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Short communication

Thin-layer chromatography with stationary phase gradient as a method for separation of water-soluble vitamins

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ABSTRACT

The group of hydrophilic vitamins play an important role in human health, and their lack or excess produces specific diseases. Therefore, the analysis of these compounds is indispensable for monitoring their content in pharmaceuticals and food in order to prevent some human diseases. TLC was successfully applied in the analysis of hydrophilic vitamins, but the most difficult problem in the simultaneous analysis of all these compounds is to find an optimum stationary phase-mobile phase system due to different chemical characteristics of analytes. Unfortunately structural analogues are difficult to separate in one chromatographic run, and this is the case in hydrophilic vitamins investigations. TLC gives the possibility to perform two-dimensional separations by using stationary phase gradient achieving the highest resolution by combining two systems with different selectivity. The goal of this work was to develop a method of analysis enabling separation of hydrophilic vitamins using TLC with adsorbent gradient. The developed method was used for identifying the water-soluble vitamins in alcoholic extracts of *Hippophae rhamnoides* and of *Ribes nigrum*.

1. Introduction

The group of water-soluble vitamins, also called hydrophilic vitamins (Fig. 1), consist of vitamin C (ascorbic acid), B complex vitamins (B_1 – thiamine, B_2 – riboflavin, B_3 – niacin, B_5 – pantothenic acid, B_6 – pyridoxine, B_9 – folic acid, B_{12} – cobalamine), and vitamin H (biotin). Hydrophilic vitamins play an important role in human health, and their lack or excess produces specific diseases. Therefore, the analysis of these compounds is indispensable for monitoring their content in pharmaceuticals and food in order to prevent some human diseases. Different methods for the analysis of water-soluble vitamins and their metabolites are reported in literature [1–6]. Most of these methods are complicated and limited to the analysis of an individual vitamin and its degradation compounds. There are only a few papers that report the simultaneous analysis of almost all hydrophilic vitamins [7–11].

TLC and HPTLC, using different stationary and mobile phases, were successfully applied in the analysis of water-soluble vitamins and their metabolites from standards and different pharmaceutical and biological samples [12]. Because hydrophilic vitamins have different chemical characteristics, the most difficult problem in the simultaneous analysis of all these compounds is to find an optimum stationary phase-mobile phase system.

TLC is the method of choice when many samples must be compared, when flexibility is important, and when rapid quantitative data are needed at low cost per sample. Unfortunately structural analogues are difficult to separate in one chromatographic run, and this is the case in hydrophilic vitamins investigations. Multidimensional chromatography is a popular method for separation of multicomponent mixtures. TLC gives the possibility to perform two-dimensional separations either by use of the same stationary phase with different mobile phase systems [13–16] or by using stationary phase gradient [17–20]. The highest resolution in separation is achieved by combining two systems with different selectivity, such as the case of separations performed on two different stationary phases coated side by side. The stationary phases are usually a normal phase with adsorption separation mechanism, and a reversed phase with partition separation mechanism [15]. Different combinations of adsorbents have been reported: silica gel + alumina, cellulose + silica gel, poliamide + silica, poliamide + cellulose [21]. Due to different mechanisms of interaction, the separations on such adsorbents combinations enable obtaining a complete resolution of very complex samples.

The goal of this work was to develop a method of separation enabling separation of water-soluble vitamins using TLC with stationary phase gradient.

