ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 353–359

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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Optimization of TLC analysis of flavonoids and phenolic acids of *Helleborus atrorubens* Waldst. et Kit.

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Received 2 May 2000; accepted 11 July 2000

Abstract

Numerical methods for the evaluation of the separation power of thirteen thin-layer chromatographic systems for splitting a methanolic extract of leaves of *Helleborus atrorubens* Waldst. et Kit. into 15 compounds (flavonoids and phenolic acids) have been investigated. For this purpose, the following mathematical approaches have been applied: calculation of the information content (*I*), determination of discriminating power (*DP*) and formation of clusters and dendrogram. The most suitable chromatographic system for the separation of investigated compounds is ethyl acetate-formic acid-water (65:15:20, v/v/v). © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Thin-layer chromatography; Flavonoids; Phenolic acids; *Helleborus atrorubens* Waldst. et Kit.; Numerical methods

1. Introduction

Helleborus atrorubens Waldst. et Kit. is an endemic species belonging to the family Ranunculaceae. This plant is distributed in Croatia and Slovenia. It is a ca. 40 cm tall species with 7–11 segments of leaves. Flowers are 4–6 cm in diameter, violet or green [1,2].

Thin-layer chromatography (TLC) on silica gel is very favourable for the qualitative analysis of flavonoids and phenolic acids [3–5].

In this paper the efficiency of different TLC systems is compared by numerical methods [6,7]. Information content can serve as an important criterion for the evaluation, selection or optimization of analytical procedures [8]. Information theory can be used to compare the quality of chromatographic systems as well as to optimize such systems [9]. The discriminating power (*DP*) is used as a measure of the effectiveness of chromatographic systems. Two compounds are chromatographically similar if the differences in their identification values do not exceed the error factor *E*. In case of a large number of compounds, complete identification is rather difficult. Thus, calculating and maximizing the *DP* values can be

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PII: S0731-7085(00)00455-6

more readily achieved by satisfying the following conditions: a rectangular distribution of R_F values, reproducibility of results and no correlations between chromatographic systems [10,11]. The numerical taxonomy methods classify the chromatographic systems according to clusters [6,12]. Numerical methods were compared by applying our computer search programs KT1 [13] on TLC data of the flavonoids and phenolic acids identified in the methanolic extract of the leaves of *Helleborus atrorubens*.

2. Experimental

2.1. Plant material and chemicals

Leaves of *Helleborus atrorubens* were collected in February 1998 in Samobor (surroundings of Zagreb). 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, from E. Merck (Darmstadt, Germany). Standards (ferulic acid, caffeic acid and chlorogenic acid) were obtained from C. Roth (Karlsruhe, Germany).

Table 1
The mobile phases studied

No.	Solvents	Ref.
1	Ethyl acetate-formic acid-acetic acid-water (100:11:11:27, v/v/v/v)	[14]
2	Ethyl acetate-formic acid-water (8:1:1, v/v/v)	[15]
3	Ethyl acetate-formic acid-water (65:15:20, v/v/v)	[16]
4	Ethyl acetate-formic acid-water (67:20:13, v/v/v)	[17]
5	Ethyl acetate-formic acid-water (88:6:6, v/v/v)	[18]
6	Ethyl acetate-formic acid-water (30:2:3, v/v/v)	[19]
7	Ethyl acetate-methanol-water (77:13:10, v/v/v)	[20]
8	Ethyl acetate-1-propanol-water-formic acid (40:40:28:2, v/v/v/v)	[17]
9	1-Butanol-acetic acid-water (4:1:5, v/v/v), upper phase	[14]
10	1-Butanol-acetic acid-water (66:17:17, v/v/v)	[17]
11	Ethyl acetate-methyl ethyl ketone-formic acid-water (5:3:1:1, v/v/v/v)	[16]
12	Ethyl acetate-formic acid-acetic acid-methyl ethyl ketone-water (50:7:3:30:10, v/v/v/v/v)	[14]
13	Ethyl acetate-methanol-formic acid-water (100:13.5:2.5:10, v/v/v/v)	[21]

2.2. Thin-layer chromatography

Extract solution: 1.0 g air-dried, powdered leaves of *Helleborus atrorubens* was refluxed with 10.0 ml methanol for 30 min, filtered, the filtrate concentrated under reduced pressure, and the residue taken up in 5.0 ml methanol [14].

Standard solution: ferulic acid, caffeic acid and chlorogenic acid (10 mg of each) were dissolved in 10.0 ml methanol.

TLC was performed on 10×20 cm TLC sheets coated with 0.25 mm layers of silica gel 60 F_{254} (E. Merck, No. 5554). After application of extract and standard solution (5 μl) the sheets were developed in paper-lined all-glass chambers (Desaga, Heidelberg-Germany) previously left to equilibrate for at least 30 min. The thirteen mobile phases used are listed in Table 1 [14–21].

