CURRENT STATE OF THE LIQUID COLUMN CHROMATOGRAPHY OF COUMARINS.

II. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF COUMARIN DERIVATIVES

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Literature information for the period 1983-1990 on the use of high-performance liquid chromatography (HPLC) in the phytochemical, biochemical, toxicological, and pharmaceutical analysis of coumarin derivatives has been generalized and systematized. Conditions for the separation of complex mixtures of coumarins are considered, and questions of the use of coumarins in the HPLC analysis of natural and synthetic organic compounds are discussed.

In the first paper of this series [1], we discussed some questions of the practical use of the liquid column chromatography in the study of natural coumarins (CMs). The present review is devoted to the HPLC of coumarins and covers its use in the field of phytochemistry, biochemistry, and pharmaceutical and toxicological analysis.

HYDROXYCOUMARINS AND COUMARINS OF SIMPLE STRUCTURE

The substantial differences in the polar properties of many hydroxycoumarins (HCMs) require the individual selection of the composition of the mobile phases (MPs). Universal from this point of view are binary MPs that consist of components mixed in many ratios and are used particularly frequently in the form of an isocratic regime. Under the traditional conditions for the preparative isolation of CMs by the HPLC method that are now obsolete (Table 1, Nos. 1-4), Bittner et al. [2] separated scopoletin (I) and the 6-hydroxy-3-methoxy derivative of pereflorin (II) [2]; Zdero et al. separated a mixture of hydroxy and methoxy derivatives of (II) [3] and coumarins with structures (III)-(V) [4]; and Jakupovic et al. separated the acetate and propionate of 9-methyllongipesin (IV) [5]. Isocratic elution with 40% aqueous methanol, which was used for the separation of 4-(3',4'-dihydroxyphenyl)- and 4-(3',4'-dimethoxyphenyl)-8-hydroxy-5,7-dimethoxycoumarin by the HPLC method [6] proved equally effective in relation to glycosides with an analogous structure of the aglycon – biosides of 4-(3',4'-dihydroxyphenyl)-5,7-dihydroxycoumarin [7]. Jensen et al. used a linear gradient of aqueous methanol in the isolation of the 7-O-primveroside of (I) – fabiatrin [8] (Table 1, Nos. 3 and 4a).

Normal-phase (NP) HPLC is used extremely rarely for the CMs of the group under consideration. Thus, the separation and isolation of (I) from isofraxidin (V) on silica gel (SG) has been described in [9] (Table 1, No. 6). At the same time, the use of NP HPLC for the separation of structurally close isomeric derivatives of ferulenol and ferprenin has been carried out by Appendino et al. [10] (Table 1, No. 7).

A large number of studies have been devoted to the HPLC of 3-substituted derivatives of 4-hydroxycoumarin (4-HCM, VIa), so-called coumarin anticoagulants and rodenticides (Table 2, Nos. 4-30). Warfarin (WFR, VIb), Phenprocoumon(PPN, VIc), and dicumarol (VIIa) in complex powders [11] and tablets [12]. In the organism, these medicinal agents (MAs) readily interact with blood plasma proteins, forming stable complexes.

By modeling the influence of proteins on the determination of WFR in blood plasma, Shibukawa et al. showed that its mixture with albumin is eluted in the form of two peaks – free and protein-bound WFR [13, 14]. Therefore, if the object of analysis consists of bio-

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TABLE 1. Conditions for the Analysis of Coumarins by High-Performance Liquid Chromatography (IC — isocratic; LG — linear gradient; UV — ultraviolet; FL — fluorescent detector)

Number	Adsorbent, column (mm)	Mobile phase	Detector, wavelength (nm)	Source of isolation	Literature
	1	2	3	4	5
		I. Hydroxycoumarins			
1	RP-8	IC:MeOH—water -	UV	Perezia megalantha	[2]
2	"	IC:MeOH—water (7:3)	UV	Dolichlasium lagascae	[3]
3	"	" (4:1)	UV	Bathriocline longipes	[5]
4a	Bondapak C_{18} (300 × 7.3)	" (4:6)	UV	Coutarea hexandra	[6, 7]
4b	Bondapak C_{18} (300 × 3.9)	IC: MeOH—water—acetic acid (50:50:1)	UV	—	[155]
5	RP-8	LG: MeOH—water (15.2% → 33.3%)	UV	Gelsemium sempervirens	[8]
6	Silica gel (100 × 4.5) (250 × 7.0)	IC: ethyl acetate—petroleum ether—acetic acid (10:9:1)	UV, 254	Tanasetum parthenium Tanacetum vulgare Artemisia vulgaris	[9]
7	Silica gel	IC: hexane—ethyl acetate (9:1, 7:3, 4:1)	Differential reflectometer	Ferula communis	[10]
		II. Furocoumarins			
8	Spherisorb ODS (100 × 4)	LG: MeOH (25% → 40%) in a mixture of THF and water (3.5:96.5)	UV	Heracleum sphondylium Heracleum montegozzianum	[68]
9	Spherisorb ODS (100 × 4)	IG: aqueous—organic mixtures	UV	Heracleum sphondylium	[67]
10a	"	IC: THF—MeCN—MeOH—water (2.9: 32.5: 5.0: 59.6)	UV, 320	Peucedanum palustre	[156]
10b	Spherisorb C_{18} (300 × 3.9)	IG: MeOH—water (65:35)	UV	—	[155]
11	LiChroprep RP-8 (310 × 25)	" (6:4)	UV	Heracleum fruit	[64]
12	LiChrosorb RP-18 (200 × 8)	" (6:4)	UV	Peucedanum oreoselinum	[76]
13	Silica gel C_{18} (300 × 3.9) (250 × 10)	IC: MeOH—water (0:1, 7:3, 1:0); MeOH—water (55:45), (48:52)	UV, 315; FL, 315/425	Citrus floveda	[74]
14	Separon $SGXC_{18}$ (250 × 4)	LG: MeOH—water (0% → 100%)	UV	Angelica silvestris	[63]
15	TSK gel LS-410 (150 × 4)	IC: MeCN—water (35:65)	UV	Cnidium monnieri	[69]
16	Silica gel C_{18}	" (37:63)	UV	Thamnosma texana	[70]
17	MCH-10 (500 × 8)	IC: MeCN—water (35:65)	UV, 286	Petroselium crispum	[72]
	MCH-10 (300 × 4)	" (50:50)	"	Coriandrum sativum	[55]
18	RP-8	IC: MeOH—water (17:3)	-	Triptilion benaventi	[71]
19	Nucleosil 5—C_{18} (250 × 4)	IC: MeOH—acetic acid—1 M $NaNO_3$ solution—water (34:1.5:0.5:64); MeOH—THF—acetic acid—1 M $NaNO_3$ solution—water (34:5:1.5: 0.5:39)	Electrochemical detector	Citrus fruit	[80]
20	Silica gel C_{18}	LG: MeOH—water (50% → 95%)	FL	Products of the photochemical decomposition of xanthoxin and bergapten	[8]
21	Silica—Senshu pak (250 × 8)	IC: hexane—ethyl acetate (3:1)	UV, 320	Angelica edulis	[75]
22	"	" (5:8)	UV, 320	Products of the reduction of nodakenetin	[77]
23	Radial-Pak B_5 (110 × 5)	" (9:1)	UV, 254, 275	Unaspis yanenensis	[79]
24	LiChrosorb Si_{60} (250 × 4)	" (8:2)	UV	Cnidium monnieri	[69]

