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Optimization of Chromatographic Conditions in Thin Layer Chromatography of Flavonoids and Phenolic Acids*

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Flavonoids are one of the largest groups of natural compounds known. They are supposed to have numerous physiological activities. There are many foods that contain flavonoids, but one of the most important sources of flavonoids is propolis. Besides flavonoids, phenolic acids are the main active substances of propolis. The list of uses and preparations of propolis is almost endless and demands an accurate analytical method to define the substances in this natural product. It can be easily analyzed by chromatographic methods, but before testing a new type of propolis it is opportune to optimize chromatographic conditions. The aim of this study is to optimize the chromatographic conditions in TLC of flavonoids and phenolic acids, as standard compounds that may be present in Croatian propolis. We compared 9 different mobile phases, using information theory and numerical taxonomy methods and applying the computer search program KT1, to find the most appropriate mobile phase, the optimal combination of two and three mobile phases for separation of standards.

Key words
flavonoids
phenolic acids
optimization
TLC
information theory
numerical taxonomy

INTRODUCTION

Flavonoids are members of a class of natural compounds with widespread occurrence in the plant kingdom. They are one of the largest groups of natural products known. Over 4000 flavonoids have been identified to date, widely distributed in the leaves, seeds, bark and flowers of plants. In plants, these compounds provide protection against ultraviolet radiation, pathogens, herbivores and are anthocyanin copigments in flowers that attract pollinating insects. They are also responsible for the characteristic red and blue colors of berries, wines and certain vegetables.[1] Flavonoids are benzo-γ-pyrone derivatives consisting of phenolic and pyrane rings (Figure 1) and are classified according to substitutions. There are six classes of flavonoids, which differ in their chemical structure – flavanols, flavones, flavonols, flavanons, isoflavons and anthocyanidins.

Most dietary flavonoids occur in food as 3-*O*-glycosides and polymers, but they can also exist in aglycon forms.

Many beneficial health effects are attributed to flavonoids, mostly due to their antioxidant and chelating

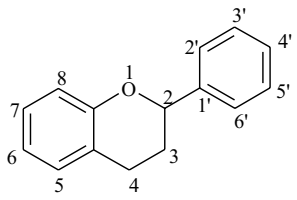

Figure 1. Base structure of flavonoids.

* Dedicated to Professor Nenad Trinajstić on the occasion of his 65th birthday.

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abilities.[2] Numerous studies have been conducted to prove flavonoids' efficacy as antimycotic, antibacterial, antiviral, anti-inflammatory, antioxidant, immune modulator, enzyme inhibitor, mutagenic and toxic agents.[3]

Many foods contain flavonoids, but one of their most important sources is propolis – a resinous substance collected by honeybees (*Apis melifera* L.) from various plants.

The use of propolis has been known since ancient times and now the list of its preparations and uses is almost endless. Its still increasing use led to the need for standardization and analysis of this natural product. The analysis is easily performed by chromatographic methods – thin layer chromatography, gas chromatography and high-performance liquid chromatography. To identify a group of compounds using these methods, it is necessary to have a set of standard substances for comparison of the analysis results of the unknown compound. Also, a common problem is to find the optimal chromatographic system to perform the analysis – to obtain the optimal separation and identification of the particular group of compounds.[5,6]

Optimization of chromatographic conditions can be done using different methods.[7–10] In our work we used information theory and numerical taxonomy[11] (applying the computer search program KT1)[12] to test (and compare) the efficacy of nine mobile phases appropriate for TLC of fifteen standard solutions of flavonoids and four standards of phenolic acids (which are a minor group of compounds in propolis, but also with potential physiological activities). The results of this study will be used to perform TLC analyses of Croatian propolis from different geographic regions, so that similarities and differences in their composition can be established.

EXPERIMENTAL

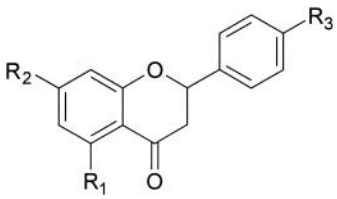
The analysis was performed on precoated 20 × 20 cm (0.25 mm thick) TLC plates K6F silica gel 60 A purchased from Whatman, USA. 10 μl of each standard solution (concentration 0.1 mg ml^{-1}, purchased from the Department of Pharmacognosy, Faculty of Pharmacy and Biochemistry, University of Zagreb) was applied as spots onto TLC sheets (standards are listed in Table I). Nine different mobile phases (Table II) were selected (according to their polarity) to establish the R_F value for every standard (all solvents were of analytical grade).

