate–$CHCl_3$–diethyl ether–hexane (5,5:11:11:72,5)	[38]
2	LiChrosorb Si60 5 (μm)	735×26	Ethyl acetate–$CHCl_3$–diethyl ether–hexane (0,6:58,5:0,9:40.0)	[27]
3	LiChrosorb Si60 (5–25 μm)	920×49 173×26	Ethyl acetate–$CHCl_3$–diethyl ether–hexane (5,5:11:11:72,5)	[36, 37]
4	Silica gel-60 (40–63 μm)	200×10	CH_2Cl_2–heptane–MeCN (50:50:1,5)	[39]
5	LiChroprep Si 6 (40–63 μm)	240×10	CH_2Cl_2–heptane–dipropyl ether (70:30:3.5)	[46]
6	LiChroprep RP-8 (40–63 μm)	310×10	MeOH–water (7:3)	[46]

A similar hypothesis has been put forward previously by Knobloch and Prochazka, who observed the chromatographic behavior of dicoumarol in paper chromatography [133] (Fig. 2a).

The retention times of WFR analogues in RP systems increase in the following order [65, 67, 71]: 9,10-dehydro- < 4-OH < 6-OH < 4'-NH-Ac < 8-OH < 4'-NH_2 < 7-OH < PPN < 4-CH_3WFR.

The main use of acidic MPs is in the separation of mixtures of CMs with phenolic compounds [58, 105, 108-110, 112, 129, 134, 136] and the analysis of drugs derived from 4-HCM and their metabolites where the necessary pH value (usually 2.0-4.2) is achieved by the addition of a formate [68], acetate [65, 67, 71, 79, 101, 102, 137], or phosphate [79, 87] buffer. Acetic acid [28, 61, 74, 75] or phosphoric acid [84] is used for the same purpose, for example, in the analysis of xanthotoxin and its metabolites [62]. A new representative of the fairly rare natural nitrogen-containing CMs – necatorin (XXIV) – has been isolated by HPLC under such conditions (Table 4, No. 9) [138]. For the analysis of WFR in the presence of other drugs, Eigendorf et al. [79] and Sidhu et al. [139] recommend MeCN with a phosphate buffer or sodium hydrogen phosphate.

Among MPs with a different composition we must mention mixtures of MeOH, MeCN, and water for the separation of CMs and phenolic compounds [104, 106, 140] and aflatoxins [141-143],

TABLE 4. Chromatographic Systems for the Separation of Coumarins: (IC) — Isocratic; (LG) — Linear Gradient NP

Number	Adsorbent, column (mm)	Mobile phase	Literature
1	Novopak C_{18} (150 × 3.9) (5 μm)	IC:MeOH–water–acetic acid (70:30:1), (80:20:1)	[93]
2	Novopak C_{18} (150 × 3.9) (5 μm)	IC:MeOH–water–acetic acid (70:30:1), (80:20:1)	[93]
		IC:THF–MeOH–water–acetic acid (5:15:80:1)	[93]
3	Novopak C_{18} (150 × 3.9) (5 μm)	IC:MeCN–water (1:1)	
		IC:MeOH–water (8:2)	[93]
	Porasil (300 × 3.9) (10 μm)	IC:Ethyl acetate–hexane (1:4), (1:3)	
4	LiChrosorb RP-18 (10 μm) (250 × 4.6)	MeOH–5% aqueous formic acid, IC: (7:93), LG: (7:93)→ (15:85) → (75:25) → (80:20), IC:(80:20)	[81]
5	Micropak C_{18} (300 × 4) (5 μm)	IC:MeOH–water–acetic acid (80:15:5)	[132]
6	LiChrosorb RP-18 (150 × 4.6) (10 μm)	IC:MeCN–ethyl acetate–ammonium acetate buffer (pH 4.9) (90:1:100)	[67]
7	Spherisorb C_{18} (150 × 4.8) (5 μm)	IC:MeOH–MeCN–0.06% acetate buffer (pH 4.0) (45:44:11)	[73]
8	Toyopearl HW-50 (55 × 5)	IC:0.05 M sodium acetate buffer (pH 5.0)	[52]
9	Silica gel C_{18} (100 × 8) (5 μm)	IC:0.05 M NaCl solution	[138]
		IC:MeOH–0.02 M phosphate buffer (pH 3.5) (11:9)	
10	LiChrosorb Si60 (250 × 4) (10 μm)	IC:n-heptane–CH_2Cl_2–diisopropyl ether (45:55:3.5)	[144]
11	Bondapak C_{18} (300 × 7.8)	IC:MeOH–water (4:6)	[20]

and also those containing more than three components, based on MeOH–THF–water, which have a high selectivity potential.