TABLE 1 (continued)

Number	Adsorbent, column (mm)	Mobile phase	Detector, wavelength (nm)	Source of isolation	Literature
25	Silica gel Si_{60} (300 × 8)	IC: cyclohexane–ethyl acetate (5:2)	UV, 254, 313	Pituranthas triadiatus	[61]
26	LiChrosorb Si_{100} (250 × 4)	" (7:3)	UV	Seseli elatum	[60]
27	Silica gel Si-5 (300 × 4)	IC: cyclohexane–iso-PrOH–AmOH (15:4:0.5)	UV	Umbelliferae roots	[57] [56]
28	"	IC: cyclohexane–iso-Pr_2O–AmOH (15:4:0.5)	UV, 254	Petroselinum crispum	[55]
29	Silica gel (100 × 5)	IC: cyclohexane–n-Pr_2O–AmOH (15:4:1)	UV, 254	Pastinaca sativa	[53] [54]
30	LiChrosorb Si_{60} (250 × 4)	IC: n-heptane–CH_2Cl_2–iso-Pr_2O (45:55:3.5)	UV	Peucedanum palustre	[58]
31	Silica gel	IC: $CHCl_3$–ethyl acetate–formic acid (99.8:0.1:0.1)	UV, 250	Apium graveolens	[51] [52]
32	Silica–Senshu pak (250 × 8)	IC: ethyl acetate–MeOH–water (35:8:2)	UV, 320	–	[78]
33	"	IC: $CHCl_3$–MeOH–water (30:10:1)	UV, 320	Peucedanum palustre	[77]
34	LiChrosorb Si_{60} (250 × 16)	IC: $CHCl_3$–ethyl acetate–hexane (20:20:60)	UV, 313	Heracleum mantegazzianum	[66]
		III. Pyranocoumarins			
35	Ultrasphere CN	IC: hexane–iso-PrOH (7:1), (5:1)	FL	Musineon divaricatum	[88]
36	Ultrasphere ODS	IC: MeOH–water (7:3), (4:1)	UV, FL	Musineon divaricatum	[88]
37	Silica–Senshu pak (250 × 8)	IC: hexane–ethyl acetate (3:1)	UV, 320	Peucedanum decursivum	[89]
38	Silica gel Si_{60} (250 × 16)	IC: hexane–CH_2Cl_2–THF (75:25:1)	UV	Eryginum campestre	[91]
39	RP-18 (250× 16) (250 × 4.6)	IC: MeOH–water (6:4)	UV	Eryginum campestre	[91]
40	LiChroprep RP-8 (270 × 25)	IC: MeOH–water	UV, 275	Polygola paniculata	[92]
41	RP-8	IC: MeOH–water (4:1)		Nassauria magelanica	[94]
42	Silica gel C_{18}	IC: MeOH–water (65:35)		Jellyfish Echinogorgia reticulata	[95]
		IV. Mixtures of Coumarins with Compounds of Different Structure			
43	Bondapak C_{18}	LG: MeCN (20% → 90%)in a mixture of water and acetic acid (99:1)	UV, 340	Artemisia	[114]
44	(300 × 3.9)	IC: solution of sodium hydrogen phosphate (pH 7.3)–dioxane (63.7:36.3)	UV, 340	Matricaria chamomilla	[115]
45	Hypersil ODS (100 × 2.1)	IC: phosphoric acid (pH 2.8)–MeCN (39:1; 4:1; 13:1; 0:1)	UV, 337	Eclipta alba	[116]
46	LiChrospher 100 CH-18/2 (125 × 4)	LG: MeCN (15% → 40%) in 0.003% phosphoric acid	UV, 254	Matriacaria chamomilla Eclita alba	[117]
47	Nucleosil C_{18} (250 × 4)	LG: phosphoric acid (1.5% → 40%) in MeCN–MeOH–water (1:1:1)	UV, 280, 320	Picea abies	[118]
48	Hypersil ODS Nucleosil C_{18}	Programmed gradient of BuOH–MeOH (5:1) in 1% acetic acid, from 8% → 80%, etc.	UV	Prunus tissues	[119]
49	Nucleosil C_{18}	LG: MeOH (5% → 60%) in 0.04 M formic acid; MeOH–water (10% → 25%)	UV, 254, 280	Zea mays	[120]
50	Bondapak C_{18} (300 x 3.9)	IC: MeOH–water (4:6); MeOH–water–acetic acid (4:6:1)	UV	Vanilla planifolia	[123]
51	LiChrospher RP-8 (250 × 4.6)	IC: MeOH–water–acetic acid (15:80.7:4.3)	UV, 350	Products of an enzymatic reaction	[124]
52	LiChrosorb RP-8 (250 × 7)	IC: THF–MeCN–MeOH–water (8:20:12:60)	UV, 275	–	[126]
53	"	IC: MeOH–water (1:1)		–	[127]
54	Silica gel C_{18} (150 × 4)	IC: MeCN–0.06 M phosphate buffer (pH 3.7) (32:68) + 5 mM tetraamylammonium bromide	UV, 254	Cinnamomum cassia	[128]
55	Bondapak C_{18} (300 × 7.8)	IC: MeCN–water (pH 3.0)	UV, 254	Glycine max.	[132]
56a	Zorbax ODS (250 × 4.6)	IC: MeOH–acetic acid	UV	Artemisia scoparia	[155]
56b	RP-8	IC: MeOH–water (1:1)	UV	Artemisia herba-alba	[134]
57	"	" (9:1)	UV	Brachuclados megalanthus	[4]
58	"	" (4:1)	UV	Dolichlasium lagasceae	[3]
59	"	" (4:1)	UV	Gypothamnium pinifolium	[135]
60	"	" (17:3)		Aphyllocladus denticulatus	[136]
61	LiChrosorb C_{18} (150 × 4.6)	IC: MeCN–MeOH–water (28.5:13:58.5); (31.8:22.7:45.5); MeOH–water (65:35)		Citrus juice	[137]