The plates were developed at room temperature in a vertical separating chamber to the height of approximately 16 cm from the start. The chamber was previously saturated with the appropriate mobile phase (saturation time was 1 hour). After drying, visualization was performed in two ways:

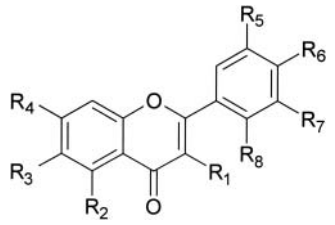
i) in short UV light (254 nm)

ii) spraying with 1 % methanolic diphenylboryloxyethylamine and 5 % ethanolic polyethyleneglycole 4000; chromatograms were interpreted in long wave UV light (366 nm).[13]

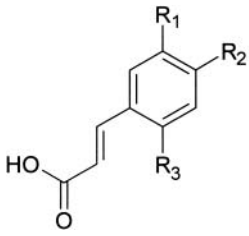
TABLE I. Standards that may be present in Croatian propolis



FLAVANONES	R_1	R_2	R_3
Flavanone	H	H	H
Naringenin	OH	OH	OH



Flavones	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
Flavone	H	H	H	H	H	H	H	H
3-Hydroxyflavone	OH							
6-Hydroxyflavone			OH					
6'-Hydroxyflavone								OH
7-Hydroxyflavone				OH				
3,6-Dihydroxyflavone	OH		OH					
3,7-Dihydroxyflavone	OH			OH				
Morin	OH	OH		OH	OH		OH	
Chrisyn		OH		OH				
Quercetin	OH	OH		OH	OH	OH		
Galangin	OH	OH		OH				
Apigenin		OH		OH		OH		
Kaempferol	OH	OH		OH		OH		



Phenolic acids	R_1	R_2	R_3
o-Coumaric acid	H	H	OH
p-Coumaric acid	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	OMe	OH	H

The R_F value is the identification characteristic in TLC and depends on the used combination of solvents. To optimize the chromatographic conditions two mathematical methods

TABLE II. Thin layer chromatographic systems studied

Chromatographic system No.	Solvent[(a)]
1	toluene:ethyl acetate:formic acid, 36:12:5
2	cyclohexane:ethyl acetate:formic acid, 30:15:5
3	toluene:ethyl acetate:acetic acid, 36:12:5
4	cyclohexane:ethyl acetate:acetic acid, 31:14:5
5	n-hexane:ethyl acetate:formic acid, 31:14:5
6	toluene:acetone:formic acid, 38:10:5
7	n-hexane:ethyl acetate:acetic acid, 31:14:5
8	petroleum ether:ethyl acetate:formic acid, 30:15:5
9	carbon tetrachloride:acetone:formic acid, 35:10:5

[(a)] Volume ratio.

have been applied: information theory and numerical taxonomy. The first approach is based on the generation of all possible combinations of the chromatographic systems studied with an estimation of the average amount of information for the selected set of compounds. It also calculates the probability of separating two compounds selected at random from a set of given substances (discriminating power, DP), as a measure of effectiveness of chromatographic systems. The second approach uses the classification of chromatographic systems according to clusters.[11] The chromatographic system can be selected from the dendrogram on the basis of the average amount of information or discriminating power.[14,15]

Generating information can be considered to be the reduction of uncertainty with respect to the composition or identity of the sample to be analyzed.[16] The Shannon equation describes the average information content (entropy); in this case, distribution of R_F values into groups with error factor E (usually $E \leq 0.05$), respecting R_F units and assuming $n_k R_F$ values in the k-th groups:

$$I(X) = H(X) = -\sum_k (n_k / n) ld(n_k / n) . \quad (1)$$

Discriminating power (DP) describes the chromatographic similarity of compounds – if the differences in their identification values (in this case R_F) do not exceed the given error factor E. For a chromatographic system, it is the probability of separating two randomly selected substances from a specific population. For a set of chromatographic systems, it defines the probability of separating two randomly selected substances in at least one of the systems:

$$DP_k = 1 - 2\,M / [N(N - 1)], \quad (2)$$

where k represents the chromatographic system, N is the number of compounds analyzed; M is the total number of matching pairs.

