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Evaluation and Selection of Optimal Solvent and Solvent Combinations in Thin-layer Chromatography of Flavonoids and of Phenolic Acids of *Zizyphus jujuba* Mill.*

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A series of simple mathematical techniques for the evaluation of solvents and solvent combinations in thin-layer chromatography have been investigated. The classification has been carried out using the numerical taxonomy techniques and information content derived from Shannon's equation. The methods have been applied to an R_F data set of flavonoids and phenolic acids identified in the methanolic extract of *Zizyphus jujuba* Mill. The most suitable chromatographic systems for the separation and future isolation of flavonoids and phenolic acids from *Zizyphus jujuba* Mill. are : ethyl acetate : formic acid : acetic acid : water, 100 : 11 : 11 : 27, and ethyl acetate : formic acid : water, 8 : 1 : 1.

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INTRODUCTION

Zizyphus jujuba Mill. is a small deciduous tree of the family Rhamnaceae, indigenous to the area extending from Southern Europe to Southeast Asia and widely cultivated in China, Korea and Japan.[1] It is also a cultivated plant in Croatia (Dalmatia and Istria).[2] This plant produces opposite small spines and two or three twigs in clusters in each node of the branch. The oval, smooth and asymmetric leaves with three costae are alternately arranged on the twig. Numerous, small, light-yellow flowers bloom in clusters at the axil in summer. The elliptic smooth and lustrous drupe is green at first to become yellowish-brown or red at maturity. The drupe contains a large seed.[1,3] The generic name, *Zizyphus*, and the specific epithet *jujuba*, both are derived from the Arabic name for this plant »zizuf«.[4]

Zizyphus jujuba Mill. is mostly used in folk medicine to heal bronchitis, diarrhea, insomnia, ulcers and wounds. Flavonoids from this plant have some influence on these pharmacological effects.[5,6]

Chemical investigations of *Zizyphus jujuba* Mill. leaves indicated the presence of flavonoids: isoquercitrin, rutin, quercetin 3-O-diglucoside, rhamnetin and eriodictyol.[7,8]

In the present paper, a strategy for a rapid selection of the optimum combination of solvents is proposed. Use was made of classification procedures based on calculation of the similarity between systems.[9] Classification is carried out using numerical taxonomy techniques.[10] Selection of optimal sets from the clusters that appear in the classification is based on the information content.[11] It is the object of this article to present such a technique. The proposed methods were applied to examine the efficiency of thirteen TLC systems for separating the seven components discovered in the methanolic extract of *Zizyphus jujuba* Mill.

EXPERIMENTAL

Materials

Extract solution: 1.0 g air-dried, powdered leaves of *Zizyphus jujuba* Mill. was refluxed with 10.0 ml methanol for 5 min, filtered; the filtrate was concentrated under reduced pressure, and the residue was taken up in 5.0 ml methanol.[12]

Reference solution: 10 mg rutin and 10 mg isoquercitrin dissolved in 10.0 ml methanol.

The thirteen systems used are given in Table I.[12-19]

In all systems, silica gel plates (20 × 20 cm, 0.25 mm thick) incorporating a fluorescent indicator, Kieselgel 60 F_{254}-Alufolien (E. Merck, Darmstadt, Art. Nr. 5554) were used. Paper liners were used in all tanks, and after addition of appropriate solvents, the systems were allowed to equilibrate for at least 30 minutes. 5 μL of the extract solution and of the reference solution was applied to the plates and the systems were allowed to run for 15 cm.

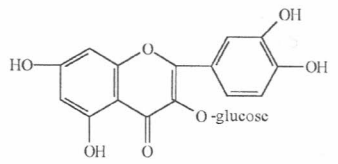
TABLE I

The thin-layer chromatographic systems studied

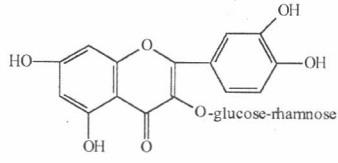
System No.	Solvent	Ref.
1	Ethyl acetate : formic acid : acetic acid : water (100 : 11 : 11 : 27)	12
2	Ethyl acetate : formic acid : water (8 : 1 : 1)	13
3	Ethyl acetate : formic acid : water (65 : 15 : 20)	14
4	Ethyl acetate : formic acid : water (67 : 20 : 13)	15
5	Ethyl acetate : formic acid : water (88 : 6 : 6)	16
6	Ethyl acetate : methanol : water (77 : 13 : 10)	17
7	Ethyl acetate : 1-propanol : water : formic acid (40 : 40 : 28 : 2)	15
8	1-Butanol : acetic acid : water – upper phase (4 : 1 : 5)	12
9	1-Butanol : acetic acid : water (66 : 17 : 17)	15
10	Chloroform : methanol : water – lower phase (6.5 : 3.5 : 1)	18
11	Ethyl acetate : methylethylketon : formic acid : water (50 : 30 : 10 : 10)	19
12	Ethyl acetate : methylethylketon : formic acid : water (50 : 30 : 30 : 10)	14
13	Ethyl acetate : formic acid : acetic acid : methylethylketon : water (50 : 7 : 3 : 30 : 10)	12