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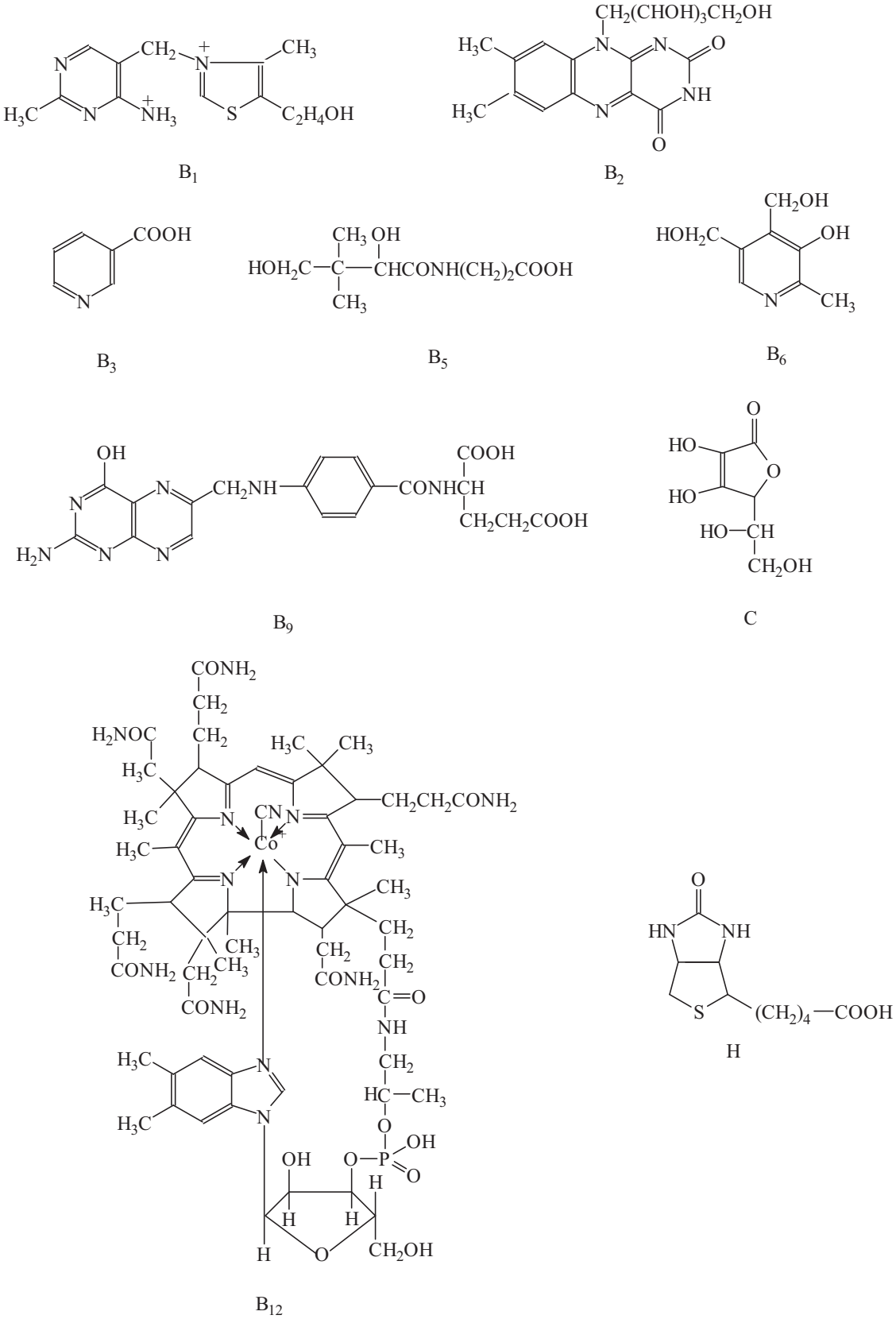

Fig. 1. The structures of water-soluble vitamins.

Table 1
The experienced mobile phases.

No.	Solvent system	Composition (v/v/v)
1	Acetone–methanol–benzene	1:2:8
2	Methanol–benzene	7:3
3	Methanol–benzene	6:4
4	Methanol–water	8:2
5	Methanol–benzene–formic acid	6:4:1
6	Chloroform–methanol–ethylacetate	5:5:2

2. Material and methods

2.1. Materials

All solvents were of pro analysis grade from "Reactivul" (Bucharest, Romania), and the TLC plates were purchased from Merck, Darmstadt, Germany.

All used vitamins were commercially pure grade for human nutrition. The separate solution of each of the water-soluble vitamin was prepared by dissolving an appropriate amount of the substance in methanol. The concentrations of standard solutions were 0.1% for vitamin C and 1% for vitamins B_1, B_2, B_3, B_6, B_9 and B_{12}.

1 g of each powdered dried fruit (*Hippophae rhamnoides* and *Ribes nigrum*) was extracted by maceration in 10 mL of ethanol:water (8:2, v/v) at room temperature for 10 days. After that, the extracts were filtered and the filtrates were stored in dark vials.

2.2. Chromatographic analysis

The solutions of standards (2 μL of vitamin B_2 and 1 μL of other vitamins) were applied as spots on the plate using an applicator device (Nanomat 4 – Camag). The analyzed vitamins standards were applied onto the silica gel F_{254} and cellulose F TLC plates at 10 mm distance from the low edge.

2.2.1. One-dimensional TLC

The plates were developed to a distance of 70 mm at ambient temperature in normal chromatographic chamber pre-saturated for 30 min with mobile phase. Detection was performed under UV lamp at 254 nm. After the development the R_F values of vitamins were determined.

