THE THIN-LAYER CHROMATOGRAPHY OF FLAVONOIDS

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The development of the chemistry of flavonoid compounds in the last 10-15 years is due,to a considerable extent,to the wide use of effective methods of separating mixtures of substances. These methods include chromatography in thin layers of adsorbents.

The first experiments on the separation of flavonoids in thin layers of adsorbent were performed in 1961 by Stahl and Schorn [1] and by Paris [2]. In recent years, this method has become widely used for the isolation, purification, and identification of biflavonoids [3, 51, 52, 61, 69] and coumestan derivatives [4, 5, 19, 29] and the identification of a number of flavones and flavonols [9, 16, 24, 33, 38, 44, 62, 64], isoflavones [26, 27, 31, 37, 63], and anthocyanins [22, 30, 36, 46, 49].

The question of the separation of flavones, flavonols, and flavanones in thin layers of silica gel has been considered most fully in the literature. As early as 1962 and 1963, thin-layer chromatography on silica gel was successfully applied to the separation of the flavonoids of citruses and the camomile [11, 12] and for the analysis of isoflavones in plant extracts [13], and in 1964 Hörhammer et al. [7] reviewed information on the chromatography of 13 different aglycones in thin layers of silica gel in the benzene–pyridine–formic acid (36 : 9 : 5) system. They established that the distance of migration of a substance depends on its polarity. Polyhydroxyflavones have lower R_f values (0.00-0.25) than oligohydroxylated and methoxylated flavones (0.3-0.5). Higher R_f values are found for flavanones, for methoxylated flavones, and for flavonols (0.5-0.75). The addition of gypsum to the silica gel does not affect the R_f values [7]. Other authors have also pointed out relationships between structure and R_f value [6, 9, 56].

Thus, for example, Stahl has reported that a number of laws can be observed in relation to chromatography in a thin layer of silica gel in the following systems: 1) butan-1-ol–glacial acetic acid–water (40 : 10 : 50) and 2) m-cresol–glacial acetic acid–water (50 : 2 : 48).

1. An increase in the number of free hydroxy groups decreases the R_f value. There may be an exception in the case of the ortho and adjacent positions of substituents, when, because of the formation of hydrogen bonds, the R_f value may rise.

2. The methylation of OH groups leads to an increase in the R_f value which is 1/3 to 1/2 times less than for the elimination of the OH groups.

3. As a rule, acetylation greatly increases the R_f value.

4. Glycosidation lowers the R_f value, but this effect (glucose) overlaps that due to the introduction of an OH group [6].

V. P. Georgievskii and A. L. Litvinenko [56], using as solvent systems various combinations of solvents with amphoteric metals (neutral solvents), methyl ethyl ketone, and acetylacetone (differentiating solvents) and mixtures of neutral solvents with acid solvents (acetic and formic acids) and also with basic solvents (formamide, dimethylformamide, ammonia) for chromatography in a thin layer of type-KSK silica gel, have established that the degree of hydroxylation has a particularly marked influence on the mobility of the aglycones, while a hydroxy group on the third carbon atom has a greater influence. In the chromatography of glycosides, the magnitude R_m [$R_m = \log (1/R_f - 1)$] is affected by the nature of the sugar component and the number of its residues in the molecule [56].

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TABLE 1. Adsorbents and Solvents Used for the TLC of Various Types of Flavonoids