Visualization of the flavonoids was attained by spraying the sheets with 1% methanolic diphenylboryloxyethylamine, followed by 5% ethanolic polyethylene glycol 4000. The chromatograms were evaluated in UV 366 nm light (flavonoids as orange-yellow and phenolic acids as blue fluorescent bands).[12]

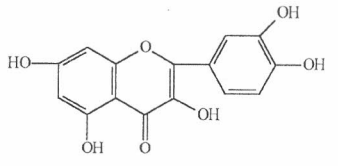
The structures of the flavonoids identified in the methanolic extract of *Zizyphus jujuba* Mill. are presented in Figure 1.



Isoquercitrin /quercetin 3-0-glucoside/



Rutin /quercetin 3-0-rhamnoglucoside/



Quercetin

Figure 1. Structures of the studied compounds.

Methods

Calculation of the information content

Extensive information has been calculated for thirteen TLC systems by Shannon's formula. Calculation of the information content will become possible if the uncertainties before and after the analysis can be expressed in a quantitative way.

Distribution of 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 the assumption of n_k R_F values in the k-th groups, the average information content (entropy) is given by the following Shannon equation.[9,20,21]

$$I(X) = H(X) = -\sum_k [n_k/n]\, ld[n_k/n]\ [bit]\ .$$

It is also assumed that the compounds with R_F values within one group cannot be identified. It is obvious that the entropy is at its highest level if there is only one R_F value, *i.e.* $H_m(X) = ld\ n$ within each group.

Determination of discriminating power (DP)

The *DP* of a set of chromatographic systems is defined as the probability of identifying two randomly selected compounds in at least one of the systems.[22-26] It must be possible to discriminate all pairs of N in order to compute the *DP* of k chromatographic systems in which N compounds are investigated. For the total number of matching pairs (M), the probability of a random selection of chromatographically similar pairs is $2M/N(N\text{-}1)$. Therefore, the *DP* of k systems is

$$DP_k = 1 - 2M/N(N-1)\ .$$

The average number of chromatographically similar compounds (T) for the chromatographic systems considered can be calculated from the following equation

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

Calculation of taxonomic distances, cluster formation and dendrogram

Taxonomy is defined as the theoretical study of classification including its elementary principles, procedures and rules.[10] Numerical taxonomy deals with the ways of classifying chromatrographic systems into taxonomic groups based on characteristic values (R_F values). The mathematical principle of this procedure is based on the formation of a matrix where the columns represent the solvent systems and the rows the substances. Classification is carried out with respect to resemblances between the solvent systems. Dissimilarity expressed as the complement of similarity is proportional to the distances of the solvent systems in the given metric space. The greater the differences in the properties of the solvent systems, the larger are their spatial distances. Taxonomic distance is inversely related to similarity. The distance $d_{j,k}$ between the solvent systems j and k is defined as

$$d_{j,k} = \left[\sum_{i=1}^{N} (X_{i,j} - X_{i,k})^2\right]^{1/2}$$

where $X_{i,j}$ and $X_{i,k}$ are the R_F values of the investigated compound i in the solvent systems j and k and N is the number of R_F values taken into account.

In the classification by taxonomy, a resemblance matrix containing either the correlation coefficient or the taxonomic distance is constructed. The reduction of this matrix is carried out by a weighted pair group method using the arithmetic average.[10] The smallest distance $d_{i,k}$ or highest correlation coefficient is selected: i and k are the most similar solvent systems and are therefore considered to form one group p'. The similarity coefficient between the new group p' and all other phases (*e.g.* q) is calculated, *e.g.* for the distance, as follows:

$$d_{j,p} = 1/2(d_{j,p} + d_{j,q}) \ .$$

The total number of rows and columns in the resemblance matrix is, therefore, reduced to one. This process is repeated until all chromatographic systems are comprised in one non-overlapping hierarchic system of groups and subgroups (clusters). The procedure for cluster formation is presented by a dendrogram.[27-29]

The three approaches were compared applying our computer search program KT 1.[27]

RESULTS AND DISCUSSION

A data set of R_F values for the separation of flavonoids and phenolic acids of a methanolic extract of *Zizyphus jujuba* Mill. into thirteen different chromatographic systems was analyzed.

