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CHROMATOGRAPHIC SEPARATION OF ISOFLAVONES

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The optimum ratios of the components of the solvent systems have been determined with the aid of the simplex-grid method of experimental planning and the separation of a number of isoflavones has been carried out with the aid of these systems in thin layers of silica gel (Silufol plates); in addition, the chromatographic constants of the functional groups have been determined, which makes it possible to obtain the ηR_f values of compounds under investigation in an analysis of plant material by a mathematical method.

The detection of isoflavones in plant material is made difficult by the fact that many of them have no characteristic color reactions, and therefore chromatographic separation on paper or in a thin layer of adsorbent, elution of the spots from the chromatograms, and the recording of the absorption spectra from the eluates in the UV region is used for their investigation [1, 2].

To determine the absorption maxima it is necessary to be convinced that the separation of the substances on the chromatograms has taken place sharply. The observance of this condition is also necessary for the identification of the isoflavones in the presence of "markers."

Of the numerous group of flavonoids, the best-studied chromatographically are the flavones, flavonols, and flavanones. It has been established that the R_f values of these compounds depend on the number of hydroxy groups and carbohydrate residues and their configuration [3, 4].

Usually the number of hydroxy groups is inversely proportional to the R_f value; on methylation, R_f falls, i.e., the following relationship exists:

$$R_f(x - OH) < R_f(x - OCH_3) < R_f(x - H) \quad [4].$$

We set ourselves the task of performing the optimization of the chromatographic separation of two-component mixtures of isoflavones in three-component systems of solvents in model experiments, using mathematical methods of experimental planning, and of determining the chromatographic constants of the functional groups of some isoflavones. Analysis of literature information on the chromatographic separation of isoflavones shows that in paper chromatography systems containing alcohols are used most frequently, and in thin-layer chromatography systems containing hydrophobic solvents: pentane, hexane, benzene, chloroform, and some others [2, 5-8].

On performing the chromatography of isoflavones in the alcoholic systems described in the literature (we always considered the separation of isoflavones in 17 systems), we established that in a number of cases the R_f value is affected by the number of hydroxy groups but not by their distribution. The methoxylation or methylation of the isoflavone nucleus leads to a change in the R_f value but, in a number of cases, it is impossible to distinguish compounds

having the same number of substituents from their R_f values. The situation is no better when using the solvent system employed most frequently: benzene-CH₃COOH-H₂O (6:7:3) [1]. In this case, a number of isoflavones remain at the start.

To optimize the chromatographic separation of two-component mixtures of isoflavones, we used the simplex grid method of experimental planning [9]. The optimality criterion used was the value of η [10]:

$$\eta = (R_{f_1} - R_{f_2}) \left[1 \pm \left(\frac{R_{f_1} + R_{f_2}}{2l} - 0.5 \right) \right] \sqrt{\frac{b_1 \cdot b_2}{a_1 \cdot a_2}}, \quad (1)$$

where a_1 , a_2 , b_1 , and b_2 are the axes of the ellipses of the two spots, mm; l is the distance from the start to the front of the chromatogram, mm; and the plus sign is taken in formula (1) if $R_{f_1} + R_{f_2}/2l < 1/2$, and the minus sign if $R_{f_1} - R_{f_2}/2l > 1/2$ [10].

The regression equation was calculated from the formula for a {3,3} grid (third-order model for a three-component system [11]). The model was converted into a function of two independent variables and, after solution of the system of equations, graphs were plotted. The values of η in all the experiments were found on a EC 1020 computer using the programming language Fortran-IV, and the other calculations were performed on a microcalculator. As an example, we give the results of the optimization of the separation of genistein, as the isoflavone most frequently encountered in plants, from other isoflavones (thin-layer chromatography, Silufol plates):

Mixture to be separated	Solvent system	Optimum ratio of the components of the system
Genistein-formononetin	CH ₃ OH-CH ₃ COOH-H ₂ O	8:4:13
	n-propanol-CH ₃ COOH-H ₂ O	3:0:1
	n-butanol-CH ₃ COOH-H ₂ O	Optimum ratios not determined
	n-pentanol-CH ₃ COOH-H ₂ O	
Genistein-prunitrin	n-butanol-CH ₃ COOH-H ₂ O	6:4:5
Genistein-sophoricoside	n-butanol-CH ₃ COOH-H ₂ O	4:4:7

We used the systems with the optimum ratios of the components also for the chromatographic separation of other isoflavones (Table 1). It was established that in this case, the mobility of the aglycones of the isoflavones is affected by the number of hydroxy groups. The methoxylation of the isoflavones lowers their mobility in alcoholic systems, and the distribution of the methoxy groups affects the R_f values.

