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OPTIMIZATION OF OVERPRESSURED LAYER CHROMATOGRAPHY OF POLAR, NATURALLY OCCURRING COMPOUNDS BY THE "PRISMA" MODEL

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SUMMARY

The separation of polar compounds, based on using the whole upper section of the "PRISMA" model for mobile phase optimization, is described. The solvent combinations in normal-phase chromatography possible with the upper part of the model are discussed. Rules for the selection of the solvents that form the prism are given, together with the final optimization strategy for overpressured layer chromatography separations. Depending on the separation problem, optimization of the mobile phase can be accomplished with or without a modifier to increase the solvent strength and/or eliminate the tailing effect.

The separation of ginsenosides is discussed as an example of the application of the model without modifier. The separation of the flavonoid glycosides from *Betulae folium* demonstrates the use of the upper section of the prism with a modifier for mobile phase optimization.

INTRODUCTION

Systematic statistical procedures for mobile phase optimization with three or four solvents have recently been developed, mainly for high-performance liquid chromatography (HPLC)[1–7]. In overpressured layer chromatography (OPLC), developed by Tyihák *et al.*[8–10], there has been no systematic method for the selection of the mobile phase for polar substances, until now.

The "PRISMA" optimization model has recently been developed in our laboratory for the optimization of the mobile phase for reversed-phase HPLC[11] and various normal-phase planar chromatographic methods like thin-layer chromatography (TLC)[12], OPLC[13], centrifugal-layer chromatography (CLC)[14] and sequential-centrifugal-layer chromatography (SCLC)[15]. So far, it has been applied to different apolar and semipolar, naturally occurring compounds, using the regular part of the "PRISMA" design. Optimization of the mobile phase with the irregular top

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triangle of the model in the separation of highly polar compounds, like ginsenosides, has been reported recently[16]. Here, we report the application of the whole irregular upper section of the "PRISMA" model and the strategy for mobile phase optimization in the normal-phase OPLC of polar compounds.

THEORETICAL

The "PRISMA" model

The "PRISMA" is a three-dimensional model, correlating the solvent strength and the selectivity of mobile phases[11]. With this optimization model, the most advantageous mobile phase composition may be systematically elaborated, and from one to five solvents can be combined to achieve a suitable separation.

The solvent strength (S_T), influencing primarily the R_F value in planar chromatography, is represented by the height of the prism. Since the solvent strengths of the three solvents selected to define the prism are different, the resulting cover plate will neither be parallel nor coincidental with the base.

If the prism is intersected at the height of the shortest edge, it gives an upper frustum and a regular prism. The lower part of the prism is defined as a platform, representing the modifier. Thus the "PRISMA" model consists of three parts: platform, regular part and irregular frustum (Fig. 1).

In normal-phase chromatography, the upper frustum is used for the optimization of mobile phases for polar and/or semipolar substances. Depending on the separation problem, the upper section can be used with or without modifier. The regular, centre portion of the prism is used in the solvent optimization for apolar and semipolar compounds, as reported earlier[12,13].

Points in the "PRISMA" model represent the composition of solvents for the mobile phase. The three top corners of the model represent the three individual solvents. When these solvents are diluted with hexane ($S_T = 0$), points along the edges stand for combinations of two solvents, points on the sides for combinations of three,

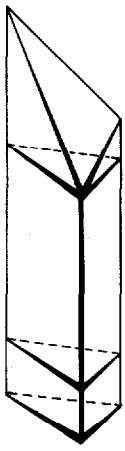


Fig. 1. The "PRISMA" optimization model.