An optimal combination of two or more systems was selected using the following procedures:

a) determination and comparison of the amount of information and discriminating power for all possible combinations of chromatographic systems,
b) classification of chromatographic systems into groups with similar separation properties and selection of the most efficient chromatographic system from each group.

The optimal combination of two or more chromatographic systems for identification of a compound by TLC has been readily determined from the taxonomic distances.[30]

Table II 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. Under the conditions most frequently used in chromatographic analysis, *i.e.* E = 0.05, the most suitable systems for separating the compounds studied are the chromatographic systems **1** (ethyl acetate : formic acid : acetic acid : water, 100 : 11 : 11 : 27) and **2** (ethyl acetate : formic acid : water, 8 : 1 : 1), because they showed the largest discriminating power (*D.P.* = 0.9048) and information content (I = 2.807). Chromatographic systems **6** (ethyl acetate : methanol : water, 77 : 13 : 10) and **11** (ethyl acetate : methylethylketon :

TABLE II

Input and output data for the D.P. and cluster formation
Input data (R_F values of the *Zizyphus jujuba* components)

Compound	Solvent system*						
	1	**2**	**3**	**4**	**5**	**6**	**7**
Phenolic acid 1	0.91	0.89	0.95	0.91	0.86	0.85	0.87
Flavonoid 1	0.66	0.54	0.76	0.78	0.31	0.53	0.82
Flavonoid 2	0.55	0.46	0.71	0.75	0.15	0.46	0.78
Phenolic acid 2	0.48	0.38	0.67	0.71	0.14	0.41	0.74
Flavonoid 3	0.44	0.26	0.58	0.64	0.11	0.35	0.69
Flavonoid 4	0.39	0.22	0.54	0.59	0.08	0.31	0.67
Flavonoid 5	0.35	0.19	0.50	0.54	0.04	0.27	0.62
Compound	Solvent system*						
	8	**9**	**10**	**11**	**12**	**13**	
Phenolic acid 1	0.71	0.68	0.82	0.90	0.88	0.92	
Flavonoid 1	0.58	0.54	0.31	0.63	0.81	0.64	
Flavonoid 2	0.53	0.53	0.30	0.61	0.80	0.62	
Phenolic acid 2	0.49	0.52	0.25	0.48	0.76	0.48	
Flavonoid 3	0.47	0.49	0.22	0.39	0.72	0.37	
Flavonoid 4	0.43	0.45	0.19	0.34	0.67	0.33	
Flavonoid 5	0.38	0.41	0.16	0.28	0.61	0.29	

Flavonoid 1 = isoquercitrin (quercetin 3-O-glucoside)
Flavonoid 3 = rutin (quercetin 3-O-rhamnoglucoside)
Flavonoids 2, 4 and 5 = derivatives of quercetin
* Copies of chromatograms can be obtained from the authors on request

TLC-system	***E* = 0.05**		***E* = 0.10**	
	D.P.	*I* (bit)	*D.P.*	*I* (bit)
1	0.9048	2.807	0.7143	2.236
2	0.9048	2.807	0.7619	2.522
3	0.8571	2.807	0.6667	2.236
4	0.8571	2.522	0.6667	1.842
5	0.7619	2.128	0.5714	1.842
6	0.9048	2.522	0.6667	2.236
7	0.8571	2.522	0.4762	1.557
8	0.8571	2.522	0.5238	1.842
9	0.6667	1.842	0.4286	1.449
10	0.8095	2.236	0.4762	1.842
11	0.9048	2.522	0.8095	2.236
12	0.8571	2.522	0.4762	1.557
13	0.8571	2.522	0.8095	2.236

Combined solvent systems – K = 2			
		***E* = 0.05**	
Combination sequence:			
1.	**6-12**	D.P. = 1.0000	T = 1.000
2.	**2-12**	D.P. = 1.0000	T = 1.000
3.	**1-11**	D.P. = 1.0000	T = 1.000
4.	**1-7**	D.P. = 1.0000	T = 1.000
5.	**12-13**	D.P. = 0.9524	T = 1.286
6.	**11-12**	D.P. = 0.9524	T = 1.286
7.	**8-13**	D.P. = 0.9524	T = 1.286
8.	**8-11**	D.P. = 0.9524	T = 1.286
9.	**6-11**	D.P. = 0.9524	T = 1.286
10.	**6-8**	D.P. = 0.9524	T = 1.286
Combined solvent systems – K = 3			
		***E* = 0.05**	
Combination sequence:			
1.	**8-12-13**	D.P. = 1.0000	T = 1.000
2.	**8-11-12**	D.P. = 1.0000	T = 1.000
3.	**6-12-13**	D.P. = 1.0000	T = 1.000
4.	**6-11-12**	D.P. = 1.0000	T = 1.000
5.	**6-10-12**	D.P. = 1.0000	T = 1.000
6.	**6-9-12**	D.P. = 1.0000	T = 1.000
7.	**6-8-12**	D.P. = 1.0000	T = 1.000
8.	**6-7-12**	D.P. = 1.0000	T = 1.000
9.	**5-12-13**	D.P. = 1.0000	T = 1.000
10.	**5-11-12**	D.P. = 1.0000	T = 1.000