Chromatographically similar compounds are also described by the value T, which represents the average number of similar substances for chromatographic systems:[13]

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

Taxonomy is defined as the theoretical study of classification, including its elementary principles; procedures and

TABLE III. Input data – R_F values of flavonoids and phenolic acids (standards)

TLC System / Standard	1	2	3	4	5	6	7	8	9
Flavanone	0.67	0.40	0.62	0.65	0.38	0.62	0.75	0.76	0.71
Naringenin	0.54	0.37	0.58	0.44	0.24	0.44	0.52	0.73	0.38
Flavone	0.88	0.62	0.92	0.86	0.66	0.85	0.91	0.92	0.80
3-Hydroxyflavone	0.77	0.51	0.80	0.76	0.56	0.66	0.82	0.83	0.64
6-Hydroxyflavone	0.67	0.39	0.61	0.62	0.36	0.56	0.75	0.80	0.62
6'-Hydroxyflavone	0.52	0.32	0.46	0.51	0.28	0.48	0.56	0.73	0.50
7-Hydroxyflavone	0.46	0.30	0.42	0.42	0.26	0.46	0.47	0.70	0.43
3,6-Dihydroxyflavone	0.54	0.36	0.51	0.52	0.34	0.46	0.56	0.72	0.45
3,7-Dihydroxyflavone	0.54	0.36	0.50	0.48	0.33	0.47	0.54	0.70	0.45
Morin	0.23	0.16	0.14	0.14	0.13	0.23	0.13	0.32	0.13
Chrysin	0.62	0.38	0.60	0.53	0.36	0.56	0.68	0.74	0.51
Quercetin	0.39	0.27	0.27	0.28	0.22	0.35	0.30	0.60	0.34
Galangin	0.65	0.44	0.64	0.57	0.37	0.60	0.72	0.85	0.77
Apigenin	0.44	0.33	0.47	0.33	0.21	0.37	0.39	0.67	0.34
Kaempferol	0.51	0.37	0.50	0.39	0.23	0.40	0.47	0.77	0.27
o-Coumaric acid	0.55	0.38	0.51	0.51	0.37	0.48	0.73	0.75	0.41
p-Coumaric acid	0.55	0.36	0.51	0.49	0.34	0.47	0.69	0.75	0.37
Caffeic acid	0.38	0.26	0.30	0.33	0.22	0.34	0.43	0.62	0.32
Ferulic acid	0.56	0.32	0.49	0.49	0.28	0.45	0.63	0.70	0.45

TABLE IV. Output data for DP and *I* in the range of error factor (*E*) for each chromatographic system

Error factor	E = 0.05		E = 0.03		E = 0.02	
TLC system	DP	I / bit	DP	I / bit	DP	I / bit
1	0.8012	2.898	0.8538	3.221	0.8947	3.537
2	0.6433	2.246	0.7836	2.735	0.8129	3.116
3	0.7719	2.926	0.8655	3.076	0.8830	3.642
4	0.8421	3.261	0.8947	3.616	0.9181	3.787
5	0.7076	2.525	0.8187	2.860	0.8655	3.287
6	0.7719	2.866	0.8480	3.011	0.8772	3.471
7	0.8713	3.406	0.9298	3.682	0.9474	3.722
8	0.7018	2.655	0.7895	3.050	0.8655	3.392
9	0.8596	3.071	0.9240	3.511	0.9415	3.787

rules.[11] Operational taxonomic units (OTU, in this case chromatographic systems) are in different ways classified into taxonomic groups based on the characteristic values of OTU. Input data are given in matrix form ($N \times t$) where N is the number of properties and t is the number of OTU's. Two OTU's with similar values (properties) are associated with only one point in space (taxonomic distance equals zero). In other words, the greater the differences in the properties, the larger is the spatial distance – taxonomic distance is inversely related to the similarity of compounds.[17] The distance $d_{j,k}$ between chromatographic systems (mobile phases) j and k is equal:

$$d_{j,k} = \left[\sum_{i=1}^{N} (X_{i,j} - X_{i,k})^2 \right]^{1/2} \quad (4)$$

and the mean taxonomic distance is:

$$\Delta_{j,k} = (d_{j,k}^2 / N)^{1/2} . \quad (5)$$

Chromatographic systems of high similarity are grouped into clusters. Cluster formation in this work was carried out by a weighted pair group method using the arithmetic average.[11] The procedure for cluster formation can be followed (and is represented) by a dendrogram.[18–20] All mathematical methods, including formation of a dendrogram were compared using computer search program KT1.[12]

RESULTS AND DISCUSSION

A data set of R_F values for the separation of flavonoids and phenolic acids that may be present in Croatian propolis was analyzed by the use of nine different mobile phases.