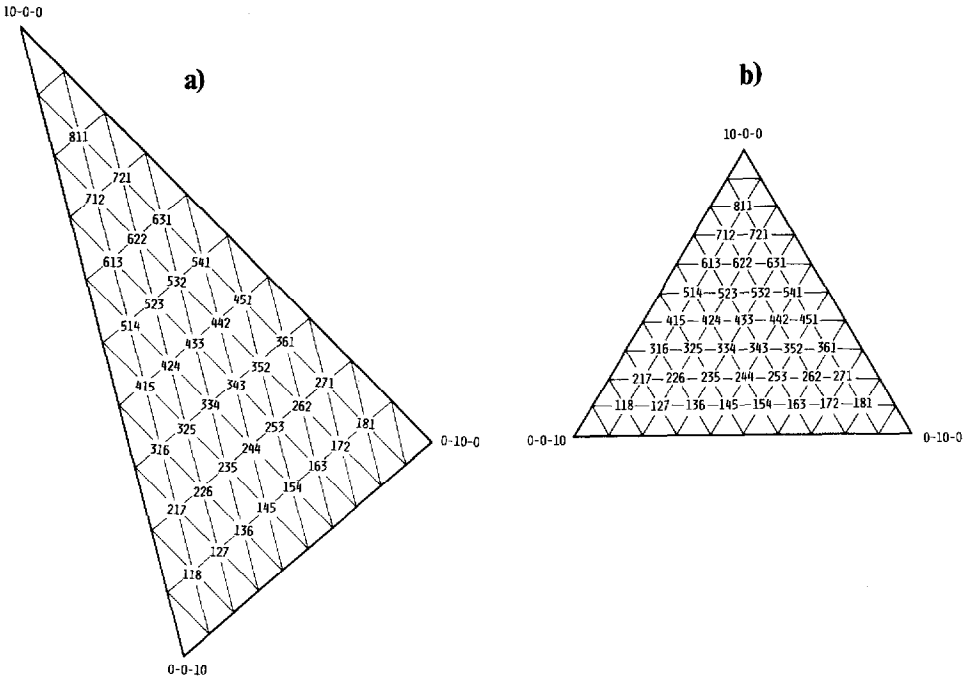


Fig. 2. (a) The selectivity points in the top triangles of the "PRISMA" model. (b) The selectivity points of the equilateral triangles of the "PRISMA" model.

and points in the interior of the prism for mixtures of four solvents. As a fifth component, a modifier can be added in a constant amount to increase the solvent strength and/or eliminate the tailing effect. The frustum surface has a special function: it represents the mobile phases without hexane. The combination of these three components A, B and C can be described with selectivity points (P_S): 100% solvent A, with the highest solvent strength is represented by P_S = 10-0-0 and the solvent with the lowest solvent strength, C, by P_S = 0-0-10. From these, all other basic selectivity points can be defined as three-digit-numbers, *e.g.* P_S = 217, which represent the volume fractions of solvents A, B and C, in this case 20% solvent A, 10% B and 70% C (Fig. 2a). Finer adjustments of the volume fractions can be expressed by three two-digit-numbers (P_S = 65-12-23).

In the upper part of the model the dilution of a solvent mixture, defined as a selectivity point in the top triangle, with hexane results in a shift along the vertical axis to the same selectivity point on a lower solvent strength level. All equilateral triangles within the prism and their selectivity points (Fig. 2b) are the projections of the top triangle of the regular part of the model and the corresponding selectivity points. Thus, the "PRISMA" model includes all possible combinations of one to five solvents for the separation of different compounds from low to high polarity.

Strategy for preliminary experiments in OPLC

Since the number of theoretical TLC plates is significantly lower than of HPLC

columns, the selection of the solvents comprising the mobile phase has a greater influence on the separation. In the Snyder[17] classification, solvents are grouped in eight classes according to three criteria: ability to donate protons, accept protons, and undergo dipole interactions. From these eight classes we have chosen 29 solvents commonly used in TLC. These are listed in Table I, together with their solvent strength values. The asterisks indicate the ten solvents that were used in the first experiments, carried out with normal TLC plates in unsaturated chambers.

