The Effect of Chromatographic Conditions on the Separation of Selected Alkaloids in RP-HPTLC

Anna Petruczynik[1], Monika Waksmundzka-Hajnos[1,*], and **Michal L. Hajnos[2]**

[1]Department of Chemistry, Medical University, Staszica 6, 20-081 and [2]Department of Pharmacognosy, Medical University, Chodszki 1, 20-093 Lublin, Poland

Abstract

Selected alkaloid standards were chromatographed on C18 W layers using various aqueous eluents containing an organic modifier and pH 3 buffer to suppress silanol ionization or an organic modifier and pH 8 buffer to suppress alkaloid ionization. Anionic ion pairs such as sodium dodecyl sulfate, octane-1-sulfonic acid sodium salt, pentane-1-sulfonic acid sodium salt, and bis(2-ethylhexyl)ortho-phosphoric acid are used to improve peak shape, efficiency, and selectivity. Amines (e.g., diethylamine, triethylamine, and tetrabutylamonium chloride) are incorporated into mobile phases to block surface silanols. The effect of chromatographic conditions on the separation of the investigated alkaloids is analyzed by the comparison of particular densitograms, asymmetry factor, or theoretical plate number. The best efficiency, peak symmetry, and separation selectivity of the investigated compounds is obtained through the addition of amine (especially diethylamine) to the mobile phases.

Introduction

Heterocyclic bases as pharmacologically active compounds widely used as pharmaceuticals and synthesized as secondary metabolites in plants are the subject of scientific interest (1–7). It is therefore necessary to analyze these organic electrolytes. Because heterocyclic bases can appear in solutions as either ionized or deionized forms, they are difficult to separate chromatographically.

The optimization in reversed phase (RP) separation and selectivity control of basic samples can be performed in a similar way, as for nonionic compounds, by a variation of solvent strength or by changing the stationary phase type (C2, C8, C18, and cyano). In applications of RP systems, for the analysis of ionic compounds, the choice of a suitable buffer (buffer capacity, solubility, stability, and interactions with sample and chromatographic system should be taken into account) is very important (8,9).

Ionic samples, especially basic compounds, can interact with the underivatized free silanols of silica-based alkyl-bonded phases. It can be observed that retention occurs by an ion-exchange process that involves protonated bases and ionized silanols. This situation leads to the increased retention, band tailing, and layer-to-layer (column-to-column) irreproducibility. It is generally desirable to minimize these silanol interactions by the appropriate choice of experimental conditions. A protonated base (BH^+) in the sample exchanges with a sodium, potassium, or other cation that is attached to an ionized silanol in the adsorbent. Silanol interactions can be reduced by the selection of a stationary phase that is designed for basic samples with a reduced number of very acidic silanols that favor the retention process. The average pK_a value of silanols is approximately 7, although the average conceals a range of acidities with some silanols likely to have $pK_a < 3$. There are several methods to reduce silanol effect (9), such as: use of a low pH mobile phase ($2.0 < pH < 3.5$) to minimize the concentration of ionized silanols; incorporation of amines into mobile phases, providing competition with the analytes for adsorbent silanol sites; or use of high pH mobile phases (e.g., $pH > 7.0$) for the separation of basic compounds, which may be deionized at higher pH values. In the analysis of basic compounds, anionic ion-pairing (IP) reagents [sulfonic acids, alkyl sulfonates, and other acids such as bis(2-ethylhexyl) phosphate (HDEHP)] are employed. When the concentration of IP reagent is gradually increased, a distinct increase in retention of the analytes is observed. In a limited range of concentrations, a linear relationship of log k (R_M) and counterion concentration is obtained (10–12). However, the linear relationships of R_M versus the concentration of the IP reagent are observed up to the moment when the saturation of surface concentration of hydrophobic counterions approaches. After that, a further increase of concentration does not lead to significant changes in retention, and a decrease of retention is sometimes observed (13,14). Changing the type and concentration of the counterion often causes a variation in separation selectivity (15,16).

Additionally, retention and selectivity in RP and IP-RP systems can be controlled by changing the type and concentration of the organic modifier in the aqueous mobile phase (15,16). The next determinant playing an important role in IP-RP chromatography of ionic compounds is the pH of the mobile phase (17,18). The pH should be selected to obtain maximum ionization of solute

* Author to whom correspondence should be addressed: email mwaks@panaceum.am.lublin.pl.

molecules and IP reagent molecules to form an IP. The pH range 7.0–7.5 is often applied to the analyses of basic solutes. The stationary phase (the length of the alkyl chains bonded to the silica support) also influences the retention of hydrophobic IPs. Similar to RP thin-layer chromatography (TLC) separations, the selectivity can be additionally moderated by solvent type (methanol, tetrahydrofuran, and acetonitrile), buffer concentration, and temperature (9,19).

