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## Selection of an optimal set of solvents in thin-layer chromatography of flavonoids and phenolic acids of *Lavandulae flos*

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A strategy for a rapid selection of a set from eleven thin-layer chromatographic (TLC) systems for the separation of flavonoids and phenolic acids identified in the methanolic extract of *Lavandulae flos* is discussed. For the selection of the optimal combination of systems, two approaches are possible. The first one is to determine and compare an amount of information and discriminating power for all possible combinations of TLC systems. The second approach is to classify the TLC systems into groups with similar separation properties and to select the best system from each group. The classification is carried out by numerical taxonomy methods. It has been shown that the most favourable TLC systems for the separation of investigated compounds are: ethyl acetate-formic acid-acetic acid-water (100:11:11:27 v/v) and ethyl acetate-formic acid-water (8:1:1 v/v).

### 1. Introduction

*Lavandula angustifolia* Mill. is a ca. 50 cm tall shrub belonging to the family Lamiaceae of widespread distribution in the Mediterranean region. It is a plant with narrow lanceolate leaves and bluish tubular-bilabiate flowers [1]. The drug *Lavandulae flos* contains an essential oil (*Lavandulae aetheroleum*), tannins, flavonoids, phenolic acids, coumarins and phytosterols [1–3]. It is applied as a mild sedative, cholagogue, spasmolytic, carminative, stomachic and diuretic drug [1].

Thin-layer chromatography (TLC) is used for the analysis of flavonoids and phenolic acids [4–6]. In this paper, the efficiency of eleven TLC systems [7–13] for the separation of flavonoids and phenolic acids identified in the methanolic extract of *Lavandulae flos* was investigated. As it is unreliable with eight separated compounds to select the best chromatogram by visual observation, we used numerical methods for this purpose [14–16].

### 2. Investigations, results and discussion

A data set of  $R_F$  values for the separation of flavonoids and phenolic acids of a methanolic extract of *Lavandulae flos* in eleven different chromatographic systems (Table 1) was analysed by three mathematical methods [17, 18].

The first method was the calculation of the information content derived from Shannon's equation [17] for each TLC system. In the second method, the discriminating power of the systems was measured individually and in combinations [18, 19]. In the third method chromatographic

systems were classified according to clusters [20–29]. The three approaches were compared by application of our computer search program KT1 [20].

**Table 1: Thin-layer chromatographic systems studied**

System No.	Solvents	Ref.
1	Ethyl acetate:formic acid:water (8:1:1 v/v)	[8]
2	Ethyl acetate:formic acid:acetic acid:water (100:11:11:27 v/v)	[7]
3	Ethyl acetate:formic acid:water (65:15:20 v/v)	[9]
4	Ethyl acetate:formic acid:water (67:20:13 v/v)	[10]
5	Ethyl acetate:formic acid:water (88:6:6 v/v)	[11]
6	Ethyl acetate:formic acid:water (30:2:3 v/v)	[12]
7	1-butanol:acetic acid:water (4:1:5 v/v), upper phase	[7]
8	1-butanol:acetic acid:water (66:17:17 v/v)	[10]
9	Chloroform:methanol:water (6.5:3.5:1 v/v), lower phase	[13]
10	Ethyl acetate:methylethylketone:formic acid:water (5:3:1:1 v/v)	[9]
11	Ethyl acetate:formic acid:acetic acid:methyl ethyl-ketone:water (50:7:3:30:10 v/v)	[7]

**Table 2: Input data –  $R_F$  values of flavonoids and phenolic acids of *Lavandulae flos* and development time (t)**

TLC system	1	2	3	4	5	6	7	8	9	10	11
t (min)	27	36	38	46	30	28	110	97	25	32	30
Compd.	$R_F$ values										
Apigenin	0.95	0.95	0.95	0.96	0.93	0.90	0.86	0.89	0.99	0.99	0.98
Luteolin	0.94	0.94	0.91	0.95	0.90	0.88	0.82	0.85	0.98	0.94	0.96
Caffeic acid	0.89	0.89	0.89	0.93	0.88	0.84	0.75	0.79	0.95	0.93	0.94
Flavonoid 1	0.82	0.83	0.83	0.86	0.75	0.71	0.70	0.75	0.91	0.83	0.85
Flavonoid 2	0.75	0.75	0.75	0.83	0.59	0.57	0.70	0.75	0.90	0.80	0.70
Flavonoid 3	0.61	0.62	0.62	0.80	0.38	0.37	0.66	0.70	0.85	0.63	0.65
Flavonoid 4	0.50	0.50	0.50	0.75	0.30	0.30	0.66	0.67	0.82	0.50	0.54
Chlorogenic acid	0.42	0.38	0.38	0.65	0.23	0.23	0.61	0.63	0.75	0.47	0.47

\* Copies of chromatograms can be obtained from the authors on request

Table 2 gives the input data ( $R_F$  values) for the investigated compounds. Table 3 gives output data for the discriminating power and the information content for each TLC system. Table 4 gives output data for combined systems  $K = 2$  and  $K = 3$  in a range of error factors ( $K$  is the number of combined systems). The error factors were 0.05 and 0.10, respectively.