Visualisation of the flavonoids and phenolic acids was achieved by spraying the sheets with 1% methanolic diphenylboryloxyethylamine followed by 5% ethanolic polyethylene glycol 4000. The chromatograms were evaluated in UV light at $\lambda = 366$ nm (flavonoids appeared as orange-yellow bands and phenolic acids as blue fluorescent bands) [14].

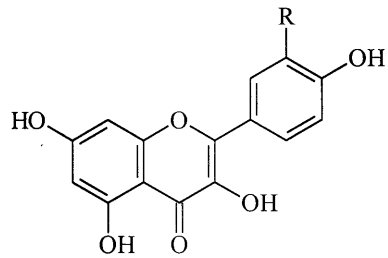
The structures of the identified flavonoids and phenolic acids are presented in Fig. 1.

2.3. Numerical methods

2.3.1. Calculation of the information content

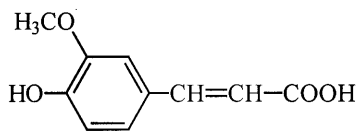
Distribution of R_F values into groups with error factor E (e.g. $E = 0.05$ or $E = 0.10$) with respect to R_F units and the assumption of $n_k R_F$ values in the kth group, the average information content (entropy) is given by the following Shannon equation [22,23]:

$$I(X) = H(X) = -\sum_k \frac{n_k}{n} ld \frac{n_k}{n} \quad \text{[bit]} \qquad (1)$$

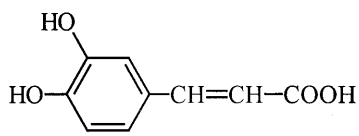


R=OH Quercetin

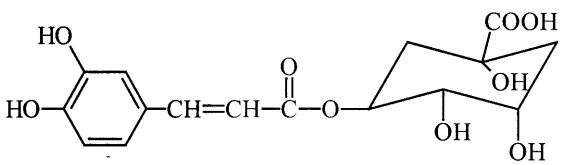
R=H Kaempferol



Ferulic acid



Caffeic acid



Chlorogenic acid

Fig. 1. Structures of the identified flavonoids and phenolic acids.

2.3.2. 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 [13,24–26]. 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 $2M/N(N-1)$. Therefore, the *DP* of k systems is:

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

The average number of chromatographically similar compounds (T) for the chromatographic systems considered can be calculated from the following equation [13]:

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

2.3.3. Computation of taxonomic distances, cluster formation and dendrogram

The optimum combinations of two or more chromatographic systems for the separation of flavonoids and phenolic acids by TLC can be readily determined from the taxonomic distances [27]. Taxonomic distance is inversely related to similarity. The greater the differences between the properties of the mobile phases, the larger are their spatial distances. Chromatographic systems with high resemblance are grouped into clusters. Cluster formation in this paper was performed by a weighted pair group method using the arithmetic average [6]. The procedure for cluster formation is presented by a dendrogram [5,13,24–26,28–31].

3. Results and discussion

A data set of R_F values of flavonoids and phenolic acids identified in the methanolic extract of leaves of *Helleborus atrorubens* (Table 2) by thirteen mobile phases (Table 1) was analyzed.

Table 3 gives output data for the discriminating power (*DP*) and information content (I) for each mobile phase. Tables 4 and 5 give output data for combined mobile phases $K=2$ and $K=3$ in a range of error factors. 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 mobile phase for separating the compounds studied is mobile phase 3 (ethyl acetate-formic acid-water, 65:15:20, v/v/v) because it

Table 2
Input data: R_F values of flavonoids and phenolic acids of the leaves of *Helleborus atrorubens* and development time (t)[a]