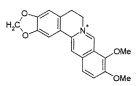
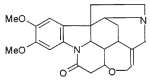
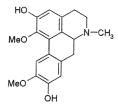
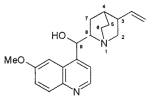
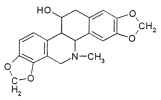
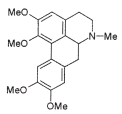
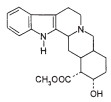
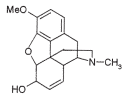
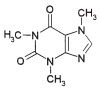
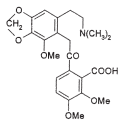
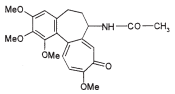
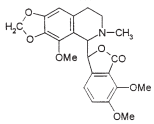
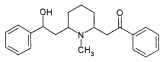
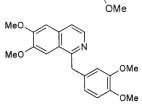
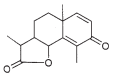
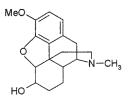
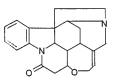
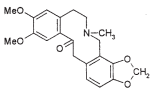
TLC is very often applied as a tool for the separation of crude plant extracts, often without preliminary purification. Plant extracts containing alkaloids are also very often separated on various layers with post-process derivatization. Various alkaloids were chromatographed by RP-TLC with aqueous mobile phases (20,21), buffered methanol (22–24), and also the addition of amines (25), IP reagents (22,23), or chiral selectors (26). RP-TLC was successfully used for the determination of the lipophilicity of some alkaloids to suggest their pharmacokinetic properties (20).

The aim of this paper was to investigate the various parameters influencing the retention, peak shape, efficiency, and separation selectivity of some alkaloids from different classes on C18 W layers. The effects of the organic modifier, buffer pH, amine type, and concentration in the mobile phase, as well as, the kind and concentration of anionic IP reagents were examined.

Experimental

TLC was performed on 10- × 10-cm glass C18 WF_{254} high-performance (HP)TLC precoated plates (E. Merck, Darmstadt, Germany). In contrary to C18 plates, C18W plates are wettable with water and enable the use of aqueous eluents containing low concentrations of organic modifiers. Samples (2 µL) of 2.5% w/v solutions of the solutes in methanol were spotted on the adsorbent layer. Plates were conditioned for 20 min in eluent vapours. Plates were developed face-down to a distance of 8 cm from the origin at 20°C ± 1°C in horizontal Teflon chambers with an eluent distributor (DS, Chromdes, Lublin, Poland). Binary mixtures of organic modifier, methanol (MeOH), acetonitrile (MeCN), tetrahydrofuran (THF), isopropanol (iPrOH), or dioxane (DX)

Table I. List of Investigated Alkaloids

Abbreviation	Name of alkaloid	Chemical structure	pK_a	Abbreviation	Name of alkaloid	Chemical structure	pK_a
Be	Berberine		> 10	Br	Brucine		8.28
Bo	Boldine		6.62	Q	Quinine		I = 5.1 II = 9.7
Chl	Chelidonine		> 10	E	Ephedrine		9.96
G	Glaucine		6.4	Y	Yohimbine		6.7
Cd	Codeine		8.21	C	Caffeine		14
N	Narceine		9.3	Co	Colchicine		12.35
Na	Narcotine		7.8	L	Lobeline		8.03
P	Papaverine		8.07	Sa	Santonine		–
Pa	Paracodine		–	St	Stychnine		8.26
Pr	Protopine		8.28	T	Theophylline		8.77

with buffered water were used as eluents. Phosphate buffer concentrations of 10 and 20mM in the pH range of 2–10 were applied. Solvents were analytical-grade from Polish Reagents (POCh, Gliwice, Poland). Amines, such as diethylamine (DEA), triethylamine (TEA), trioctylamine, triisopentylamine (TIPA), and tetrabutylammonium chloride (TBA-Cl), purchased from Fluka AG (Buchs, Switzerland) were used as mobile phase components. Reagents such as: sodium dodecyl sulfate (SDS), sodium octane sulfate, sodium pentane sulfate, HDEHP, and tri-*n*-butyl phosphate purchased from Fluka AG were used as anionic IP reagents. The location of the spots was determined under UV light (λ = 254 nm). The investigated alkaloids are listed in Table I. Chromatograms were scanned at λ = 254 nm by a Camag TLC Scanner 3 (Muttenz, Switzerland) assisted by computer program Cats 4. For videoscaning, a TLC Camag Reprostar 3 apparatus with Videostore computer program was used.