The influence on MPs on the chromatographic behavior of CMs in MP- and RP-HPLC has been discussed in detail by Biegenowska and Glowniak [130, 144]. They report that the highest R' values on NP–SG are possessed by CMs the structures of which contains a diol fragment, and this not only in the benzole nucleus as, for example, in columbianetin (XXVI) and khellactone (XXVII), but also in the alkyl substituent — meranzin hydrate (XXVIII) and oxypeucedanin hydrate (XXIX). In this CS (Table 4, No. 10), the elution of CMs by nonpolar solvents saturated hydrocarbons), takes place in the order of a decrease in those lipophilic properties. Nevertheless, it may change under the influence of various organic modifying agents for MPs [145]. For example, the unusual order of elution of CMs changes greatly on the use of such modifying agents for MPs containing heptane as groups of cyclic ethers with electron-accepting oxygen-atoms: THF and dioxane. The values of R' least "resistant" to their action are those of meranzin (XXX) and of O-preynl-(XXI). On the addition of MeCN or iso-PrOH, in contrast to CH_2Cl_2, to an MP, more pronounced changes are undergone by derivatives with a free 7-OH group — (XXI), demethylsuberosin (XXXII), osthenol (XXXIII), ostruthin (XXXIV), and scopoletin (XXXV) — than 7-methoxy derivatives — herniarin (XXXVI), osthole, O-phenyl-(XXI), and (XXX) [144]. Attention is attracted to the fact that the replacement of a 7-OH group by a methoxy group also exerts an influence on the order of elution of glycosides of 4-phenyl-(XXI) in an RP system (Table 4, No. 11). Thus, the 5-O-glycoside with a free 7-OH is eluted earlier than the 7-OCH_3 analogue [20]. The same sequence is observed for derivatives of (XXVI) [33], in both cases a faster elution of the apiosyl glucosides than of the diglucosides was observed.

The weak acidic properties of the HCMs favor the formation of ion pairs with quaternary organic ammonium bases in the form of which their separation carried out in the IP-HPLC method. With a rise in the concentration of the ion-pair reagent in the MP, there is a linear increase in R' for 4-HCM derivatives — for example, for difenacoum (XXXVII) in the interval of 0-10 mM tetrabutylammonium phosphate at pH 7.5 [63, 87]. The IP-HPLC of coumarin with phenolic compounds has been carried out in an acidic MP (pH 3.7) [107]. As compared with RP-HPLC, the order of elution of some CMs in this case may acquire a somewhat different form. In the separation of hydroxy analogues of WFR, the WFR derivatives hydroxylated in the α-benzyl nucleus are eluted first, and then, successively, those hydroxylated in positions 6, 7

and 8 of the coumarin nucleus. Enantiomers of warfarin alcohol having a second asymmetric C_{11} atom are separated completely at the beginning of elution, while those containing in addition to this a 4'-OH group appear at the end [63]. In the case of the separation of coumarin rodenticides, the order of elution corresponds completely to that in RP-HPLC [28, 29]: WFR < coumatetralyl < bromadialone < difenacoum < brodifacoum.

Chromatography on unmodified silica gel (NP systems) gives real competition with RP-HPLC only in methods of separating furocoumarins. The first place in NP systems is taken by eluents based on saturated hydrocarbons: hexane (HX) cyclohexane (CHX), and heptane (HP), most frequently associated with esters such as ethyl acetate (EtAc), chlorinated hydrocarbons such as dichloromethane (DCM), and chloroform (Chlf), or alcohols — MeOH, PrOH, AmOH.

With respect to FCMs [14, 96, 97, 146] and for CMs of other groups the most universal MP is HX–EtAc. At an HX content of 75-90% a successful separation has been achieved of more than 15 isomers of ferulenol (XLI) and ferprenin (XLII) [16] and esters of dihydroxanthyletin (XLIII) [96], and also a mixture of edultin (XLIV) and columbianadin (XLV) that was undifferentiated in the RP system [127]. However, Thompson and Brown [93] did not succeed in separating a mixture of bergapten, phellopterin, and imperatorin. An MP with the composition HX–iso-PrOH was used for separating HCMs [118] and PCM isomers [12]. The combination of CHX and EtAC [44, 56] and a mixture of CHX with iso-PrOH and a small amount (2.5-5%) of AmOH [55, 122, 126, 147, 148] are effective for multicomponent mixtures of FCMs. An MP based on HX–DCM with additions of 0.7-2.0% of acetic acid has been used for the separation of 4-HCM derivatives [28, 87] and one containing THF for PCM isomers [43]. In this case, for the FCMs iso-PrOH (3.5-5.0%) was used as an MP modifying agent [46, 144], while the selectivity of this MP exceeded that of an analogous MP used by Glowniak et al. [39] that contained 1.5-3.0% of MeCN.

