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Application of numerical methods in the investigation of the flavonoids of Christ's thorn (*Paliurus spina-christi* Mill.)

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The efficiency of ten thin-layer silica gel chromatographic systems for separating flavonoids is compared by using the information theory and numerical taxonomy. The design of the most effective series of chromatographic systems is discussed in terms of individual discriminating powers of the systems and the inter-system correlation. The most favourable chromatographical systems for the future quantitative analysis of the flavonoids from *Paliurus spina-christi* Mill. are: 1 (ethyl acetate:formic acid:acetic acid:water, 100:11:11:27), 2 (ethyl acetate:formic acid:water, 8:1:1) and 10 (chloroform:methanol:water, 6.5:3.5:1).

Keywords: Paliurus spina-christi Mill., flavonoids, information content, discriminating power, numerical taxonomy

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Paliurus spina-christi Mill. (*Rhamnaceae*) is a perennial thorny shrub widely spread on dry and rocky places in the Mediterranean region and Asia. In Croatia, this plant grows along the Adriatic coast and on the islands (1). Christ's thorn is used in folk medicine against diarrhoea and rheumatism (2, 3).

Chemical investigations of *Paliurus spina-christi* Mill. fruits indicated the presence of flavonoids: isoquercitrin, rutin and hyperoside (2, 4 – 6). Six flavonoid glycosides were isolated from the leaves of Christ's thorn. Three flavonoids were identified as isoquercitrin, rutin and quercetin-3-O-rutinoside-7-O-rhamnoside (7, 8). Other flavonoids are kaempferol-O-triglycoside and flavonoid C/O-triglycosides (8).

In phytochemical analysis some chromatographic separations are more important than the others (9).

Thin-layer chromatography (TLC) on silica gel is very favourable for the qualitative and quantitative analysis of flavonoids (10). The choice of mobile phases for separation

*Taken, in part, from the Master Thesis of Ž. M.

** Correspondence

of flavonoids is made according to the polarity of the flavonoid compound and the legality, which is introduced for the paper chromatography:

$$R_F\ (R\text{-}CH_3) > R_F\ (R\text{-}H) > R_F\ (R\text{-}OCH_3) > R_F\ (R\text{-O-sugar})$$

Experiments were carried out with six flavonoids by TLC methods in ten systems, which are representative of the systems in current use. The effectiveness of chromatographic systems is measured in terms of the selectivity and probability of separating two flavonoids, randomly selected from a specific population (11). The measure of selectivity is the information content, and the discriminating power is the measure of probability. Classification of chromatographic systems into groups with similar separation properties and selection of the most efficient TLC-systems from each group are carried out by the methods of numerical taxonomy (12 – 14).

MATERIALS AND METHODS

Materials

The ten systems used are given in Table I (15 – 22).

In all systems, silica gel plates (20 × 20 cm, 0.25 mm thickness) incorporating a fluorescent indicator, Kieselgel 60 F_{254} – Alufolien (E. Merck, Darmstadt, Art. Nr. 5554) were

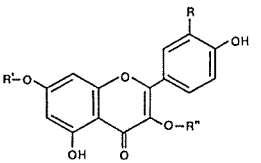
Table I. The thin layer chromatographic systems studied

System No.	Solvent	Ref.
1.	Ethyl acetate:formic acid:acetic acid:water (100:11:11:27)	15
2.	Ethyl acetate:formic acid:water (8:1:1)	16
3.	Ethyl acetate:formic acid:water (65:15:20)	17
4.	Ethyl acetate:formic acid:water (67:20:13)	18
5.	Ethyl acetate:formic acid:water (88:6:6)	19
6.	Ethyl acetate:methanol:water (77:13:10)	20
7.	Ethyl acetate:1-propanol:water:formic acid (40:40:28:2)	18
8.	Ethyl acetate:methylethylketon:formic acid:water (50:30:10:10)	21
9.	1-Butanol:acetic acid:water (66:17:17)	18
10.	Chloroform:methanol:water (6.5:3.5:1)	22

used. Paper liners were used in all tanks, and after addition of the appropriate solvents, the systems were allowed to equilibrate for at least 30 minutes. The investigated flavonoids were dissolved in methanol; 5-μL quantities were applied to the plates and the systems were allowed to run for 15 cm.