Under the conditions most frequently used in the chromatographic analysis, i.e.,  $E = 0.05$ , the most suitable systems for separating the compounds studied are systems 1 (ethyl acetate-formic acid-water 8:1:1 v/v) and 2 (ethyl acetate-formic acid-acetic acid-water 100:11:11:27 v/v) because they showed the largest discriminating power ( $DP = 0.964$ ) and a high information content ( $I = 2.750$ ). At  $E = 0.10$ , chromatographic system 2 is better than system 1, because of its higher discriminating power ( $DP_2 = 0.821$ ;  $DP_1 = 0.786$ ) and information content ( $I_2 = 2.500$ ;  $I_1 = 2.250$ ).

Combining two chromatographic systems with the error factor  $E = 0.05$ , combinations of systems 2 and 10, and 1 and 10 are the best, because they showed the highest DP value (1.000) and the smallest number of chromatographically similar compounds ( $T = 1.000$ ). At  $E = 0.10$  sys-

tem 2 was included in the third and fourth combination ( $DP = 0.893$ ,  $T = 1.750$ ). Applying the combinations of three TLC systems at  $E = 0.05$  all combination sequences have the maximal discriminating power ( $DP = 1.000$ ) and a minimal number of chromatographically similar compounds ( $T = 1.000$ ). TLC system 2 is also found in the first ten combinations regardless of the error factor.

The same results were obtained by cluster formation (Table 5) and from a dendrogram (Fig.). Systems 1 and 2 are very similar, because the distance between them is very small and they belong to the same cluster. In order to obtain the optimal combination of two systems according to the dendrogram system 2 should be chosen from cluster I and one system should be chosen from cluster II (system 5 or 6). At  $E = 0.05$  system 5 and system 6 showed good results ( $DP = 0.929$ ,  $I = 2.750$ ).

If the criterion for the evaluation of systems is the development time, system 1 was found to be very suitable because it had a shorter development time ( $t_1 = 27$  min) than system 2 ( $t_2 = 36$  min).

The proposed calculations point to the conclusion that for the TLC analysis of the investigated compounds, the chro-

**Table 3: Output data for DP and I in a range of error factors (E) for each TLC system**

Error factor	E = 0.05		E = 0.10	
	DP	I (bit)	DP	I (bit)
TLC-system				
1	0.964	2.750	0.786	2.250
2	0.964	2.750	0.821	2.500
3	0.929	2.750	0.786	2.500
4	0.821	2.750	0.607	1.906
5	0.929	2.750	0.821	2.500
6	0.929	2.750	0.821	2.156
7	0.750	2.000	0.429	1.299
8	0.786	2.500	0.500	1.561
9	0.750	2.250	0.393	1.406
10	0.893	2.500	0.821	2.156
11	0.893	2.750	0.786	2.156

**Table 4: Output data for DP and T for combined TLC systems – K = 2 and K = 3**

Combination sequence	E = 0.05			E = 0.10		
	TLC systems	DP	T	TLC systems	DP	T
<b>K=2</b>						
1.	2, 10	1,000	1,000	3, 6	0.893	1.750
2.	1, 10	1,000	1,000	3, 5	0.893	1.750
3.	10, 11	0.964	1.250	2, 6	0.893	1.750
4.	8, 11	0.964	1.250	2, 5	0.893	1.750
5.	7, 11	0.964	1.250	10, 11	0.857	2.000
6.	7, 10	0.964	1.250	7, 10	0.857	2.000
7.	6, 10	0.964	1.250	6, 11	0.857	2.000
8.	6, 8	0.964	1.250	6, 10	0.857	2.000
9.	6, 7	0.964	1.250	6, 7	0.857	2.000
10.	5, 10	0.964	1.250	5, 11	0.857	2.000
<b>K=3</b>						
1.	8, 10, 11	1,000	1,000	3, 6, 7	0.929	1.500
2.	7, 10, 11	1,000	1,000	3, 5, 7	0.929	1.500
3.	6, 8, 10	1,000	1,000	2, 6, 7	0.929	1.500
4.	6, 7, 10	1,000	1,000	2, 5, 7	0.929	1.500
5.	5, 8, 10	1,000	1,000	7, 10, 11	0.893	1.750
6.	5, 7, 10	1,000	1,000	6, 7, 11	0.893	1.750
7.	3, 8, 10	1,000	1,000	6, 7, 10	0.893	1.750
8.	3, 7, 10	1,000	1,000	5, 7, 11	0.893	1.750
9.	2, 10, 11	1,000	1,000	5, 7, 10	0.893	1.750
10.	2, 9, 10	1,000	1,000	4, 10, 11	0.893	1.750

**Table 5: Formation of clusters**

Cluster	Solvent	Solvent	Distance
1	2	3	0.011
2	1	2	0.017
3	3	4	0.025
4	4	5	0.036
5	1	6	0.038
6	1	6	0.042
7	2	5	0.058
8	1	4	0.121
9	1	2	0.145
10	1	2	0.255