Mixtures not containing saturated hydrocarbons, such as chloroform with additions of MeOH [146, 98], formic acid, or ethyl acetate [149, 150] are less selective, which may, in addition, lengthen the separation procedure. It is therefore desirable to use the MPs described above or those with a complex composition proposed by Zogg et al. [27] (Table 3, No. 2). It has been established that in the PLCC of coumarin glycosides (rutarensin, edgeworosides B and C) the latter are eluted in chloroform fractions containing 10-20% of MeOH [151]. Some MPs used in PLCC (NP systems) may contain a small amount of water (0.04-5.0%) [27, 95, 98, 146]. It is considered that this leads to a faster equilibration of the column and to an increase in the reproducibility of the method [22].

CHROMATOGRAPHIC SYSTEMS WITH IMMOBILIZED LIGANDS

Recently, polymeric adsorbents of the affinity type with phenolic and polyphenolic ligands have been proposed for the separation and isolation of CMs. The separation of the peaks of CMs and their R' values on adsorbents of the affinity type are approximately 1.5 times greater than on an unmodified matrix. A lowering of the efficiency of separation with a pronounced rise in the concentration of ligand (>50 μM/ml of packed adsorbent) has been observed for the first time in LCC. The order of elution is, on the whole, analogous to that in RP systems (RP–SG), although the capacity exceeds that of the well known SG–C_{18} [46, 50-52]. In the low-pressure LCC regime it is possible to separate in a short time not less than 5-6 structurally close CMs and SCMs (5 × 75 mm column) in a total amount of 0.5-0.6 mg.

It is known that the formation of specific complexes with proteins plays an important role in the realization of mechanisms of the biological activity of many drugs. Thus, Fitos and Somonyi [47] have used the receptor properties of HSA for the separation of enantiomers of WFR by affinity chromatography [47]. The elution of the WFR from the immobilized HSA was carried out with solutions of compounds competing with the WFR for binding with the protein (diazepam derivatives). It was found that the separation of the R- and S-isomers took place not only on the use of optically inactive clonazepam but also with S-uxepam (4,5-dyhydrodiazepam), while R-uxepam caused no separation. Somewhat later, Szinai et al. used this sorbent for the preparative isolation of the entiomers and then for a study of their pharmokinetics [47a].

In the HPLC of enantiomers of WFR on an adsorbent with immobilized BSA, Wainer and Chu showed an improvement in their separation by elution with certain groups of compounds (for example, trichloroacetic acid) which bind with the same sections of the biopolymer-ligand as

WFR [48]. Völker and De Vries [48a] have described the use of this acid as a component part of the eluent in the HPLC of enantiomers of phenprocoumon on the sorbent Nucleosil Chiral 2R.

The separation of racemic WFR into enantiomers on an adsorbent with α_1-acid glycoprotein was achieved by Hermansson [49]. Chromatography on a chiral-phase polyamide with immobilized acetylquinine has been used for the same purpose [152]. A polymeric adsorbent with a chiral phase having the structure of poly(ethyl S-2-(acroylamino)-3-phenylpropionate) has been used for the preparation of isomeric photodimers of coumarin [153]. The use of affinity chromatography for the analysis of aflatoxins has been described in [154].

A method of separating the FCMs (III), (V), and (VI) proposed Gazdag et al. [155] and later modified by Cepeda-Saez et al. [156] on an adsorbent with immobilized β-cyclodextrins (β-CDs) – Cyclobond I – is of interest. The mechanism of separation is connected with the formation of specific adducts of bergapten with the β-CDs of the "host-guest" type (because of which it is retained most strongly on the adsorbent), and this has been confirmed by spectral studies [157]. R- and S-Naphthyethylcarbamate and derivatives of β-CDs obtained by Armstrong et al. have proved effective for the separation of enantiomers of the coumarin rodenticide coumachlor. An important advantage of this stationary phase is the possibility of working both in RP and NP HPLC systems [157a]. It has been established by the HPLC method that carboxymethyl and hydroxypropyltrimethylammonium ethers of β-CDs form complexes with warfarin [157b].