TABLE 2. Conditions for the Analysis of Coumarin Derivatives by High-Performance Liquid Chromatography in Medicinal Preparations and Biological Materials (IC — isocratic; LG — linear gradient; UV — ultraviolet; FL — fluorescent detector)

No.	Adsorbent, column (mm)	Mobile phase	Detector, wavelength (nm)	Literature
	1	2	3	4
1	Chromsep C_{18} (100 × 3)	IC: MeCN—0.05 M ammonium acetate buffer, pH 4.25 (88:12)	UV	[43]
2	Bondapak (300 × 3.9)	IC: MeOH—water—AcOH (20:30:0)	UV, 280	[45]
3	LiChrosorb Si_{60} (250 × 4)	IC: n-hexane—iso-PrOH (15:1)	UV, 280	[46]
4	Nucleosil $5C_{18}$ (150×4)	IC: MeOH—water (3:7)	UV, 280	[46]
5	Hypersil ODS	IC: MeCN—0.1 M ammonium formate, pH 3.5 (23:77)	UV, 254	[35]
6	Bondapak (300 × 3.9)	IC: MeOH—water—AcOH—THF (10:65:0.1:35)	UV, 311	[11]
7	LiChrosorb RP-8 (150 × 4.6)	IC: 0.1% AcOH—MeCN—ethyl acetate (100:9:1) + 1 N ammonia solution to pH 4.9	UV, 303	[24] [19]
8	"	LG: MeCN—water (40% → 100%) + 0.5% acetic acid	UV, 313	[18]
9	LiChrosorb RP-18	IC: MeOH—dilute H_3PO_4 (75:25)	FL	[28]
10	"	IC: MeCN 0.1% ammonium acetate, pH 4.8 (3.5)	UV	[44]
11	RP-18 (150 × 3.2)	IC: MeCN—phosphate buffer, pH 2.3 (1:2); MeOH—0.5 M ammonium chloride solution—2 M ammonium acetate buffer (60:40:2)	—	[50]
12	Silica gel C_{18} (250 × 4.5)	IC: MeCN—0.05 M sodium dihydrogen phosphate (3:7, 4:6; 5:5, 6:4)	UV, 310	[49]
13	Silica gel C_{18} (250 × 4.5)	IC: MeOH—THF—5 mM ammonium phosphate, pH 7.5—1 M tetrabutylammonium phosphate (30:7:62:1)	Photodiode, 310	[36]
14	Silica gel C_{18} (150 × 4.6) (250 × 4.6)	IC: THF—0.1 M phosphate buffer, pH 6.0	Photodiode, 310	[13]
15	Bondapak C_{18} (250 × 3.9)	IC: 1.5% ammonium acetate, pH 4.7—MeCN (69:31)	UV, 310	[38]
16	"	IC: MeCN—0.1 M ammonium dihydrogen phosphate (63:37)	Fl 320/390	[16]
17	"	IC: MeOH—water—acetic acid (56:40:4)	UV, 254, 280	[15]
18	Bondapak C_{18} (250 × 3.9)	IC: MeOH—water—acetic acid (30:66.5:3.5)	UV, 313	[21]
19	Nucleosil chiral-2R	IC: n-heptane—dioxane—trichloroacetic acid (100:25:0.1)	FL	[30]
20	Spherisorb ODS (150 × 4.8)	IC: MeCN—MeOH—acetate buffer, pH 4.0 (11:44:45)	UV, 300	[17]
21	Spherisorb ODS (250 × 4.6)	IC: MeOH—5 mM phosphate buffer, pH 3.0 (2:8)	UV, FL	[27]
22	Spherisorb (250 × 4.6)	IC: hexane—acetic acid—CH_2Cl_2 (8.0:1.84:90.16)	UV, FL	[27]
23	Spherisorb (250 × 4.6)	IC: hexane—acetic acid—CH_2Cl_2 (34.3:65.0:0.7)	FL, 310/390	[25]
24	Sperisorb ODS (250 × 4.6)	LG: MeOH—0.25% acetic acid (65% → 84% → 95%)	FL, 310/390	[25]
25	Hypersil ODS (250 × 4.6)	IC: MeOH—water (76.5:23.5) + 5 mm tetrabutylammonium phosphate	UV, FL	[27]
26	Hypersil APS (250 × 4.6)	IC: MeCN—0.04 M tris-HCl buffer, pH 7.5 (15:85)	UV, FL	[27]
27	Hypersil ODS (250 × 4.6)	LG: MeOH—water (58% → 78% → 88%) + 5 mM tetrabutylammonium phosphate	FL 310/390	[26]
28	Ultrasphere C_{18} (100 × 5)	IC: dioxane—water (4:6) pH 4.2	UV, 305	[47]
29	Ultrasphere C_{18} (250 × 4.6)	IC: 1.5% ammonium acetate buffer, pH 4.7—MeCN (69:31)	UV, 310	[39]
30	Nova-Pak (150 × 4.6)	IC: 0.25 M ammonium acetate buffer, pH 4.85—MeCN (72:28)	—	[37]
31	Supelcosil (150 × 4)	IC: MeOH—water (6:4)	UV	[83]
32	Silasorb (64 × 2)	IC: hexane—chloroform (7:3)	UV, 240	[81]
33	Cyclobond I (250 × 4.6)	IC: 0.01 M solution of β-cyclodextrin in MeOH—water (1:4)	FL, 317/490	[154]
34	Nucleosil C_{18} (250 × 4)	IC: MeCN—0.05% phosphoric acid (1:1)	UV, 220	[85]
35	Nucleosil C_{18} (250 × 4.6)	LG: MeOH—MeCN (0.9% → 10%) in 5 mM phosphate buffer, pH 2.8	Scintillation	[86]