The structures of the flavonoids analyzed in this work are presented in Table II. Isoquercitrin and rutin were isolated from the leaves and fruits of *Paliurus spina-christi* Mill. (6, 7) and quercetin-new (quercetin-3-O-rutinoside-7-O-rhamnoside) from the leaves of the same plant (8). Quercitrin, hyperoside and robinin are the well known standards of flavonoids.

Table II. Structures of the studied compounds



Compound number	Name of the compound	R	R'	R"
1	Isoquercitrin	OH	H	Glucose
2	Rutin	OH	H	Rhamnose and glucose
3	Quercetin-new	OH	Rhamnose	Rhamnose and glucose
4	Quercitrin	OH	H	Rhamnose
5	Hyperoside	OH	H	Galactose
6	Robinin	H	Rhamnose	Rhamnose and galactose

Application of the chromatographic systems summarized in Table I allows us to generate a data set of R_F values for the separation of these flavonoids into ten different chromatographic systems. They were detected by the Naturstoff reagent – Polyethylenglycol 4000-$UV_{365\ nm}$ (15).

Methods

There are three possible approaches to the selection of a prepared combination of two or more chromatographic systems. The first is to consider every possible combination of two or more chromatographic systems and to calculate the information content for each combination. Fundamental aspects of information theory and its usefulness for chromatographic analysis are discussed in view of the applications described so far in the literature (11, 13, 23 – 26).

Distributing R_F values into groups with error factor E (*e.g.*, E = 0.05 or E = 0.10) with respect to R_F units and assuming $n_K R_F$ values in the k-th groups, the average information content given by the Shannon equation is (11, 13, 23 – 29):

$$I(X) = -\sum_k (n_k/n)\ \mathrm{ld}\ (n_k/n)$$

The second approach is to classify the chromatographic systems into dissimilar groups and to select from each group (with similar separation characteristics) the best system according to the discriminating power (DP). The discriminating power for a series of chromatographic systems is defined as the probability that two flavonoids selected at random from a large population would be discriminated in at least one of the systems (13, 28 – 30). To compute the discriminating power (DP_k) of k chromatographic systems in which N flavonoids are investigated, we must be able to discriminate all pairs of N's. For the total number of matching pairs, M, the probability of chromatographically similar pairs at random selection is: $2M/N(N-1)$. DP_k for the k systems is therefore given by:

$$DP_k = 1 - \frac{2M}{N(N-1)}$$

The average number of chromatographically similar compounds for chromatographic systems considered is calculated from the following equation (13, 30):

$$T = 1 + (N-1)(1 - DP_k)$$

The third approach classifies chromatographic systems according to clusters (12, 13, 31, 32). A chromatographic system can be selected from a dendrogram on the basis of an average amount of information (33, 34). Classification is carried out by using the numerical taxonomy method. The three approaches were compared applying our computer search programs KT 1 (13).

RESULTS AND DISCUSSION

A data set of R_F values for the separation of flavonoids into ten different chromatographic systems was analyzed.

Table III gives the input and output data for the information content and the discriminating power for each TLC system and for combined systems in a range of error factors. The error factors were 0.05 and 0.10, respectively.

The most suitable systems for separating the studied flavonoids (Table II) are chromatographic systems: 1 (ethyl acetate:formic acid:acetic acid:water, 100:11:11:27), 2 (ethyl acetate:formic acid:water, 8:1:1) and 10 (chloroform:methanol:water, 6.5:3.5:1) regardless of the error factor. Under the conditions most frequently used in chromatographic analysis (35), *i.e.* E = 0.05, chromatographic systems 1, 2 and 10 have an identical discriminating power and information content, but at E = 0.10 chromatographic system 2 seems to be more favourable than chromatographic systems 1 and 10 due to its greater DP and larger amount of information.