The first ten preliminary experiments are carried out with neat solvents from Table I. If the R_F values of the compounds are too low ($R_F < 0.2$), the solvent strength is increased by adding modifiers (*e.g.* water or acetic acid). One to four of these solvents, which may be diluted with hexane, are selected. If the selected two or three solvents are not miscible with hexane, a third or fourth solvent must be chosen in

TABLE I

SOLVENTS FOR OPLC OPTIMIZATION

Group	*Solvent*	*Solvent strength*
–	*n*-Hexane	0
I	*n*-Butyl ether	2.1
	Diisopropyl ether	2.4
	Methyl *tert.*-butyl ether	2.7
	Diethyl ether*	2.8
II	*n*-Butanol	3.9
	2-Propanol*	3.9
	1-Propanol	4.0
	Ethanol*	4.3
	Methanol	5.1
III	Tetrahydrofuran*	4.0
	Pyridine	5.3
	Methoxyethanol	5.5
	Dimethylformamide	6.4
IV	Acetic acid*	6.0
	Formamide	9.6
V	Dichloromethane*	3.1
	1,1-Dichloroethane	3.5
VI	Ethyl acetate*	4.4
	Methyl ethyl ketone	4.7
	Dioxane*	4.8
	Acetone	5.1
	Acetonitrile	5.8
VII	Toluene	2.4
	Benzene*	2.7
	Nitrobenzene	4.4
VIII	Chloroform*	4.1
	Nitromethane	6.0
	Water	10.2

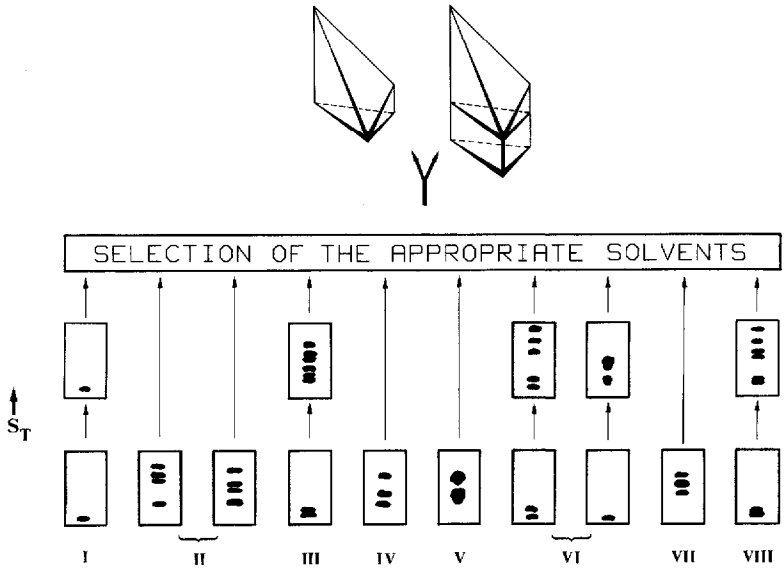


Fig. 3. The strategy of preliminary experiments for OPLC of polar compounds. The first row of ten plates are the first preliminary experiments carried out with neat solvents from Group I–VIII indicated with the asterisk on Table I. In a further experiment (2nd row of plates) the solvent strength (S_T) was increased, where necessary, to bring the substances in the suitable R_F range.

which the sample compounds do not migrate. This solvent can be used for a prerun. In our experience, this is one way to eliminate the so-called "disturbing" zone in OPLC[19] which destroys the separation and is caused by air and/or gas adsorbed on the large surface and within the silica plate. Using the selected solvents, the appropriate part of the prism is constructed as shown in Fig. 3.

If more than three solvents appear to be suitable for constructing the prism, several "PRISMA" combinations can be tested. If a solvent from one group gives a favourable separation, other solvents from this solvent group can then be tested.