fluids, the preparation of the sample for HPLC must necessarily include, in addition to the concentration of the CM, a stage of breaking down the MA-protein complex. Methods are known for the quantitative HPLC analysis of neodicoumarin (VIIb) [15], WFR [16], and chlorine-containing and other analogues of WFR [17, 18] in blood plasma (Table 2, Nos. 17, 16, 20, and 8). The use of HPLC for the determination of the pharmacokinetic parameters of WFR [19-21], of PPN [22], and of a new MA with an antiallergic action – picumast (VIII) and its metabolites M_1 and M_2 [23] – has been described. A method developed by Thijssen et al. for the analysis of acenocoumarin (ACN, VId) has also been proposed for the determination of its amino- and acetamido metabolites (Table 2, No. 7) [24].

Hunter [25, 26], analyzing coumarin rodenticides – WFR, coumatetralyl (IXa), diphenacoum (IXb), brodifacoum (IXc), and bromadiolone (X) – showed that the separation of (IXa) and the isomers of (X) and also of WFR and (X) [25], and WFR and (IXa), (IXb), and (X) [26] is readily achieved by isocratic elution (Table 2, No. 23). However, the separation of a mixture of all the above-mentioned compounds was possible only with the use of a programmed linear gradient (Table 2, Nos. 24 and 27). Several variants of HPLC (NP, reversed-phase (RP), ion-pair) were tried with the same aim and were used together with ion-exchange chromatography for the separation of enantiomers of (X) in [27] (Table 2, Nos. 21, 22, 25, 26). Methods for determining residual amounts of (X) in biomaterial have been developed by Morin et al. [28] (Table 2, No. 9).

Because of differences in pharmacological activity and in pharmacokinetic parameters, the quantitative determination of the individual enantiomers of coumarin anticoagulants is of great importance for therapeutic monitoring. The HPLC methods proposed for the separation of the enantiomers of FPN and WFR [29], including the use of chiral [14, 30, 31] and affinity [32-34] sorbents, may be the optimum means of obtaining such information. A procedure is known for the quantitative determination of the R- and S-isomers, developed by Ueland et al. [35], that does not require their separation.

Wong and Davis, using ion-pair TLC for the products of the transformation of WFR in a suspension of cells of the fungus Cunninghamella elegans, which has been used as microbiol model of the metabolism of mammals, revealed the 6-, 7-, 8-, 4'-, and 3'-hydroxy metabolites of WFR and some others. In this work they separated a mixture of 11 compounds [36] (Table 2, No. 13). Some of them have been analyzed by Spink et al. [37] by RP HPLC (Table 2, No. 30). Under similar conditions, Davis and Rizzo determined WFR and SPN in the presence of metabolites with a ketonic structure that had been detected in the cells of Aspergillus niger [8], and in an investigation of the mechanism of the formation of 4'-hydroxy metabolites of WFR they separated enantiomers of the intermediates in the synthesis of 4'-deutero-WFR: 4-bromo- and 4'-deuterocyclocoumarol (XI) (Table 2, No. 29) [39], representing structural analogues of natural derivatives of isotriptospinocoumarin (XLIV).

The nature of the enzymatic reduction of the keto groups of ACN [40] and WFR [41] in cytosol and human and animal microsomes and of the biotransformation of ACN in the human organism under the influence of MA [42] have been studied by the HPLC method. The determination of scoparone (XII) and its metabolites (I) and isoscopoletin (XIII) have been described [43, 44]. Metabolites of coumarin (umbelliferone(XIV), (VIa), and O-coumaric and o-hydroxyphenylacetic acids) [45] and 7-vinyloxycoumarin (XV) have been identified [46]. Conditions for the performance of HPLC are given in Table 2, No. 10, 1-4.

In order to eliminate possible errors in the identification of CMs from their retention times, the internal-standard (IS) method is frequently used. As the ISs are used not only CMs close in structure to those being analyzed but also other compounds (Table 3). For example, WFR is eluted between PPN and 4-methyl-WFR, and Parisi et al. used these two compounds as ISs in the determination of WFR [47]. Shaikh [48] used umbelliferons as IS in the determination of aniline as an impurity in arsanitic acid.