Adsorbent and flavonoid	Solvent system and literature source
Silica gel	
Flavones	Benzene–pyridine–HCOOH–H_2O (36 : 9 : 5) [7]; benzene–dioxane–CH_3COOH (90 : 25 : 4) [17]; benzene–acetone (4 : 1 and 9 : 1) [17, 20]*; benzene–ethyl acetate (3 : 1) [21]; $CHCl_3$–3% CH_3OH [23]; 6-10% CH_3COOH in $CHCl_3$ [39]; 45% ethyl acetate in C_6H_6 [39-53]
O-Glycosides of flavones	Ethyl acetate–methyl ethyl ketone–HCOOH–H_2O (50 : 30 : 10 : 10) [22], methanol–CH_3COOH–H_2O (18 : 1 : 1) [62]; butan-2-ol–ethyl acetate–dimethylformamide–H_2O (10 : 6 : 3 : 2) [62]
C-Glycosides of flavones	Ethyl acetate–CH_3COOH–H_2O (100 : 20 : 10) [32]; ethyl acetate–methanol (8 : 2) [33]
Biflavones	$CHCl_3$–CH_3COOH–acetone (75 : 25 : 10) [28]; benzene–pyridine–HCOOH (36 : 9 : 5) [3]; toluene–ethyl formate–HCOOH (5 : 4 : 1) [3]; benzene–ethyl acetate–CH_3COOH (8 : 5 : 2) [3]; benzene–pyridine–ethyl formate–dioxane (5 : 1 : 2 : 2) [3]
Flavonols	Benzene–pyridine–HCOOH (36 : 9 : 5) [7, 16]; toluene–ethyl formate–HCOOH (5 : 4 : 1) [22, 16]; ethyl acetate–methanol (8 : 2) [33]; ethyl acetate–HCOOH–H_2O (70 : 15 : 15) [30]; ethyl acetate–toluene–CH_3OH (8 : 6 : 1) [35]
O-Glycosides of flavonols	Ethyl acetate–methyl ethyl ketone–HCOOH–H_2O (50 : 30 : 10 : 10) [22]; ethyl acetate–HCOOH–$CHCl_3$ (2 : 1 : 2 and 3 : 3 : 1) [22]; ethyl acetate–methanol–H_2O (100 : 20 : 10) [32]; (100 : 16.5 : 13.5) [35]; ethyl acetate–ethanol–toluene (4 : 2 : 2) [38]; ethyl acetate–HCOOH–H_2O (10 : 2 : 3) [66]
Flavanones	Benzene–pyridine–HCOOH (36 : 9 : 5) [7]; $CHCl_3$–CH_3OH–CH_3COOH (7 : 1 : 1) [60]
Isoflavones	$CHCl_3$–ethanol (3 : 1) [31]; $CHCl_3$–11% CH_3OH (9 : 1) [27, 25]; $CHCl_3$–ethanol (1 : 1) [31]; ethyl acetate–CH_3OH–H_2O (100 : 16.5 : 13.5) [35]
Coumestans	Benzene–isopropanol–CH_3OH (95 : 5 : 1) [19]; isopropanol–conc. NH_4OH (2 : 1) [29]
Dalbergenones	Benzene–acetone (4 : 1) [18]
Anthocyanins	Butan-1-ol–HCOOH–H_2O (85 : 5 : 10) [30, 36]; ethyl acetate–HCOOH–H_2O (70 : 15 : 15) [30]; ethyl acetate–HCOOH–H_2O (85 : 6 : 9) [30, 36]
Leucoanthocyanins	Ethyl acetate–$CHCl_3$–HCOOH (3 : 3 : 1) [34]
Silica gel + cellulose	
Anthocyanins	H_2O–HCOOH–HCl (8 : 1 : 4) [46]; acetone–HCl (0.5 N) (1 : 3) [46]
Cellulose	
C-Glycosides of flavones and free flavones	Ethyl acetate–HCOOH–H_2O (10 : 2 : 3) [44]; 20% CH_3COOH [55]; butan-1-ol–CH_3COOH–H_2O (6 : 1 : 2) [55]; isopropanol–H_2O (22 : 78); isopropanol–HCOOH–H_2O (2 : 5 : 5) [55]; CH_3COOH–HCl–H_2O (5 : 1 : 5) [55]
Coumestans	Isopropanol–conc. NH_3 (2 : 2) [5]; 50% CH_3COOH [5]; 15% CH_3COOH [54]
Anthocyanins	Conc. HCl–CH_3COOH–H_2O (3 : 1 : 20) [36]; conc. HCl–CH_3COOH–H_2O (280 : 45 : 325) [36]
Leucoanthocyanins	Ethyl acetate–$CHCl_3$–HCOOH (3 : 3 : 1) [34]
Polyamide	
Flavones	Benzene–ethyl methyl ketone–methanol (90 : 5 : 5) [40, 62]; 80% CH_3OH [42]; ethanol–H_2O (3 : 2) [42]
O-Glycosides of flavones	Benzene–methanol (1 : 1) [62]; $CHCl_3$–CH_3OH–butan-2-ol (5 : 3 : 1) [62]; methanol–H_2O (1 : 1) [62]; nitromethane–methanol (5 : 2) [62]

TABLE 1 (continued)