Final optimization strategy in OPLC

After the selection of the solvents, the upper section of the "PRISMA" model can be used either alone, or together with the platform, representing the modifier. If no modifier is necessary and the upper section of the "PRISMA" is used, the subsequent optimization strategy resembles the strategy for apolar substances[13]. First, the solvent mixture corresponding to the centre of the irregular triangle (P_S = 333) and the selectivity points near the edges (P_S = 811, 181, 118) are tested. Depending on the results of these four experiments, the selectivity is further optimized by choosing new points in the triangle near or between the selectivity points giving the best resolution. This gives the optimum selectivity point on the frustum surface. If the solvent strength is too high, it can be reduced by adding hexane, giving a selectivity point for the optimum mobile phase within the upper part of the prism.

For the separation of highly polar substances, water can be chosen as the solvent with the highest solvent strength. Then, the selectivity points in normal-phase

chromatography can only be selected in the lower part of the irregular top triangle and a very small change in the selectivity points can result in an extreme change in the separation of the compounds. Since changes of the selectivity points must generally be made in small steps, they should be characterized by three two-digit-numbers (*e.g.* 10-45-45).

In case a modifier is used, the upper section of the "PRISMA" model is used, together with the platform. If three solvents are selected, the amount of modifier is kept constant in the next experiments; again, the selectivity points P_S = 333, 811, 181 and 118 are tested, and a suitable selectivity point is chosen for further experiments. If necessary, the solvent strength can be either decreased by diluting with hexane or increased by adding a suitable amount of modifier for a final optimization.

If high and/or different amounts of water were found in the preliminary experiments, *e.g.* in the separation of highly polar compounds, water can be used either as modifier or as the solvent with the highest solvent strength in the top triangle. Thus, one of the three organic solvents selected can be kept constant, acting as modifier, while the prism is constructed with the two remaining solvents plus water.

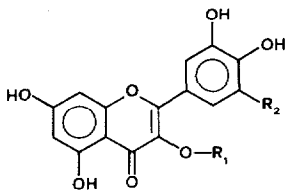
EXPERIMENTAL

The separations were carried out with silica gel $60F_{254}$ TLC and HPTLC plates (Merck, Darmstadt, F.R.G.).

The flavonoid glycosides (Table II) and the ginsenosides had been isolated and identified at the School of Pharmacy, ETH Zurich. Solutions of between 0.5 and 1.2 mg/ml methanol were used. Samples were applied with a Linomat III TLC spotter from Camag (Muttenz, Switzerland). OPLC was carried out with a Chrompres-10 overpressure layer chromatograph from Labor MIM (Budapest-Esztergom, Hungary). Impregnation of the plates on all four sides was performed with Impress polymer suspension from Labor MIM. Two channels were scraped out of the silica, one at the solvent inlet, the other at the solvent outlet at 18 cm distance. Reagent-grade solvents were used for the separations. For the visual detection of the ginsenosides, vanillin sulphuric acid was used[20]. The densitograms were taken with a Shimadzu 920 scanner (Shimadzu, Kyoto, Japan).

TABLE II

STRUCTURES OF INVESTIGATED FLAVONOID GLYCOSIDES



R_1	R_2	*Symbol*
– Ara(fur)	– H	Q-Ara(f)
– Rha	– H	Q-Rha
– Ara(pyr)	– H	Q-Ara(p)
– Gal	– H	Q-Gal
– Gal	– OH	My-Gal
– Glucur	– H	Q-Gluc
– Rha-Glc	– H	Rutin

RESULTS

Optimization of the ginsenoside separation without modifier

The eight most important ginsenosides from *Panax ginseng* C. A. Meyer (Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2) were separated as a model of the application of the top triangles of the "PRISMA" model, as reported recently[16]. On the basis of the preliminary experiments, two organic solvents, methanol and methyl ethyl ketone were selected. As the third solvent, water was chosen, because the ginsenosides are highly polar compounds. Since the solvent strengths of the selected single solvents were very different, small changes in the selectivity points had a large effect on the separation. The optimum separation on TLC plates was achieved with a mobile phase of water, methanol and methyl ethyl ketone (8:22:70) (P_S = 08-22-70). This mobile phase was transferred without any modification to OPLC after a prerun with methyl ethyl ketone to eliminate the disturbing zone[19]. With this single solvent the ginsenosides did not migrate, but the disturbing adsorbed air and/or gas could be expelled from the plate within 5 min, and then the separation was started. A densitogram of