In addition to CC with chemically immobilized ligands, we must mention adsorbents with "impregnated" ligands. Thus, the separation of racemic mixtures of CMs is possible through a mechanism of complex-formation with metal ions. In this way, on CG treated with a 5-10% solution of silver nitrate Wu et al. separated the (–)-isomer of omphalocarpin (XLVI) from a mixture with the (+)-isomer and murracarpin (XLVII) [24, 25]. Optically active murrayatin (XLVIII) has been isolated by Baric et al. [158].

PROGNOSIS OF THE CHROMATOGRAPHIC BEHAVIOR OF COUMARINS

The search for an MP of suitable composition for increasing selectivity is usually carried out empirically. The majority of authors use simple, previously known, MPs. Only comparatively recently in the LCC of coumarins have attempts been made to connect the structure of CMs and the composition of the mobile phase with chromatographic behavior. Many authors have reported a linear dependence of $\log R'$ on the proportion of modifying agent increasing the eluting power of the MP [130, 144]. Nevertheless, by careful measurements of R' under the conditions of RP-HPLC (MP: MeOH–water). Düren and Diehl have established that for a number of CMs this relationship is described by a quadratic equation. The "lipophilicity" parameters of 7-hydroxy, 7-alkoxy-, and 7-alkoxy-4-alkylcoumarins expressed in terms of $\log R'$ rise in proportion to the length of the chain of the substituent (C_1–C_{12}) [92].

Taking the specific nature of solvents into account through "molecular binding capacity" and "solvate selectivity" indices based on Synder's well known classification (in terms of eluting strength) [159] has enabled Vuorela and Lehtonen [145] to predict the R' values of the FCMs (VI, XVIII, XX, XXIX, and XLV), oxypeucedanin (XLIX), isobyakangelicin angelate, and ostruthol (L) in aqueous solutions of MeCN, dioxane, THF, MeOH, etc. However, the calculations of these indices require the use of a computer and, in the majority of cases, the values of R' found (particularly at $R' > 15$) do not agree with those observed [145].

The optimization of the composition of an MP for solving applied problems is more convenient with the aid of the PRISMA three-dimensional model [94, 95a, 160]. Thus, after 4-5 control experiments Vuorela et al. determined the necessary components of a complex mixture (THF, MeCN, MeOH, and water) for the separation of the same model mixture of FCMs and their isolation from an extract of Peucedanum palustre containing them [95a]. The use of this method for methoxy derivatives of psoralen and angelicin has been discussed in detail by Nyredy et al. [94] and by Härmäkä et al. [160, 161], and a full description of this principle is given in a monograph by Schoenmakers [88].

In conclusion, we may mention that from information given in the present review clearly suggests future developments in the use of liquid column chromatography in the field of coumarin studies. The main tendency of the modern stage of the development of this method appears in the wide use of chemically modified adsorbents, including those created by the classical strategy of adsorbents of the affinity type.

NOTATIONS

(I) Benzo-α-pyrone (coumarin); (II) Xanthotoxol; (III) Xanthotoxin; (IV) Sphondin; (V) Isopimpinellin; (VI) Bergapten; (VII) Sibiricin; (VIII) Frutinone A; (IX) Frutinone B; (X) Angelicin; (XI) Isobergapten; (XII) Pimpinellin; (XIII) Psoralen; (XIV) Esculetin; (XVII) Osthole; (XVIII) Isoimperatorin; (XIX) Phellopterin; (XX) Imperatorin; (XXI) Umbelliferone; (XXII) Hydrangetin; (XXIII) Isofraxidin; (XXIV) Necatorin; (XXV) Bergaptol; (XXVI) Columbianetin; (XXVII) Khellactone; (XXVIII) Meranzin hydrate; (XXIX) Oxypeucedanin hydrate; (XXX) Meranzin; (XXXI) 3-Methoxypereflorin; (XXXII) Demethylsuberosin; (XXXIII) Osthenol; (XXXIV) Ostruthin; (XXXV) Scopoletin; (XXXVI) Herniarin; (XXXVI) Difenacoum; (XLI) Ferulenol; (XLII) Ferprenin; (XLIII) Xanthyletin; (XLIV) Edultin; (XLV) Columbianidin; (XLVI) Omphalocarpin; (XLVII) Murracarpin; (XLVIII) Murrayatin; (XLIX) Oxypeucedanin; (L) Ostruthol.

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