Table III gives the R_F values for the investigated compounds. Table IV presents the output data of the discriminating power (DP) and information content (I) for each chromatographic system in a range of three error factors E = 0.05, 0.03, 0.02. Output data for combined systems (2 and 3 mobile phases, K = 2 and K = 3) are presented in Tables V and VI. The error factors were in the same range as error factors for DP.

After determination and comparison of the DP and I values for all TLC systems, the one with the largest discriminating power and information content is considered to be the best. Under the conditions commonly used in chromatographic analysis (E = 0.05), DP and I values for all systems were too low, so we had to determine a new set of values in a range of error factors E = 0.03 and E = 0.02. Among these 9 chromatographic systems with error factor E = 0.02, the most suitable TLC system for separating the studied compounds was system number 9 – carbon tetrachloride : acetone : formic acid, 35:10:5 (vol.) with the largest discriminating power (DP = 0.9415) and information content (I = 3.787). The TLC system number 7, with a slightly lower information content (I = 3.722) and larger discriminating power (DP = 0.9474) than the chromatographic system 4, was also suitable for analysis.

Using the error factor in a range of E = 0.05, the best combination of two chromatographic systems was shown to be the combination of TLC systems 7 and 9 (DP =

TABLE V. Output data for DP and *T* for combined TLC systems (K = 2, E = 0.05; 0.03; 0.02)

Combination sequence	TLC systems	E = 0.05		E = 0.03		E = 0.02	
		DP	T	DP	T	DP	T
1	7 – 9	0.9766	1.421	0.9942	1.105	0.9942	1.105
2	3 – 7	0.9532	1.842	0.9883	1.211	0.9942	1.105
3	4 – 9	0.9474	1.947	0.9825	1.316	0.9883	1.211
4	3 – 9	0.9474	1.947	0.9766	1.421	0.9883	1.211
5	1 – 9	0.9415	2.053	0.9766	1.421	0.9883	1.211
6	5 – 9	0.9357	2.158	0.9766	1.421	0.9883	1.211
7	4 – 7	0.9357	2.158	0.9766	1.421	0.9825	1.316
8	7 – 8	0.9298	2.263	0.9766	1.421	0.9825	1.316
9	2 – 9	0.9298	2.263	0.9708	1.526	0.9825	1.316
10	8 – 9	0.9240	2.368	0.9708	1.526	0.9825	1.316

TABLE VI. Output data for DP and *T* for combined TLC systems (K = 3, *E* = 0.05; 0.03; 0.02)

Combination sequence	TLC systems	*E* = 0.05		*E* = 0.03		*E* = 0.02	
		DP	*T*	DP	*T*	DP	*T*
1	7 – 8 – 9	0.9883	1.211	1.0000	1.000	1.0000	1.000
2	3 – 7 – 9	0.9883	1.211	1.0000	1.000	1.0000	1.000
3	2 – 7 – 9	0.9883	1.211	0.9942	1.105	1.0000	1.000
4	1 – 7 – 9	0.9883	1.211	0.9942	1.105	1.0000	1.000
5	6 – 7 – 9	0.9825	1.316	0.9942	1.105	1.0000	1.000
6	4 – 7 – 9	0.9825	1.316	0.9942	1.105	1.0000	1.000
7	5 – 7 – 9	0.9766	1.421	0.9942	1.105	1.0000	1.000
8	5 – 8 – 9	0.9708	1.526	0.9942	1.105	1.0000	1.000
9	4 – 5 – 9	0.9708	1.526	0.9942	1.105	1.0000	1.000
10	3 – 7 – 8	0.9708	1.526	0.9942	1.105	1.0000	1.000

TABLE VII. Formation of clusters

Cluster	TLC system	TLC system	Distance
1	4	6	0.0461
2	1	3	0.0545
3	2	4	0.0621
4	1	3	0.0687
5	1	5	0.0929
6	1	3	0.1271
7	1	3	0.2074
8	1	2	0.2994

0.9766, *T* = 1.421). In the case when two experiments are carried out, one in the TLC system number 9 and the other in the TLC system number 7, all substances will differ according to the R_F value. In a range of the error factor value 0.02, combinations of systems 3 and 7 have the same values of discriminating power and the number of chromatographically similar substances (DP = 0.9942, *T* = 1.105) as the most suitable combination (7 and 9).