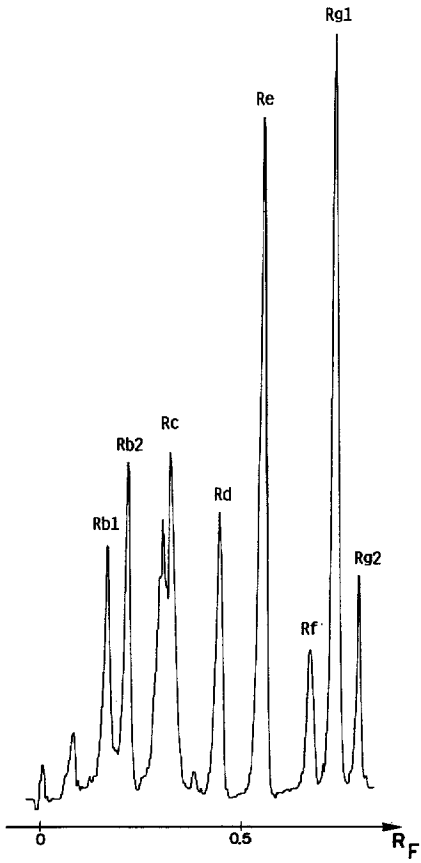


Fig. 4. Densitogram of a Ginseng extract. Separation on a HPTLC plate by OPLC; distance 17 cm; time 60 min; cushion pressure 10 bar; starting pressure 1 bar.

an extract at 520 nm is shown in Fig. 4. As can be seen, the separation was excellent for the ginsenosides in the high and middle R_F range and good for the identification of Rb1 and Rb2.

Optimization of the flavonoid separation with a modifier

The separation of the seven main flavonoid glycosides from *Betulae folium* (Ph. Helv. VI) is an example of the use of the upper section of the "PRISMA" model with a modifier for mobile phase optimization[11]. The structures of the investigated compounds are given in Table II.

The preliminary experiments were either carried out with water-saturated solvents or 3–5% water was added as modifier. From these experiments, methyl *tert.*-butyl ether from Group I, acetic acid from Group IV and chloroform from Group VIII were chosen to construct the prism and 3% water was added as modifier. In the first step of the optimization process, the four basic selectivity points P_S = 333, 811, 181, and 118 were tested. For P_S = 181, no modifier was used, because it was not water-miscible. From the results of these experiments, the further selectivity points near the corner of methyl *tert.*-butyl ether chosen were P_S = 217 and 127. With a solvent combination of P_S = 217, all seven flavonoid glycosides could be separated.