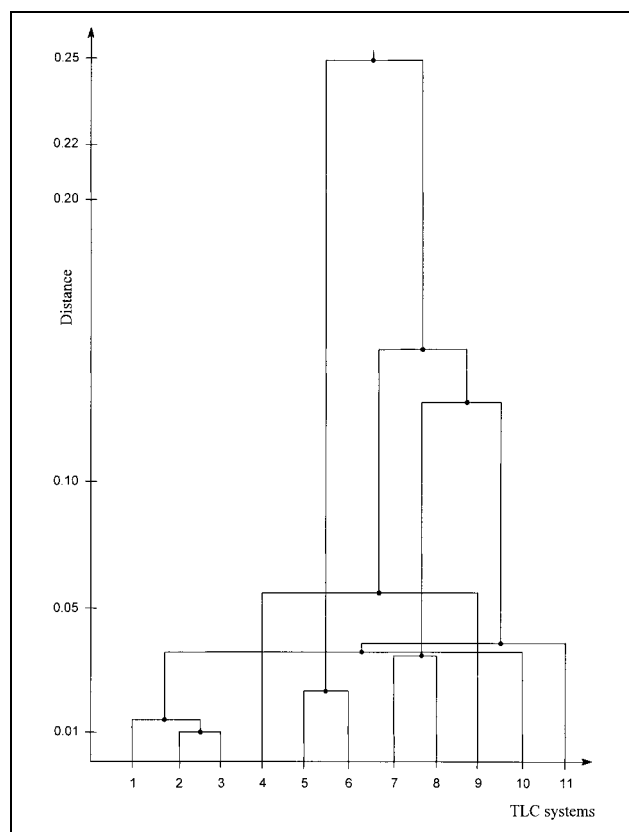


Fig.: Dendrogram for eleven TLC systems

matographic systems ethyl acetate-formic acid-acetic acid-water (100:11:11:27 v/v) and ethyl acetate-formic acid-water (8:1:1 v/v) are the most favourable. These results are in good agreement with those of two previous papers [26, 29].

### 3. Experimental

#### 3.1. Materials

Flowers of *Lavandula angustifolia* were collected in August 1996 in Nako-vanj (Croatia). A voucher specimen was retained at the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia.

All solvents were of analytical grade purchased from E. Merck (Darmstadt, Germany). Standards (apigenin, luteolin, caffeic acid and chlorogenic acid) were obtained from C. Roth (Karlsruhe, Germany).

Extract solution: 1.0 g air-dried, powdered flowers of *Lavandula angustifolia* was refluxed with 10.0 ml MeOH for 30 min and filtered. The filtrate was concentrated under reduced pressure, and the residue was taken up in 5.0 ml MeOH [7].

Standard solution: Apigenin, luteolin, caffeic acid and chlorogenic acid (10 mg of each) were dissolved in 10 ml MeOH.

#### 3.2. Methods

##### 3.2.1. Thin-layer chromatography

TLC was performed on precoated 10 × 10 cm TLC silica gel 60 F<sub>254</sub> sheets (thickness of layer 0.25 mm). Extract solution (5 µl) and 5 µl of the standard solution were applied 15 mm from the bottom of the sheet as 10 mm wide bands. The sheets were developed for 8 cm in paper-lined all-glass chambers (Desaga, Heidelberg-Germany) previously left to equilibrate for at least 30 min. Visualisation of the flavonoids and phenolic acids was achieved by spraying the sheets with 1% methanolic diphenyl-boryloxyethylamine, followed by 5% ethanolic polyethylene glycol 4000. The chromatograms were evaluated in UV light at λ = 366 nm (flavonoids appeared as orange-yellow bands and phenolic acids as blue fluorescent bands) [7].

##### 3.2.2. Numerical methods

###### 3.2.2.1. Calculation of the information content

The entropy (average information content) is given by Shannon's equation [17, 20]:

$$I(X) = H(X) = -\sum_k \frac{n_k}{n} \log_2 \frac{n_k}{n} \quad (\text{bit}) \quad (1)$$

###### 3.2.2.2. Determination of discriminating power (DP)

The DP of a set of chromatographic systems is defined as a probability of identifying two randomly selected compounds in at least one of the systems. For the total number of matching pairs (M), the probability of a random selection of chromatographically similar pairs is 2M/(N(N-1)). The DP of k systems in which N compounds are investigated is therefore [18, 22]:

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

The average number of chromatographically similar flavonoids or phenolic acids (T) for the chromatographic systems considered can be calculated from the eq. (3) [19, 25]:

$$T = 1 + (N-1)(1 - DP_k) \quad (3)$$

###### 3.2.2.3. Cluster formation and dendrogram

Cluster formation in this study was performed by a weighted pair group method using the arithmetic average [20]. Chromatographic systems with

high resemblance are grouped into clusters. The procedure for cluster formation is presented as a dendrogram [22-29].

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