FUROCOUMARINS

A fairly large number of MPs for NP HPLC intended mainly for the separation of derivatives of psoralen (XVI) and of angelicin (XVII) are known (Table 2, Nos. 21-34). The separation of simple mixtures of methoxy derivatives of these furocoumarins (FCMs) for example, a mixture of (XVI), begapten (XVIII), xanthotoxin (XIX) and isopimpinellin (XX) has been described by Beier et al. [51, 52]. Under conditions developed by Berenbaum et al., the separation of (XVIII)-(XX), imperatorin (XXI), and sphondin (XXII) was achieved in 6 min [53, 54] (Table 1, Nos. 31, 29). Under similar conditions but using longer columns (up to

TABLE 3. Internal Standard for the High-Performance Liquid Chromatography of Coumarins

Compounds analyzed	Internal standard	Literature	Compounds analyzed	Internal standard	Literature
Metabolites of WFR	Phenprocoumon	[39]	Aflatoxin M_1	Aflatoxin B_2	[97]
"	9,10-Dihydro-WFR, warfarin alcohol	[36]	Furocoumarins	Ethyl salicylate	[69]
WFR	Phenprocoumon, 4-methyl WFR	[47]	"	Ethyl vanillate	[80]
WFR in a mixture with albumin	4'-Hydroxywarfarin alcohol	[13]	Coumarin and phenolic compounds	Vanillin	[126]
Metabolites of WFR	Deutero-6-hydroxy-WFR	[37]	WFR	Naproxen	[16, 21]
FPN	p-Chloro-FPN	[18]	Neodicoumarin	Carbamazepine	[15]
Psoralen	Bergapten	[83]	Bromadiolone	Chlorophacinone	[28]
Xanthotoxin	4,5',8-Trimethylpereflorin	[85]	Psoralen	2,4-Dinitrobenzene	[81]
Bergapten	Psoralen	[154]			

300 mm), Chaudhary et al. [55], and also Ceska et al. [56, 57], separated, in addition to some of the above-mentioned compounds, (XVI), (XVII), and (XXV) [56] and (XVI), isoimperatorin (XXIII), oxypeucedanin (XXIV), oxypeucedanin hydrate (XXV), and byakangelicin [57] (Table 1, Nos. 27, 28). Other variants of the separation of such mixtures of FCMs have also been used [58, 59] (Table 1, No. 30). Locar and Delben [60] separated compounds (XIX), (XX), (XXII), and (XXV), and also xanthotoxol (XXVII), phellopterin (XXVIII), and the pyranocoumarin seselin (XXIX), the quantitative estimation of which was made the basis of a chemosystematic study of Seseli populations. Ashkenazy et al. [61] analyzed FCMs under similar conditions (Table 1, Nos. 25, 26). Thus, the MPs used in these methods permit the separation of 8- to 9-component mixtures of FCMs in 20 min [55-57]. However, the separation of alkoxy derivatives of FCMs with the aid of the programmed elution gradient described by Enriquez et al. [62] and by Kopesky et al. [63] takes longer, the time for the separation of 4- to 5-component mixtures reaching 25-50 min (Table 1, No. 14).

RP HPLC has proved to be more selective than NP HPLC in the separation of the complex of FCMs (XVIII-XXI), (XXVIII), and pimpinellin (XXX) contained in various Heracleum species [64]. By this method, Khetwal et al. [65] separated and isolated the FCMs from Heracleum brunonisi. Selective eluents and several variants of separation and detection for the HPLC of FCMs from other Heracleum species have been described [66-68] (Table 1, Nos. 11, 34, 8).

Sagara et al. achieved the separation of a 10-component mixture of FCMs (RP HPLC) containing, in addition to the most common ones (XVIII-XXI) and alloimperatorin (XXXI), derivatives of columbianetin (XXXIIa) — its acetylated and other analogous: edultin (XXXIIb) and columbianadin (XXXIIc)[69]. In the preparative RP HPLC regime, Oertli et al. [70] separated a mixture of these compounds with heraclein (XXXIII) and some HCMs: thamnosmin (XXXIV) and herniarin (XXXV) (Table 1, Nos. 15, 16). The conditions for the isolation by preparative HPLC of oxypeucedanin [55], a mixture of epimeric derivatives of 2',3'-dihydro-4,3:4',5'; furocoumarin — isotriptiliocoumarin (LIXa) [71] — the furoisocoumarins coriandrin (XXXVI) and 2,3-dihydro(XXXVI) [72], a mixture of columbianetin glycosides (columbianin) (XXXVII), and the O-diglucoside and O-apioglucoside of (XXXIIa) [73], and some others [74] have been described (Table 1, Nos. 17, 18, 13). McHale et al. [74] separated (XXXVII) and its analogue with an open dihydrofuran ring (XXXVIII) [74].

Derivatives of (XXXIIa) are found fairly frequently in the form of esters of organic acids. The separation of the esters formed by (XXXIIa) with angelic, senecionic, and acetic acids was achieved by Kawasaki et al. [75]. Lemmich and Gylle [76] isolated the diester of (XXXIIa) with angelic and isovaleric acids (Table 1, Nos. 12, 21).

Asahara et al. [77] and Motano et al. [78], using the NP HPLC method, isolated and analyzed the products of the reduction of nodakenetin (XXXIX), which was detected in the Chinese MA "Qian-Hu" and also the decurosides (III)-(V) contained in it (the 6'-O-apioglucoside of (XXXIX), the 6'-O-glucoside of 3-hydroxy-(XXXIX) [77], and the 6'-O-maltoside of (XXXIX)

[78]) (Table 1, Nos. 32, 33). At the same time, it must be mentioned that the chromatography of CM glycosides on NP sorbents in the HPLC regime is found extremely rarely.

HPLC has been used as the concluding stage in the identification and quantitative determination of psoralen [79] and bergaptol [80] in citrus fruit, and also for monitoring the quality of psoralen [81] and of xanthotoxin [82] in MAs with a photosensitizing action and for the therapeutic monitoring of psoralen [83] (Table 1, Nos. 23, 19; Table 2, Nos. 32, 31). The determination of xanthotoxin in blood plasma has been described [84, 85] (Table 2, No. 34). With the aid of HPLC, Mays et al. have separated and identified more than 10 metabolites of (XIX) consisting of 5,8-dihydroxy and 5,8-dioxopsoralen and 5-hydroxy-(XIX) and their sulfuric acid conjugates, and also (XXVII), 7-hydroxy-8-methoxy-6-coumarylacetic acid, and other compounds [86] (Table 2, No. 35). Decout et al. have developed conditions for the analysis of the products of the photochemical decomposition of xanthotoxin and bergapten, consisting of two chromophores linked by 2-12 methylene units [87] (Table 1, No. 20).