Cluster formation			
Cluster	Solvent	Solvent	Distance
1.	11	13	0.0131
2.	7	12	0.0169
3.	8	9	0.0270
4.	3	4	0.0429
5.	1	9	0.0478
6.	2	5	0.0600
7.	3	5	0.0774
8.	1	2	0.1017
9.	3	5	0.1034
10.	1	4	0.1230
11.	1	2	0.2300
12.	1	2	0.3568

formic acid : water, 50 : 30 : 10 : 10) are also suitable because of their slightly lower information content (I = 2.522) and identical discriminating power as systems **1** and **2**. At E = 0.10, the chromatographic system **2** seems to be the most appropriate one due to its amount of information (I = 2.522).

Combining two chromatographic systems with the error factor E = 0.05, all systems have a similar discriminating power (*D.P.* = 0.9524 – 1.0000). The number of compounds with similar chromatographic properties is at the minimum (T = 1.000 and 1.286). With this error factor, systems **1** and **2** turned out to be the best because of the highest discriminating power (*D.P.* = 1.0000) and a low number of chromatographically similar substances (T was only 1.000).

Applying the combination of three chromatographic systems at the same error factor (E = 0.05), all the compounds can be simultaneously positively identified. The discriminating power for the any combination from the first ten is the largest (*D.P.* = 1.0000) and the number of chromatographically similar substances is the least (T = 1.000).

The same result was obtained by cluster formation of chromatographically similar systems. In order to obtain the optimal combination of two chromatographic systems according to the dendrogram (Figure 2.), system **2** should be chosen from cluster 6 and system **1** from cluster 5.

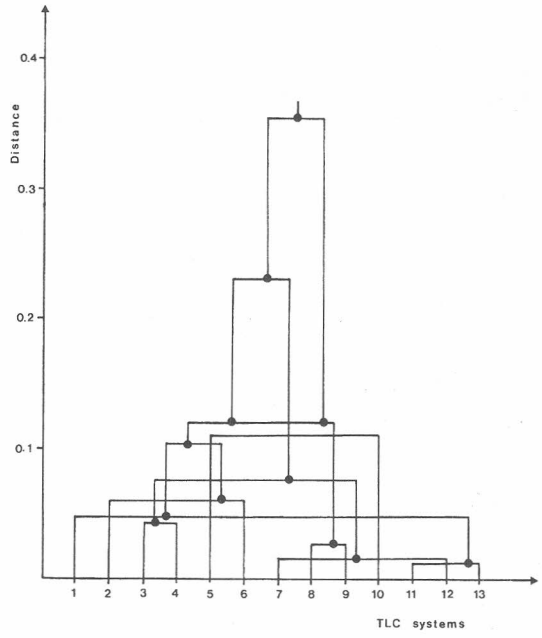


Figure 2. Dendrogram for thirteen TLC systems.

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SAŽETAK

Vrednovanje i izbor kromatografskih razvijača u tankoslojnoj kromatografiji flavonoida i fenolnih kiselina čičimaka – *Zizyphus jujuba* Mill.

Marica Medić-Šarić, Željan Maleš, Gordana Stanić i Slavko Šarić

Uporabljeni su odgovarajući matematički postupci za vrednovanje razvijača i kombinacija razvijača u tankoslojnoj kromatografiji. Klasifikacija razvijača je provedena metodama numeričke taksonomije uz dodatne kriterije: izračunavanje koeficijenta *DP*, i srednjeg vlastitog sadržaja informacije. Metode su primjenjene na eksperimentalnim podacima (R_F vrijednostima) za flavonoide i fenolne kiseline dokazane u metanolnom ekstraktu čičimaka – *Zizyphus jujuba* Mill.

Najprikladniji kromatografski razvijači za odvajanje i buduću izolaciju flavonoida i fenolnih kiselina čičimaka su: etilacetat : mravlja kiselina : octena kiselina : voda, 100 : 11 : 11 : 27 i etilacetat : mravlja kiselina : voda, 8 : 1 : 1.