Mobile phase[b]	1	2	3	4	5	6	7	8	9	10	11	12	13
t [min]	122	124	166	156	98	111	101	255	280	317	114	111	96
Compound	R_F values												
Ferulic acid	0.96	0.91	0.97	0.88	0.88	0.90	0.40	0.86	0.73	0.70	0.91	0.88	0.83
Caffeic acid	0.93	0.88	0.92	0.86	0.83	0.86	0.38	0.82	0.69	0.67	0.89	0.86	0.81
Flavonoid I	0.75	0.55	0.86	0.84	0.48	0.37	0.33	0.79	0.64	0.62	0.83	0.79	0.71
Phenolic acid III	0.72	0.48	0.81	0.81	0.42	0.35	0.30	0.77	0.57	0.56	0.72	0.67	0.51
Phenolic acid IV	0.70	0.46	0.77	0.79	0.32	0.33	0.29	0.75	0.54	0.54	0.69	0.63	0.48
Flavonoid II	0.60	0.44	0.72	0.75	0.27	0.32	0.23	0.73	0.52	0.52	0.66	0.59	0.42
Chlorogenic acid	0.53	0.42	0.61	0.71	0.23	0.24	0.21	0.68	0.49	0.49	0.54	0.51	0.38
Flavonoid III	0.49	0.36	0.56	0.67	0.17	0.17	0.17	0.65	0.44	0.46	0.50	0.46	0.35
Phenolic acid VI	0.47	0.32	0.52	0.64	0.14	0.14	0.15	0.63	0.40	0.44	0.48	0.43	0.33
Flavonoid IV	0.43	0.29	0.40	0.61	0.10	0.11	0.12	0.59	0.36	0.41	0.44	0.39	0.30
Phenolic acid VII	0.39	0.21	0.33	0.55	0.07	0.10	0.08	0.53	0.33	0.38	0.37	0.28	0.25
Flavonoid V	0.30	0.15	0.28	0.46	0.05	0.05	0.05	0.51	0.29	0.34	0.26	0.19	0.18
Flavonoid VI	0.19	0.10	0.24	0.31	0.02	0.03	0.03	0.48	0.24	0.30	0.13	0.11	0.14
Flavonoid VII	0.14	0.04	0.21	0.26	0.01	0.01	0.02	0.45	0.21	0.27	0.10	0.09	0.06
Flavonoid VIII	0.06	0.01	0.13	0.16	0.00	0.00	0.00	0.38	0.17	0.23	0.04	0.04	0.02

[a] Flavonoids I and II = derivatives of kaempferol. Flavonoids III–VIII = derivatives of quercetin.
[b] Copies of chromatograms can be obtained from the authors on request.

had the largest discriminating power ($DP_3 = 0.952$) and a high information content ($I_3 = 3.774$). Mobile phase 12 (ethyl acetate-formic acid-acetic acid-methyl ethyl ketone-water, 50:7:3:30:10, v/v/v/v/v) is also suitable, because of its slightly lower discriminating power ($DP_{12} = 0.933$) and identical information content ($I_{12} = 3.774$). For $E = 0.10$ mobile phase 11 (ethyl acetate-methyl ethyl ketone-formic acid-water, 5:3:1:1, v/v/v/v) seems to be the most appropriate because of the largest discriminating power ($DP_{11} = 0.857$) and the highest information content ($I_{11} = 3.190$).

Combining two mobile phases with the error factor $E = 0.05$, mobile phase 3 comes in the first four combinations ($DP = 0.971$, $T = 1.400$). At $E = 0.10$ mobile phase 3 is included in the first combination, while mobile phase 11 is found in the first two combinations ($DP = 0.895$, $T = 2.467$).

Applying the combinations of three mobile phases at $E = 0.05$ all combination sequences have an identical values of discriminating power ($DP = 0.981$) and an identical number of chromatographically similar compounds ($T = 1.267$). Mobile phase 3 comes in the eight combinations. At $E = 0.10$ mobile phases 3 and 11 often come in the first three combinations ($DP = 0.914$, $T = 2.200$).

The same results were obtained by cluster formation (Table 6) and from the dendrogram. In order to obtain the optimal combination of two

Table 3
Output data for DP and I in a range of error factors for each mobile phase

Mobile phase	$E = 0.05$		$E = 0.10$	
	DP	I (bit)	DP	I (bit)
1	0.923	3.640	0.838	3.057
2	0.914	3.507	0.790	2.790
3	0.952	3.774	0.838	2.923
4	0.895	3.374	0.742	2.740
5	0.866	3.240	0.723	2.340
6	0.838	2.790	0.704	2.149
7	0.828	2.840	0.638	1.966
8	0.866	3.374	0.685	2.473
9	0.895	3.507	0.742	2.689
10	0.876	3.240	0.685	2.289
11	0.933	3.640	0.857	3.190
12	0.933	3.774	0.828	3.107
13	0.904	3.640	0.800	2.923

Table 4
Output data for *DP* and *T* for combined mobile phases-K = 2

Combination sequence	$E = 0.05$			$E = 0.10$		
	Mobile phases	*DP*	*T*	Mobile phases	*DP*	*T*
1	3, 13	0.971	1.400	3, 11	0.895	2.467
2	3, 4	0.971	1.400	1, 11	0.895	2.467
3	2, 3	0.971	1.400	11, 13	0.885	2.600
4	1, 3	0.971	1.400	5, 11	0.885	2.600
5	5, 12	0.961	1.533	3, 13	0.885	2.600
6	5, 11	0.961	1.533	1, 12	0.885	2.600
7	4, 5	0.961	1.533	1, 3	0.885	2.600
8	3, 12	0.961	1.533	6, 11	0.876	2.733
9	3, 11	0.961	1.533	3, 12	0.876	2.733
10	3, 7	0.961	1.533	2, 11	0.876	2.733