PYRANOCOUMARINS

The use of HPLC for the analysis of pyranocoumarins (PCMs) is of particular interest, since they include a large proportion of the new coumarins discovered recently.

A detailed scheme of the isolation of esters formed by khellactone (XL) and organic acids (angelic, isovaleric, acetic, etc.), many of which are isomers, is given in a paper by Swager et al. [88]. For the primary purification of the PCMs these authors used LCC on Florisil (magnesium silicate), Sephadexes LH-20 and LH-60, and also LP silica gel C_{18} and the sorbent Bio-Beads S-X8 (weakly cross-linked styrogel). The concluding stage of the separation included the use of MP HPLC in a flow of hexane–isopropanol and RP HPLC with elution by aqueous methanol (Table 1, Nos. 35, 36). In this way, about 20 compounds were isolated.

Sakakibara et al. [89] obtained a purified mixture (the above-mentioned acids esterified with 3',4'-dihydroxy-3',4'-dihydroxanthyletin (XLI)) in two stages: LCC on silica gel and preparative HPLC in a flow of hexane–ethyl acetate (Table 1, No. 37). In the case of the LCC on polyamide and Sephadex of ionized derivatives of (XL) and also of rutaretin (sulfates) it is recommended to add to the eluent (aqueous methanol) 0.1% of ammonium carbonate, and then to conduct their further separation on ion-exchange resins of the type of Dowex (K^+) and Lewatit SP 1080 (H^+) [90].

Erdelmeier and Sticher, using preparative RP and NP HPLC successively, isolated a mixture of derivatives of aegelinol (3'-hydroxy-(XLI)), and then separated agasyllin (XLIIa) and grandivittin (XLIIb), which are isomers. The stages of isolation were monitored on an analytical column [91] (Table 1, Nos. 38, 39). The new compound 3'-O-acetyl-4'-O-benzoyl-(XL) [92] and the products of the synthesis of esters of (XL) (praeruptorin, etc.) [93] have been isolated by the HPLC method (Table 1, No. 40). Bittner et al. have isolated derivatives of 2',3'-dihydroxy-3,4-dihydropyranocoumarin, so-called triptiliocoumarins (XLIII) [71] and isotriptospinocoumarins (XLIV) (Table 1, No. 1) [94]. Xanthyletin (XLV) and xanthoxyletin (XLVI) have been isolated from a marine organism (the medusa Echinogorgia reticulata) for the first time [95], (Table 1, No. 42).

MYCOTOXINS WITH ISOCOUMARIN AND COUMARIN STRUCTURES

Derivatives of isocoumarin (XLVII) and of coumarin, such as the aflatoxins (AFTs), are found among the highly toxic natural compounds mycotoxins, which are products of the vital activity of certain fungi.

A large number of methods are known for checking the quantitative level of AFTs in materials under investigation, which are most frequently fruit products [96], including dairy products [97, 98]. In view of the low concentrations of AFTs, special stages of enriching the samples for HPLC analysis are necessary. For this purpose use is made of Sep-Pak cartridges filled with RP [97, 99] or NP [100, 101] silica gels. It has been established that among organic solvents the best extractant for AFTs from plant materials is acetonitrile [102].

As Tomlins et al. have shown, the best results on the extraction and concentration of AFTs among alkyl (C_2, C_8, and C_{18}), cyclohexyl, and phenyl silica gels are achieved with the uses of the last-mentioned type, which is also recommended by these authors for the purification of AFTs in the preparation of the samples for HPLC [103]. At the present time, this sorbent is being used successfully for the solid-phase extraction of AFTs [104].

A comparison has been made of the results of certain control procedures for determining AFT B_1 in peanuts [105, 106]. The most promising method is acknowledged to be that using immunoaffinity chromatography for concentrating the AFTs (EASI-EXTRACT column).

In spite of the close structures of the AFTs, practically no difficulties connected with the separation by the HPLC method arise. In the overwhelming number of investigations, RP sorbents are used for this purpose (Table 4, Nos. 2-12). Methods for the simultaneous determination of AFT G_1 (XLVIII), G_2 (XLIX), B_1 (L), and B_2 (LI) in various species of Aspergillus – HPLC on a NP sorbent, used by Leitao et al. [107] and RP HPLC by Simonella et al. [101] – are equal to one another in efficiency (Table 4, Nos. 1, 2) and are not inferior to the method given in [96].

Bodine et al., investigating the transformation of AFT B_1 under the influence of enzymes, the so-called S-fraction of animal livers (in vitro), identified its metabolites – AFTs M_1 (LII) and Q_1 (LIV) and aflatoxicol – by HPLC [108]. Mention must be made of the possibility of an unforeseen increase in the time for the analytical procedure where standard methods for the HPLC of AFTs are used for separating their metabolites. This is connected with the fact that some of them (such as M_1, Q_1, and P (LIII)), and sterigmatocystin, are less polar than their precursors. In this case, the process can be optimized by converting the metabolites into hemiacetals which, like the AFTs, are more polar and are eluted more rapidly. This method has been described by Orti et al., who used it in the analysis of AFT B_1 and its metabolites in urine [109] (Table 4, Nos. 3, 4).