In order to optimize the chromatographic separation and to choose the proper mobile phase, several eluents were tested (Table 1).

2.3. Stationary phase gradient

In this step, the plates were cut into narrow strips as follows: 1.5 cm × 9 cm silica gel plates and 1.5 cm × 8 cm cellulose plates. The silica gel strips were connected face-to-face (1 mm overlap) with cellulose strips along the shorter side (1.5 cm) of the strip (Fig. 2). Silica gel was scraped from an area of 1 cm × 1.5 cm before connecting making sure that edges would be without irregularities resulting from partial loss of adsorbent, because such irregularities may lead to deformation of the zones during their transfer to the second layer. It is important that both plates are turned face to face and pressed together, the layers should overlap. To achieve a close contact between the layers, the strips were compressed together with clamps. The plates were then developed using mixture of methanol–benzene–formic acid, 6:4:1 (v/v/v), as mobile phase. The spots were detected under UV illumination at λ = 254 nm.

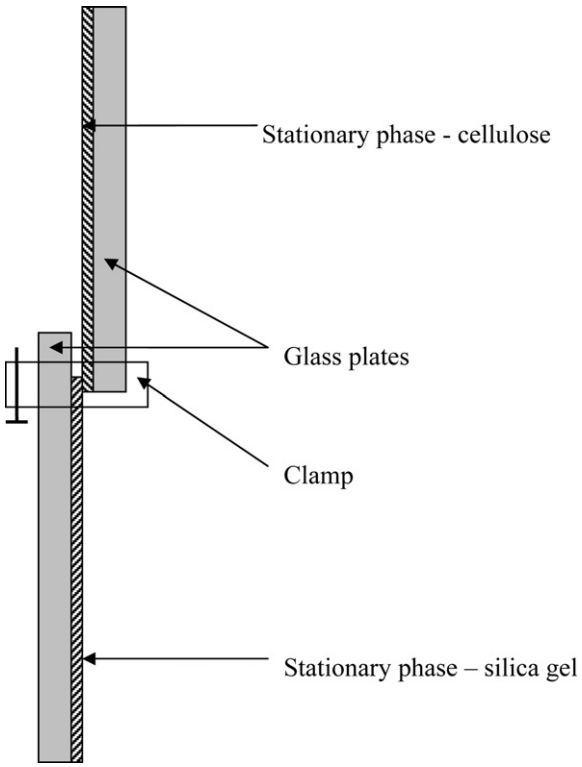

Fig. 2. Diagram of a side view of the connected layers.

3. Results and discussion

In the first step, the separation of vitamins using different mobile phases (Table 1) and different stationary phases (silica gel and cellulose) was tried. One development enables obtaining partly separated spots as it can be seen from the experimental R_F values. Gradient adsorbent TLC with the use of silica gel plates and cellulose proved even to produce greater selectivity differences when compared to two-dimensional thin-layer chromatography on one adsorbent. Due to these advantages the TLC separation with adsorbent gradient was attempted in order to enable a complete separation of a mixture of hydrophilic vitamins.

For a large number of separated species group selectivity can be assessed by using the correlation plots of retention data (R_F) from two chromatographic systems with different adsorbents. In this case, correlation plots of retention data for pairs of chromatographic layers (silica gel and cellulose) with different eluents were used to choose the best mobile phase for separation of the water-soluble vitamins (Fig. 3). The best combination of R_F coefficients was chosen to separate the vitamins. Complete separation of all vitamins was possible by using gradient adsorbent with silica gel and cellulose and the mixture of methanol–benzene–formic acid, 6:4:1 (v/v/v), as mobile phase. The experimental values for retention factors were: $R_{F,B9} = 0.05$, $R_{F,B12} = 0.12$, $R_{F,B1} = 0.20$, $R_{F,C} = 0.41$, $R_{F,B2} = 0.63$, $R_{F,B3} = 0.69$, $R_{F,B6} = 0.81$.

The developed TLC method was applied to analyze the hydrophilic vitamins contained in alcoholic extract of *H. rhamnoides* and of *R. nigrum*. The following vitamins were identified in the analyzed fruits extracts: C, B_1, B_2, B_6 and B_9.