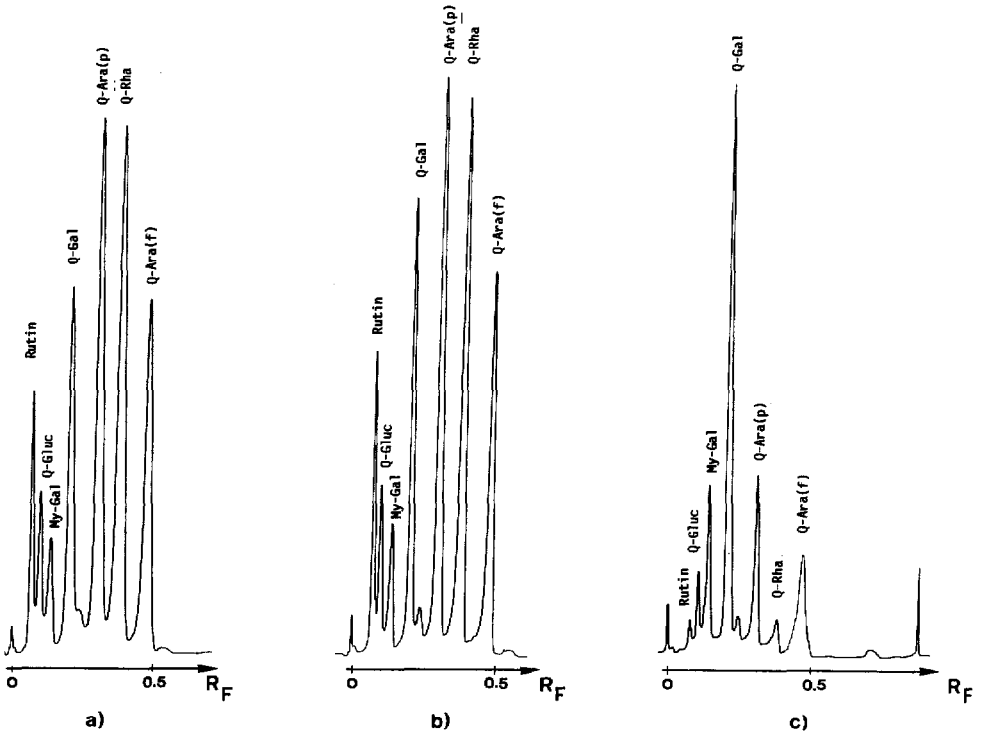


Fig. 5. Densitograms of flavonoid glycosides in *Betulae folium*. (a) Separation on a TLC plate by OPLC; distance 17 cm; time 8.5 min; cushion pressure 10 bar; starting pressure 0.5 bar. (b) Separation on a HPTLC plate by OPLC; distance 17 cm; time 8 min; cushion pressure 10 bar; starting pressure 0.5 bar. (c) Separation of a crude extract on a HPTLC plate by OPLC; distance 17 cm; time 8 min; cushion pressure 10 bar; starting pressure 0.5 bar.

To reduce the tailing effect and bring the compounds into the appropriate R_F range, the amount of water was increased to 7.5%. With this mobile phase, all seven flavonoid glycosides could be separated within a 9-cm distance on the TLC plate within 1 h.

To reduce the separation time and increase the resolution, the OPLC technique was employed. The same mobile phase was used for both TLC and OPLC. The separation on a TLC plate (Fig. 5a) and on a HPTLC plate (Fig. 5b) by OPLC within a 17-cm distance is shown in Fig. 5 together with a densitogram of a crude plant plant extract (Fig. 5c) at 254 nm. The OPLC separations on the TLC and HPTLC plates were completed in 8.5 and 8 min, respectively. If, instead of the extremely high flow-rate, a more appropriate flow-rate had been chosen, the resolution would be improved to produce a baseline separation of all compounds, but for screening and/or routine analysis of plant extracts, the resolution of the investigated flavonoid glycosides is adequate.

DISCUSSION

The upper portion of the "PRISMA" model was used for mobile phase optimization in normal-phase chromatography of polar compounds. The selection of the solvents forming the prism is similar to the strategy for apolar and semipolar compounds[12], but the solvent strength of solvents must be increased, instead of being decreased, to bring the sample compounds into the desired R_F range.

From the results of the preliminary tests, the further experiments can be carried out with or without modifier. With the selected solvents, the optimization process is similar to that for apolar and semipolar substances[13], but by changing the selectivity points, the solvent strength also changes, and this can result in extreme changes in resolution[16]. Therefore, changes in the selectivity points must be made in smaller steps, and must be described by three two-digit-numbers. Generally, the mobile phase from the preliminary TLC assay can be used for the OPLC separation without modification. If necessary, a preliminary development with a solvent in which the compounds do not migrate can be carried out to eliminate the disturbing zone. It is advantageous to select such a solvent during the preliminary assay.

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