A special test for determining AFT B_1 in plant products has been proposed by Ruffell and Trinder [100]. Its chromatographic stages include absorption of the extract in a cartridge with silica gel and, after elution, chromatography in a minicolumn 2/3-filled with alumina (activity grade III, upper layer) and then with Florisil. Detection is performed from the fluorescence after irradiation with UV light and its comparison with a control. The total time of analysis is 30 min, and the limit of detection 3 μg/kg [100]. Different variants of the separation of AFTs M_{2a} and B_2 [97] and for the analysis of M_1 [98, 110] have been proposed (Table 4, Nos. 5-7).

In order to increase the sensitivity of the determination of AFT M_1, Beaver [111] converted it into M_{2a}, which has a higher intensity of fluorescence (Table 4, No. 8). A completely automated method of determining AFT M_1, likewise based on HPLC, has been described by Tuinstra et al. [99]. Etsuko et al. also consider chromatography to be the fastest method for determining AFTs [102].

TABLE 4. Conditions for the High-Performance Liquid Chromatography of Aflatoxin and Other Mycotoxins (IC – isocratic: LG – linear gradient; UV – ultraviolet; FL – fluorescence detector)

No.	Absorbent, column, mm	Mobile phase	Detector, wavelength (nm)	Literature
1	LiChrospher (250 × 4)	IC: toluene–ethyl acetate–MeOH–formic acid (90:6:2:23)	FL	[107]
2	Supelcosil LC C_{18} (250 × 4.6)	IC: MeCN–iso-PrOH–acetic acid–water (10:10:2:78)	FL 352/442	[101]
3	Sepralyte C_{18} (250 × 2.1)	IC: THF–MeOH–water (5:35:60)	FL	[109]
4	Spherisorb C_8 and C_{18} (linked columns, each 4.0 × 150)	IC: THF–MeOH–water (5:35:60)	FL	[109]
5	Nova-Pak C_{18} (100 × 5)	LG and IC with MeOH–water mixtures	FL 358/428	[97]
6	Spherisorb ODS (250 × 4.9)	IC: MeOH–MeCN–water (3:1:6)	FL 364/434	[110]
7	LiChrosorb RP-18 (250 × 4.6)	IC: MeOH–MeCN–water (1:4:15)	FL 360/385	[98]
8	Silica gel C_{18}	IC: MeOH–MeCN–water (2:2:6)	FL	[111]
9	Chromspher C_{18} (200 × 3)	IC: MeCN–water (1:1)	UV	[99]
10	Spherisorb C_{18} (125 × 4.6)	IC: MeOH–water + H_3PO_4 to pH 3.0	FL	[112]
11	Supelcosil C_{18} (250 × 4.6)	IC: MeCN–water (0% → 100%)	UV, 254	[113]
12	Bondapak (250 × 4)	IC: MeOH–water (4:6)	UV, 365	[96]
13	LiChrosorb (250 × 4)	IC: $CHCl_3$–MeOH (100:0.8)	FL	[155]

Palmisano et al. [112] have developed a method for analyzing mycotoxins similar in structure to the AFTs – alternariol (LVa) and its methyl ether (LVb) – together with alternotoxins I and II (Table 4, No. 10). Hallok et al. [113] have used HPLC as the concluding stage in the isolation and purification of a phytotoxin – O-demethyldiaporthin (LVI) (Table 4, No. 11).

ANALYSIS OF COUMARINS IN THE PRESENCE OF COMPOUNDS OF OTHER CLASSES

In many cases, the preparation of total CM fractions for subsequent detailed analysis by HPLC methods is a fairly complex problem. While many alkyl- and alkoxy-CMs, FCMs, and PCMs can frequently be isolated practically free from impurities even by a liquid extraction method, extracts of HCMs usually contain a large number of phenolic compounds (PCs) with similar solubilities and require additional purification. A preliminary analysis of the level of CMs in such materials has been described by many authors. The problem that they have solved of separating various PCs in a single stage is of particular interest, since it opens up new possibilities for HPLC in phytochemistry and perfects the technique of performing the analysis of complex mixtures with the aid of programmed elution gradients. In view of the good solubility of PCs in polar solvents, preference is usually given to RP systems with MPs consisting of MeOH (or MeCN) and water (Table 1, Nos. 43-61).

A method of separating HCMs (esculin (I), (XII), (XIV), etc.) and flavonoids has been described by Tamma et al. [114]. A rapid quantitative determination of (XIV), (XXXV), and the 7-O-glucoside and 7-O-acetylglucoside of apigenin in an alcoholic extract of Matricaria chamomilla with the aid of HPLC has been proposed by Pietta et al. [115]. Schülz and Albroscheit [116], using HPLC and combining separation and identification (UV spectrum) at the same time, have successfully analyzed an aqueous extract of this plant which, in addition to HCMs ((XIV) and (XXXV)) contained hydroxybenzoic acids (HBAs), caffeoylquinic acids, and flavonoid aglycons. HBAs (protocatechuic, 4-hydroxybenzoic) and derivatives of coumestrol (LVIIa) – wedelolactone (LVIIb) and demethylwidelolactone (LVIIc) – were separated in [117] (Table 1, No. 46). A method of separating a mixture of glycosides of flavonoids, HCMs (skimmin) and various phenolic acids and other PCs used by Strack et al. [118] was also effective for the analysis of the corresponding aglycons obtained after hydrolysis.

Variants of the composition of MPs tested in the HPLC of the phenolic components of Prunus tissues, including (I) and (XXXV), proposed by Treutter [119], permit the analysis of a broad spectrum of PCs. It is recommended to analyze mixtures of such HCMs as (I) and (XIV) [120] or of coumarin and indole-3-ylacetic acid [121] with HBAs and hydroxycinnamic acids in a flow of aqueous MeOH containing formic acid (1-2%) (Table 1, No. 49). The latter may be replaced by acetic acid in the determination of (I), (XIV), and 4-methyl-(XIV) in the presence of analogues of cinnamaldehyde which are present in oak wood and alcoholic beverages [122] and also in the separation of coumarins and 4-hydroxybenzaldehyde derivatives [123] (Table 1, No. 50). This tpe of MPs is used for the analysis of esculetin (LVIII) in the presence of its biochemical precursors – cis- and trans-caffeic acids [124]. The proposed HPLC method, thanks to its high resolving capacity, proved to be more informative than the known method of separation on DEAE-cellulose used by Sato et al. [125], in which it is impossible to separate isomers of caffeic acid and to perform the quantitative comparison of the components of the mixture.