Table 1 gives the value $\eta R_f = R_f \times 100$ and the mobility of each compound in relation to the ηR_f value of the standard [12]. Isoflavone was taken as the standard.

To calculate the contribution of each of the functional groups to the value of ηR_f , which is the sum of the ηR_f values of the individual functional groups, we used the formula

$$\eta R_f = \eta R_{f_{st}} + \sum \sigma^{xp}, \quad (2)$$

where $\eta R_{f_{st}}$ is the value characterizing the mobility of isoflavone, and σ^{ch} is the ηR_f value of each functional group of the compound being chromatographed [12].

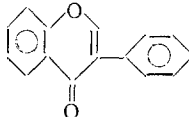
The results of the calculations are presented in Table 2.

It may be mentioned that the σ^{ch} values of the same type of functional groups are affected by their positions and by the nature of the solvents. Systems (I-VI), in which the ratio of the components was established by the use of the simplex-grid method of experimental planning can be used successfully for the thin-layer chromatography of a number of isoflavones.

By using the chromatographic constants for the given systems of solvents it is possible to calculate the ηR_f values of a number of isoflavones.

We have carried out the harmonization of the calculated figures with the amounts of isoflavones in tinctures of the *Ononis arvensis* and the fruit of the Japanese Pagoda tree, and a 60% ethanolic extract from the epigeal part of *Genista divergens* as a result of the use of values of the constants and the colorations of the spots in UV light without the employment of "markers" the isoflavones formononetin, ononin, sophoricoside, and genistein were identified.

TABLE 2. Chromatographic Constants σ^{ch} of the Functional Groups of Some Isoflavones

Functional group and its position	σ^{ch} in the following systems							
	I	II	III	IV	V	VI	VII	VIII
	65	85	92	86	94	90	88	92
5-OH	-4	2	3	0	4	3	3	9
7-OH	12	-1	-1	0	0	2	-2	-14
4'-OH	11	-1	-12	0	-9	-3	-8	-26
6-OCH ₃	11	-2	2	2	0	6	1	9
7-OCH ₃	4	-4	-1	-1	-2	1	0	-3
8-OCH ₃	10	-3	-4	2	-5	-6	-6	-8
3'-OCH ₃	0	-1	0	0	4	-5	5	9
4'-OCH ₃	1	-4	-7	-2	-8	-4	-6	-14
7-CH ₃	11	-2	-1	0	-1	2	2	5
5-D-Glucopyranosyloxy	-2	5	-3	-10	-9	-19	-80	-63
7-D-Glucopyranosyloxy	13	1	-12	-1	-7	4	2	-65
4'-D-Glucopyranosyloxy	6	-3	-21	-11	-20	-24	-3	-1
8-C-Glucopyranosyl	-4	-9	-10	-12	-10	-21	-86	-59

EXPERIMENTAL

In all cases, chromatography was performed on Silufol plates. In order to eliminate the influence of a number of factors on the chromatographic process, we took as constant magnitudes the length of the run of the solvent (120 mm), the diameter of the spot of the solution of the substance deposited on the starting line (2 mm), and the molar concentration of the solution ($5 \cdot 10^{-3}$ M solution of the isoflavones in ethanol). Chromatography was performed in "saturated" chambers. A chamber was considered saturated if the R_f values of three chromatograms obtained successively remained constant.

The values of ηR_f for each isoflavone were determined from the mean values of six parallel experiments.

SUMMARY

The optimum ratios of the components in solvent systems have been obtained with the aid of the simplex-grid method of experimental planning, and chromatographic separation of various isoflavones has been carried out in these systems. The chromatographic constants of functional groups have been found, which makes it possible to determine the ηR_f values of substances being analyzed in a plant raw material by calculation.

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