Adsorbent and flavonoid	Solvent system and literature source
O-Glycosides of flavonols	$CHCl_3$–CH_3OH–methyl ethyl ketone–acetylacetone (25 : 10 : 5 : 1) saturated with water [41]; CH_3OH–CH_3COOH–H_2O (19 : 1 : 1) [8]; ethanol–H_2O (3 : 2) [9]; ethyl acetate–CH_3OH–H_2O (100 : 16.5 : 13.5) [43, 44]; H_2O–ethanol–acetylacetone (4 : 2 : 1) [9, 42, 45]; H_2O–ethanol–methyl ethyl ketone–acetylacetone (10 : 3 : 3 : 1 and 13 : 3 : 3 : 1) [41, 45]
Anthocyanins	n-Pentanol–n-propanol–CH_3COOH–H_2O (3 : 2 : 2 : 1) [36]
Polyamide + cellulose (25 : 15)	
O-Diglycosides of flavonols	Ethanol–H_2O (95 : 5) [47]
Polyacrylonitrile + Perlon [Nylon-6] (7 : 2) wetted with phosphate buffer	
Anthocyanins and anthocyanidins	n-Pentanol–n-propanol–CH_3COOH–H_2O (3 : 2 : 2 : 1) [49]
Silicic acid	
Coumestans	$CHCl_3$–diethyl ether (1 : 1) [4]; CH_3COOH–H_2O (1 : 1) [4]

*The silica gel was buffered with sodium acetate.

Where a polyamide adsorbent is used, as has been shown by Egger [9], it is not only the aglycone but the degree of its glycosidation that affects the R_f value. Using mixtures of ethanol and water (60 : 40), water, ethanol, and acetylacetone (40 : 20 : 10), and water–ethanol–ethyl methyl ketone–acetylacetone (65 : 15 : 15 : 5), with 14 glycosides the aglycones of which were kaempferol, quercetin, and myricetin as examples, he showed that the nature of the sugar does not determine the R_f value. 3-Monosides of quercetin, kaempferol, and myricetin have the same R_f value and can easily be separated from 3-biosides, and the latter from 3,7-diglycosides.

Ribereau-Gayon [19] has reported that in this case it is particularly easy to separate in terms of R_f values 3-glucosiduronic acids from other glycosides: 3-monoglucosiduronic acids, 3-monosides, 3-biosides, 3,7-dimonosides, and 3-biosido-7-monosides with R_f 0.05, 0.22, 0.37-0.51, 0.63, and 0.65-0.67, respectively.

Some authors have reported the successful use of thin-layer chromatography on polyamide in the analysis of flavonoid compounds [8, 10, 14, 15].

. Adsorbents used at the present time, in addition to silica gel and polyamide, are cellulose [5, 36, 44, 54], Magnesol [50], silicic acid [4], polyvinylpyrrolidone [57], and polyacrylonitrile [49].

The technique of preparing the adsorbents has been described in a number of handbooks and reviews [6, 58, 59].

The great differences in the polarity of the flavonoid compounds make it necessary to use various systems of solvents. In Table 1 we give the results of the use of various systems of solvents in chromatography with the most used layers of adsorbent. For the chromatography of the more hydrophobic compounds (flavones, biflavones, coumestans, isoflavones) the main components of the mixture are lipophilic solvents (benzene, toluene, and chloroform) in combination with ketones (acetone, methyl ethyl ketone), with alcohols (ethanol, methanol, propanol, and isopropanol), and with ethers and esters; and for glycosides and flavonols water-saturated ethyl acetate is used in combination with an acid and alcohol or with an acid alone.

Systems in which toluene, benzene, chloroform, or an acid (formic or acetic) is added in various proportions to the main component (ethyl acetate) are also widely used. Four-component systems have proved very satisfactory in the separation of flavone glycosides and anthocyanins [22, 62, 49].

The chromatograms are examined in visible or UV light before and after treatment with various reagents. Of the reagents the most frequently used are: diazonium salts [3, 6]; metal salts, mainly a 25% aqueous solution of basic lead acetate and a 10% solution of antimonious chloride in chloroform [6, 7, 44]; Benedict's reagent [48]; alkalis and acids [6]; and iodine vapor [58]. To detect 3-hydroxyflavanones, Barton [60] proposed to mix silica gel with zinc dust and, after chromatography, to spray the chromatograms with hydrochloric acid. To identify polymethoxylated compounds it is possible to use an 0.5% aqueous solution of potassium permanganate [21]. Stahl recommends β-aminoethyl diphenylborate, which was proposed by Neu [6].

In recent years, ready-prepared plates with a thin layer of silica gel have been well recommended [65, 68, 69].

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