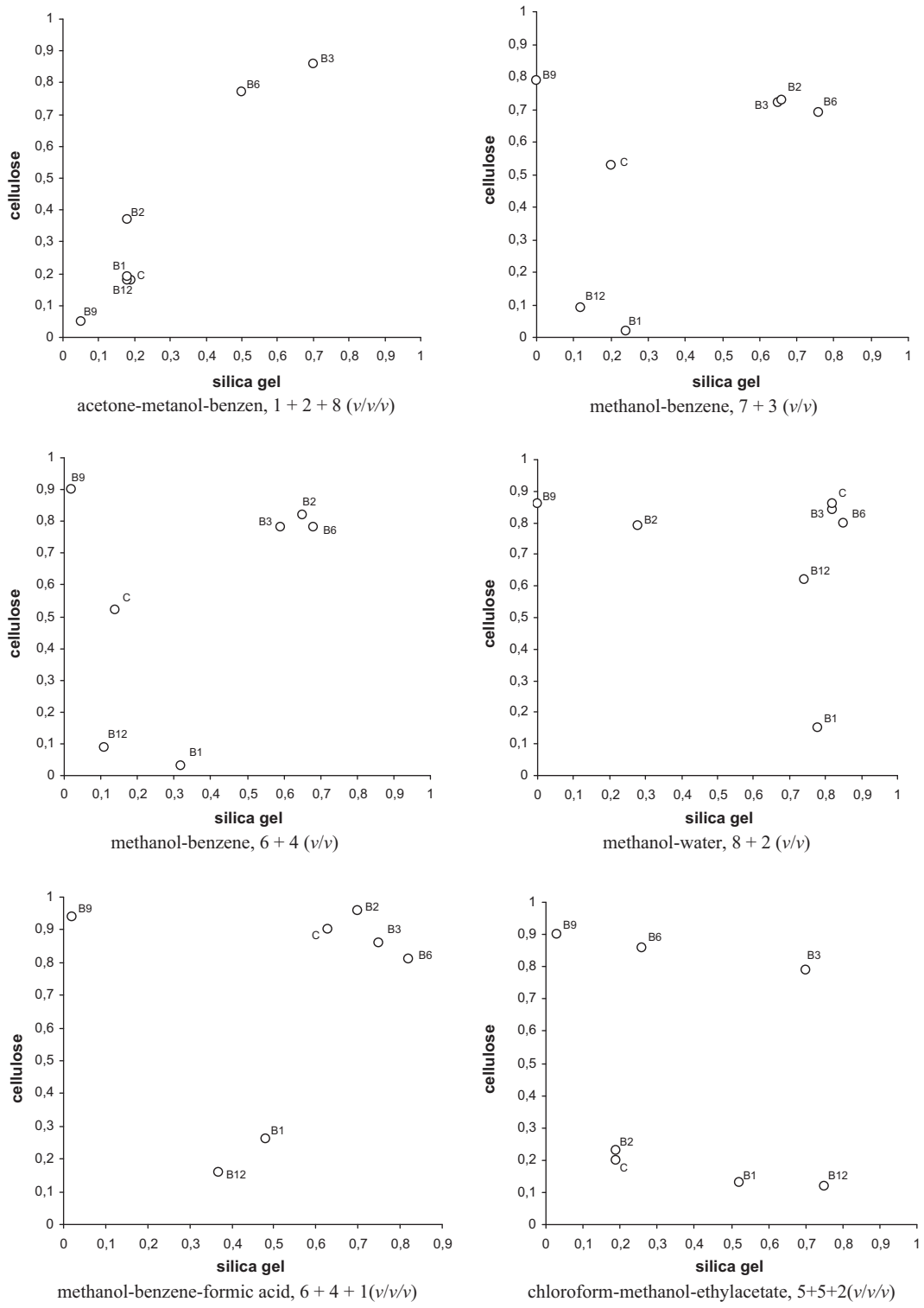

Fig. 3. Correlation between R_F values obtained by mono-dimensional TLC on silica gel and cellulose plates eluted with different mobile phases.

4. Conclusions

Thin-layer chromatography with stationary phase gradient is an effective method for separation of large groups of compounds, especially if the two chromatographic systems are of very different selectivity (e.g. if adsorption and repartition mechanisms are combined). A complete separation of the water-soluble vitamins investigated was possible by TLC with stationary phase gradient using silica gel and cellulose plates.

TLC with stationary phase gradient is relatively a simple and inexpensive method enabling combination of a large variety of stationary and mobile phases for analysis of various mixtures.

Another advantage of the proposed method is the fact that spots from a plate developed on stationary phase can be transferred to

the second plate, without the scraping of bands, extraction, and re-spotting.

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