Combining two chromatographic systems with the error 0.05 or 0.10, all the systems have the maximum discriminating power (DP = 1.00 or 0.933, respectively) and the number of chromatographically similar compounds is minimum (T = 1.000 or 1.333, respectively). Consequently, the proposed combinations enable reproducibility of the results

Table III. Input and output data for the DP and cluster formation

```
**********************
*                    *
*     INPUT DATA     *
*                    *
**********************
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COMPOUND	SOLVENT SYSTEM 1	2	3	4	5	6	7	8	9	10
1. ISOQUERCITRIN	.63	.60	.69	.77	.56	.62	.90	.85	.72	.64
2. RUTIN	.38	.33	.44	.62	.24	.50	.82	.70	.58	.51
3. QUERCETIN-NEW	.19	.15	.29	.44	.07	.30	.73	.56	.48	.39
4. QUERCITRIN	.80	.71	.85	.86	.69	.76	.95	.90	.75	.72
5. HYPEROSIDE	.60	.50	.62	.68	.90	.59	.87	.80	.63	.59
6. ROBININ	.26	.20	.34	.49	.12	.38	.79	.61	.51	.44

1. ISOQUERCITRIN = QUERCETIN-3-O-GLUCOSIDE
2. RUTIN = QUERCETIN-3-O-RUTINOSIDE
3. QUERCETIN-NEW = QUERCETIN-3-O-RUTINOSIDE-7-O-RHAMNOSIDE
4. QUERCITRIN = QUERCETIN-3-O-RHAMNOSIDE
5. HYPEROSIDE = QUERCETIN-3-O-GALACTOSIDE
6. ROBININ = KAEMPFEROL-3-O-RHAMNOGALACTOSIDE-7-O-RHAMNOSIDE

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**********************************
*                                *
*          SYSTEMS               *
*                                *
**********************************
```

TLC-SYSTEM	D.P.	I(bit)	E= .05
1	1.0000	2.585	
2	1.0000	2.585	
3	.9333	2.585	
4	.8667	2.252	
5	.9333	2.585	
6	.9333	2.585	
7	.8667	2.252	
8	.9333	2.585	
9	.8667	2.252	
10	1.0000	2.585	

TLC-SYSTEM	D.P.	I(bit)	E= .10
1	.8667	2.252	
2	.9333	2.585	
3	.8000	2.252	
4	.7333	1.918	
5	.8667	2.252	
6	.8000	2.585	
7	.4000	1.459	
8	.6667	1.918	
9	.6000	1.918	
10	.8667	2.252	

Table III. Continued

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**************************************
*                                    *
*          COMBINED SYSTEMS - K = 2  *      E=   .10
*                                    *      -------
**************************************

      COMBINATION SEQUENCE  :

 1.     SYSTEM      2-10     D.P.= .933      T= 1.33
 2.     SYSTEM      2- 9     D.P.= .933      T= 1.33
 3.     SYSTEM      2- 8     D.P.= .933      T= 1.33
 4.     SYSTEM      2- 7     D.P.= .933      T= 1.33
 5.     SYSTEM      2- 6     D.P.= .933      T= 1.33
 6.     SYSTEM      2- 5     D.P.= .933      T= 1.33
 7.     SYSTEM      2- 4     D.P.= .933      T= 1.33
 8.     SYSTEM      2- 3     D.P.= .933      T= 1.33
 9.     SYSTEM      1- 2     D.P.= .933      T= 1.33
10.     SYSTEM      6- 8     D.P.= .866      T= 1.66


**************************************
*                                    *
*      COMBINATION SYSTEMS K = 3     *      E=   .10
*                                    *      ---------
**************************************

      COMBINATION SEQUENCE  :

 1.     SYSTEM      2- 9-10     D.P.= .933      T= 1.33
 2.     SYSTEM      2- 8-10     D.P.= .933      T= 1.33
 3.     SYSTEM      2- 8- 9     D.P.= .933      T= 1.33
 4.     SYSTEM      2- 7-10     D.P.= .933      T= 1.33
 5.     SYSTEM      2- 7- 9     D.P.= .933      T= 1.33
 6.     SYSTEM      2- 7- 8     D.P.= .933      T= 1.33
 7.     SYSTEM      2- 6-10     D.P.= .933      T= 1.33
 8.     SYSTEM      2- 6- 9     D.P.= .933      T= 1.33
 9.     SYSTEM      2- 6- 8     D.P.= .933      T= 1.33
10.     SYSTEM      2- 6- 7     D.P.= .933      T= 1.33