Table 5
Output data for *DP* and *T* for combined mobile phases-K = 3

Combination sequence	$E = 0.05$			$E = 0.10$		
	Mobile phases	*DP*	*T*	Mobile phases	*DP*	*T*
1	5, 12, 13	0.981	1.267	3, 5, 11	0.914	2.200
2	5, 11, 13	0.981	1.267	1, 3, 12	0.914	2.200
3	3, 12, 13	0.981	1.267	1, 3, 11	0.914	2.200
4	3, 11, 13	0.981	1.267	3, 11, 13	0.904	2.333
5	3, 5, 13	0.981	1.267	3, 11, 12	0.904	2.333
6	3, 4, 13	0.981	1.267	3, 6, 11	0.904	2.333
7	3, 4, 7	0.981	1.267	2, 3, 11	0.904	2.333
8	3, 4, 5	0.981	1.267	1, 11, 12	0.904	2.333
9	2, 3, 13	0.981	1.267	1, 3, 13	0.904	2.333
10	2, 3, 7	0.981	1.267	11, 12, 13	0.895	2.467

mobile phases according to the dendrogram (Fig. 2.) mobile phase 3 or 11 should be chosen from cluster I and one mobile phase should be chosen from cluster II (mobile phase 5, 6 or 7). Mobile phase 5 (ethyl acetate-formic acid-water, 88:6:6, v/v/v) is better than mobile phase 6 (ethyl acetate-formic acid-water, 30:2:3, v/v/v) and 7 (ethyl acetate-methanol-water, 77:13:10, v/v/v) because of its largest discriminating power ($DP_5 = 0.866$; $DP_6 = 0.838$; $DP_7 = 0.828$) and information content ($I_5 = 3.240$; $I_6 = 2.790$; $I_7 = 2.840$).

4. Conclusion

As it is unreliable to select the best chromatogram with 15 separated compounds (eight flavonoids and seven phenolic acids) identified in the methanolic extract of the leaves of *Helleborus atrorubens* by visual observation, we used for this purpose the numerical methods. Three phenolic acids were identified as ferulic acid, caffeic acid and chlorogenic acid, while the investigated flavonoids were proved as derivatives of kaempferol and quercetin.

The best chromatographic system is shown to be system 3 (ethyl acetate-formic acid-water, 65:15:20, v/v/v), but system 11 (ethyl acetate-methyl ethyl ketone-formic acid-water, 5:3:1:1, v/v/v/v) is also suitable.

The results obtained by applying numerical methods (calculation of the information content,

Table 6
Formation of clusters

Cluster	Mobile phase	Mobile phase	Distance
1	9	10	0.038
2	1	10	0.039
3	5	6	0.040
4	2	10	0.055
5	1	9	0.056
6	1	3	0.084
7	3	6	0.092
8	2	6	0.128
9	1	2	0.151
10	3	4	0.186
11	1	2	0.195
12	1	2	0.350

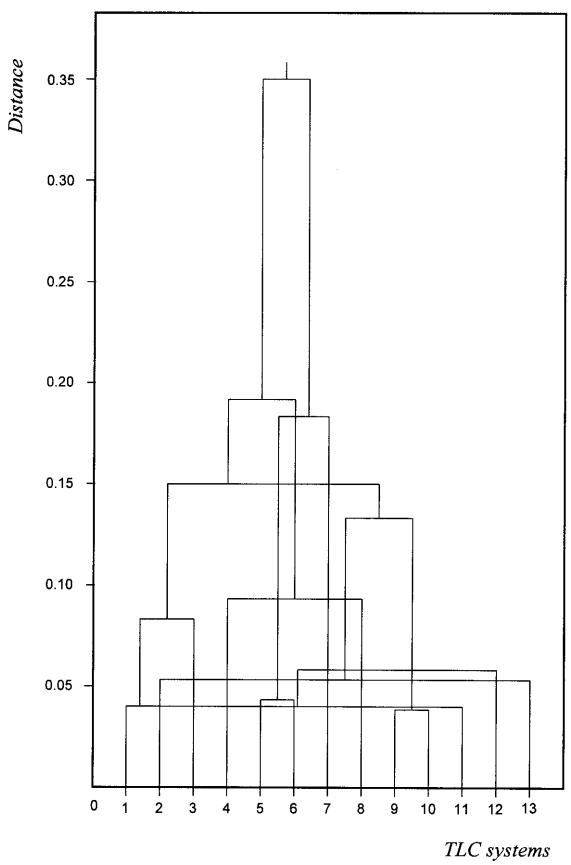


Fig. 2. Dendrogram for thirteen TLC systems.

determination of discriminating power and formation of clusters and dendrogram) are useful mathematical tools in the classification and combination of chromatographic systems.

Acknowledgements

This work was supported by the Ministry of Science and Technology of the Republic of Croatia (Project No. 006242).

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