For the separation of a mixture of coumarin with cinnamic acid, cinnamyl alcohol and their derivatives, in addition to RP HPLC, as used by Ascher [126] and Mazza [127], it is possible to use the ion-pair variant described by Sagara et al. [128] (Table 1, Nos. 52-54). Lander et al. [129] have described a method for preparing for HPLC a sample containing coumarin, quinine, β-asarone and quassine which consists in solid-phase extraction on a phenyl sorbent and elution by ethers in neutral and alkaline media.

The determination of coumestrol and of isoflavan glycosides (genistein, daidzein) in soybeans and the products of their processing has been carried out by the HPLC method [130] and the change in these compounds in a suspension culture of Glycine max. caused by the bacterium Pseudomonas syringae has been analyzed [131]. A mixture of these glycosides and coumestrin (LVIId) has been separated by semipreparative HPLC in a flow of aqueous MeCN by Le-Van [132] (Table 1, No. 55). However, under the conditions of this regime, coumarins and aromatic aldehydes of the cinnamaldehyde type are scarcely differentiated. For example, scopoletin was eluted from an extract of oak wood together with sinapyl, syringyl, and coniferyl aldehydes, vanillin, etc. in 30-40% aqueous MeOH [133]. A mixture of scopoletin and germacrolides has been isolated in the same way [134].

Elution with aqueous MeOH in the separation of coumarin fractions from other compounds of lactone structure has been shown to be more effective in investigations by Zdero et al. [3, 4, 135, 136] (Table 1, Nos. 57-60). Thus, 4-hydroxy-5-methyl-3-prenylcoumarins, cyclobrachycoumarins (LIX), chromones, and hydroxyketones from Brachyclados megalantus [4] have been separated by TLC, a mixture of isolycoserone (LX), 5-methyl-8'-oxoferulenol, and 3-hydroxy-5-methyl-2-8'-oxofarnesylcoumaran-3-one from Gypothamnium pinifoliun [135], epilycoserone (LXI), and some coumarins of the cyclolycoserone (LXII) group have been separated from sesquiterpene lactones (aphyllocladone, cladinol, etc.) [136], and a mixture of 8-methoxy- (II) and three derivatives of α-isocedrene (dolichlasin, etc.) [3] has been separated. Van Beek and Blaakmeer have described the RP HPLC of a mixture of isoaurapten (LXIII), marmin (LXIV), and 5-alkoxy-FCMs with such lactones as limonin and nomilin [137] (Table 1, No. 61).

CHROMATOGRAPHY OF COUMARINS MODIFIED BY ORGANIC COMPOUNDS

One of the methods for increasing the sensitivity of analytical methods of LCC is the modification of the compounds to be determined directed to increasing the responses of the detector and lowering the limit of their detection. In this connection, fairly wide use is made of the fluorescent properties of synthetic analogues of some natural HCMs. Therefore, in addition to the CM derivatives considered above we must mention a large group of compounds with complex structure the coumarin fragment of which plays the role of a fluorophore.

Among the widely used fluorescent probes that form covalent bonds with various functional groups under mild conditions, an important place is occupied by derivatives of 4-methyl- and 4-bromomethyl-CMs having 7-methoxy [138-140], 6,7-dimethoxy [141], 7-acetoxy [142, 143], or 6,7-methylenedioxy [144] groups. The use of these reagents has been described in the analysis of valproic acid [145], nonsteroid antiinflammatory drugs (aspirin, ibuprofen, etc.) [144], and 5-fluorouracil [138, 139]. The products of the reactions of carboxylic acids [142, 144], such as fatty acids, with 4-bromomethyl-7-methoxycoumarins [146] or 7-diethylamino-4-hydrazinocoumarin [147] may be regarded as close analogues of known CM derivatives which undoubtedly are of interest from the point of view of structural aspects in chromatographic separation. The use of luminarine (LXV) offers great prospects for the analysis of primary (with hydrocarbon chains 4-12 C atoms long) and secondary alkylamines. Tod et al. have shown that the limits of detection of the products of pentylamine (LXV) by fluorescence and by chemiluminescent detection are 1 and 6 fmole, respectively [148]. 3-(7-methoxycoumarin-3-carbonyl)- and 3-(7-dimethylaminocoumarin-3-carbonyl)oxazol-2-ones [149] and 7-methoxycoumarin-3-carbonyl chloride [150] are also recommended as fluorescent labels for determining primary amines.

As another group of covalently modified CMs we may mention the products of the interaction of CMs with nucleic acids (NAs) or with structural fragments of them. Conditions for the HPLC of photoadducts of FCMs with NAs have been described [151]. By chromatography on hydroxyapatite, Vedaldi et al. established the nature of the interaction of the trimethylallopsoralen derivatives with DNA [152]. The mechanism of the formation of an adduct of AFT B_1 with an oligodeoxynucleotide has been determined with the aid of HPLC [153].

Cepeda-Saez et al. [154] have given an example of a selective increase in the intensity of fluorescence (sixfold) of bergapten on the formation of a complex with β-cyclodextrin present in the mobile phase, which does not occur for psoralen and xanthotoxin.

In conclusion it must be mentioned that in spite of the wide introduction of HPLC in all directions of the analysis of coumarin derivatives, its main role appears most clearly in the analysis of biological materials containing various multicomponent mixture of coumarin derivatives having closely similar structures.

Thus, in recent years a clear tendency has been observed to the unification both of the most concrete procedures for the HPLC of coumarins and also of the preliminary stages of their purification and concentration. This will permit in future a considerable simplification of the isolation and investigation of coumarins, including those of unknown structure.

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