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*                      *
*   CLUSTER FORMATION  *
*                      *
************************

 CLUSTER    SOLVENT    SOLVENT    DISTANCE


    1          6          10        .0480
    2          4           9        .0587
    3          2           5        .0618
    4          1           3        .0665
    5          1           4        .0885
    6          3           5        .1132
    7          1           2        .1476
    8          2           3        .1718
    9          1           2        .3252
```

and an even distribution of R_F values. Here again, systems 2 (ethyl acetate:formic acid:water, 8:1:1) and 10 (chloroform:methanol:water, 6.5:3.5:1) come first.

In a series of three systems, regardless of the error factor, the chromatographic systems 2 and 10 are usually found in the first combinations. The same results were obtained by the cluster analysis of chromatographically similar systems. According to the dendrogram (Fig. 1), one system should be chosen from cluster 1 (system 6 or 10), although both chromatographic systems have the same amount of information (I = 2.58), but chromatographic system 10 is better because it has a higher DP value (DP = 1.000). In the same way, in cluster 3, chromatographic system 2 is better while in cluster 4, chromatographic system 1 is more convenient.

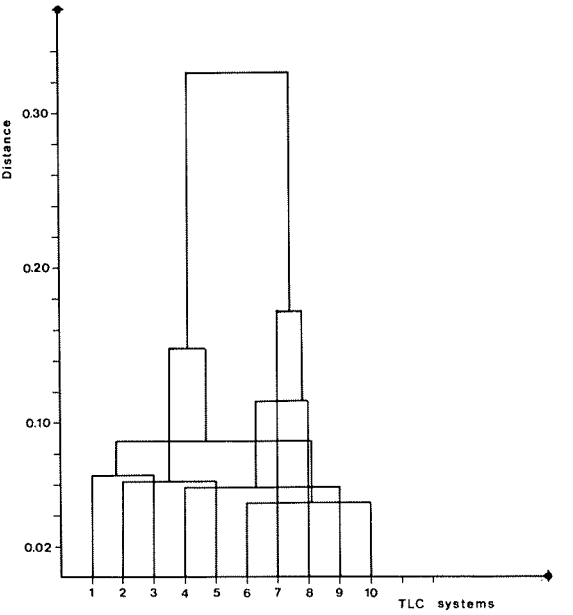

Fig. 1. Dendrogram for ten TLC systems.

The proposed calculations point to the conclusion that for the future quantitative analysis of the flavonoids from *Paliurus spina-christi* Mill., chromatographical systems 1 (ethyl acetate:formic acid:acetic acid:water, 100:11:11:27), 2 (ethyl acetate:formic acid: :water, 8:1:1) or 10 (chloroform:methanol:water, 6.5:3.5:1) are the most useful.

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S A Ž E T A K

Primjena numeričkih metoda u istraživanju flavonoida drače (*Paliurus spina-christi* Mill.)

ŽELJAN MALEŠ, MARICA MEDIĆ-ŠARIĆ i DANICA KUŠTRAK

Ispitana je efikasnost 10 uobičajenih razvijača koji se uporabljuju za odjeljivanje flavonoida u tankoslojnoj kromatografiji. Selektivnost ispitivanih razvijača utvrđena je određivanjerm srednjeg vlastitog sadržaja informacije, a vjerojatnost da će dva slučajno izabrana flavonoida iz razmatrane skupine biti razlikovana jednim ili kombinacijom razvijača, određivanjem koeficijenta DP. Klasifikacija kromatografskih razvijača u klastere (dendrogram) provedena je metodama numeričke taksonomije. Najprikladniji razvijači za buduću kvantitativnu analizu flavonoida drače (*Paliurus spina-christi* Mill.) su: 1 (etil acetat:mravlja kiselina:ledena octena kiselina:voda, 100:11:11:27), 2 (etil acetat:mravlja kiselina:voda, 8:1:1) i 10 (kloroform:metanol:voda, 6.5:3.5:1).

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