Four series of three systems for *E* = 0.05 have the same DP and *T* values and all of them include TLC systems 7 and 9 (combinations 7 – 8 – 9, 3 – 7 – 9, 2 – 7 –9 and 1 – 7 – 9 with the discriminating power values of 0.9883 and the number of chromatographically similar substances of 1.211). With the error factor *E* = 0.02, all combinations of three systems become equally suitable for analysis with the values of DP = 1.000 and *T* = 1.000.

Cluster formation (Table VII) is graphically presented by the dendrogram (Figure 2).

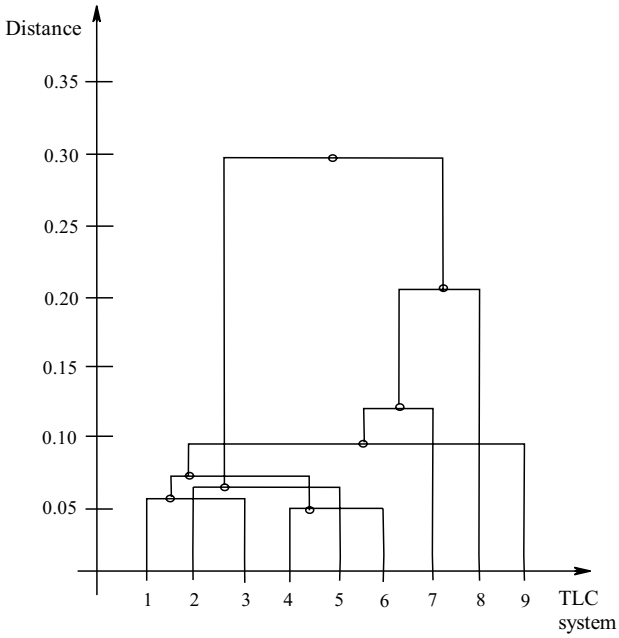


Figure 2. Dendrogram for 9 TLC systems.

CONCLUSIONS

The numerical techniques applied allow a rational classification and selection of TLC systems most suitable for a given analysis. Mathematical methods provide a rapid solution to the problem of evaluating the most efficient chromatographic system and the optimal choice of the combination of systems to enable identification of a particular group of compounds, in this case flavonoids and phenolic acids.

In our study, the most suitable TLC system for analysis was shown to be petroleum ether : acetone : formic acid, 35:10:5 (vol.) with the largest discriminating power (DP = 0.9415) and information content (*I* = 3.787). The system n-hexane : ethyl acetate : acetic acid, 31:14:5 (vol.), with a slightly lower information content (*I* = 3.722) and larger discriminating power (DP = 0.9474), was also proven suitable.

The results of this study will be used in the analyses of Croatian propolis.

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SAŽETAK

Optimiranje kromatografskih uvjeta u tankoslojnoj kromatografiji flavonoida i fenolnih kiselina

Marica Medić-Šarić, Ivona Jasprica, Asja Smolčić-Bubalo i Ana Mornar

Flavonoidi predstavljaju veliku grupu prirodnih spojeva s brojnim poznatim fiziološkim učincima. Sastavni su dio različitih namirnica, a jedan od najvažnijih izvora je propolis. U propolisu su glavne aktivne supstancije, osim flavonoida, fenolne kiseline. Uporaba propolisa i pripravaka na njegovoj bazi danas je vrlo raširena, a zahtjeva brižan odabir analitičke metode za utvrđivanje sastavnica pojedinoga prirodnoga produkta. Lako se mogu analizirati kromatografskim metodama, ali prije ispitivanja nekoga novoga uzorka propolisa potrebno je optimirati uvjete kromatografskoga procesa. U ovome je radu optimiran TLC postupak za identifikaciju standardnih spojeva iz reda flavonoioda i fenolnih kiselina, a koji su nazočni u hrvatskome propolisu. Ispitivanje je provedeno za 9 različitih kromatografskih razvijača, rabeći programski paket KT1. Pomoću metode numeričke taksonomije i određivanjem srednjega vlastitoga sadržaja informacije odabran je odgovarajući razvijač i optimalna kombinacija dva ili tri razvijača za jednoznačno razlikovanje svih ispitivanih standardnih supstancija.