Results and Discussion

Alkaloids, including 11 isoquinoline alkaloids and 10 alkaloids from other groups, were chromatographed on C18 W layers under various conditions. The use of the more traditional C18 plates was impossible because of the instability of layers in experimental conditions. In eluent systems containing an organic modifier (80% methanol or acetonitrile) in water as mobile phases, investigated alkaloids (except caffeine, theophylline, colchicine, and ephedrine) were strongly retained on C18 W layers. Under these conditions, alkaloids are present in ionized and neutral forms and, as ions, interact strongly with residual surface silanols. This causes poor peak (spot) shape and peak tailing. Table II shows the retention parameters, asymmetry factors, and number of theoretical plates for alkaloids obtained in this system. As it is seen in the values presented, most alkaloids in this system are strongly retained, and spots are wide and often asymmetric. In order to obtain better peak shapes, higher efficiency, decreased retention, and improved separation selectivity, the effect of series parameters such as the pH of the mobile phase, amine type and concentration, and type and concentration of anionic IP reagents were examined.

Effect of eluent pH

Figure 1 presents dependence of R_M versus pH of eluent over the range of pH 2–10 units. Retention of alkaloids does not change over the pH range of 2–6 units. More visible differences in retention factors were obtained with the highest mobile phase pH, especially with a pH greater than 8. Retention for the alkaloids (ephedrine, theophylline, santonine, and colchicines) does not change in the pH range investigated. These alkaloids are

Table II. R_f, A_S, and *N*/m Values for Investigated Alkaloids Obtained on RP18 W Plates in Eluent Systems*,†

	80% MeOH			80% MeOH (pH = 3)			80% MeOH (pH = 8)		
Alkaloid	R_f	A_S	*N*/m	R_f	A_S	*N*/m	R_f	A_S	*N*/m
Berberine	0			0.2	0.67	1600	0.16	0.13	800
Boldine	‡0.01			0.43	0.53	1400	0.4	0.53	1100
Chelidonine	‡0.02			0.47	0.58	900	0.45	0.58	500
Emetine	‡			‡			‡		
Glaucine	0.1			0.31	0.63	300	0.33	0.59	500
Codeine	‡0.02			0.32	0.48	400	‡0.29		
Narceine	0.24	0.5	900	two spots			two spots		
Narcotine	0.1	0.7	200	0.34	0.58	1000	0.36	0.52	1300
Papaverine	‡0.08			0.37	0.53	800	0.34	0.56	600
Paracodine	0.08	0.82	100	0.33	0.59	1200	0.3	0.64	1000
Protopine	0			0.35	0.39	1100	0.37	0.43	1400
Brucine	‡0.01			‡0.11			‡0.13		
Quinine	0			0.27	0.91	200	0.28	0.63	200
Ephedrine	0.73	1	1900	‡0.74			‡0.75		
Yohimbine	‡0.05				0.51	0.53	2200	0.5	0.86
1300									
Caffeine	0.54	1.08	400	0.54	1	8800	0.54	1.08	7900
Colchicine	0.56	1.07	300	0.55	1	6400	0.56	1	5500
Lobeline	‡0.04				0.4	0.4	1600	0.37	48 1600
Santonine	0.58	1.42	500	0.59	2	11900	0.58	2.31	10300
Strychnine	‡0.03				0.22	0.95	400	0.24	0.82
500									
Theophylline	0,65	0,93	600	0.64	1	16300	0.63	0.92	13900

* (1) MeOH–H_2O, (80:20); (2) MeOH–H_2O–buffer pH 3 (20:10:10); and (3) MeOH–H_2O–buffer pH 8 (80:10:10).
† Abbreviations: retardation factor (R_f), asymmetry factor (A_S), and theoretical plate number for 1 m of column (*N*/m).
‡ Very wide peak.

strongly basic (pK_a > 10), and in this range of pH values they are only in ionized form. At high pH values, the ionization of most alkaloids is suppressed, which leads to an increase in interactions between the neutral alkaloid molecules and hydrophobic adsorbent surface. Additionally, neutral molecules of the the compounds investigated do not strongly interact with residual silanols, which decreases the retention and causes the improved peak shape. This is also shown by the highest values of theoretical plate numbers in buffered systems. However, only in a few cases is peak symmetry fair (0.9 < A_s < 1.5) (9). Spots of alkaloids were more compact at pH 3, where the silanol ionization was suppressed. It minimizes ion interaction of the groups with alkaloid ions and results in the highest efficiency; in most cases (17 out of 23 cases), the numbers of theoretical plates are higher (see Table II). Regardless, most of them still have wide spots. Addition of buffer at pH 3 decreases the retention of alkaloids in comparison to systems methanol + water. However, most of the peaks are still asymmetric and wide (Table II).

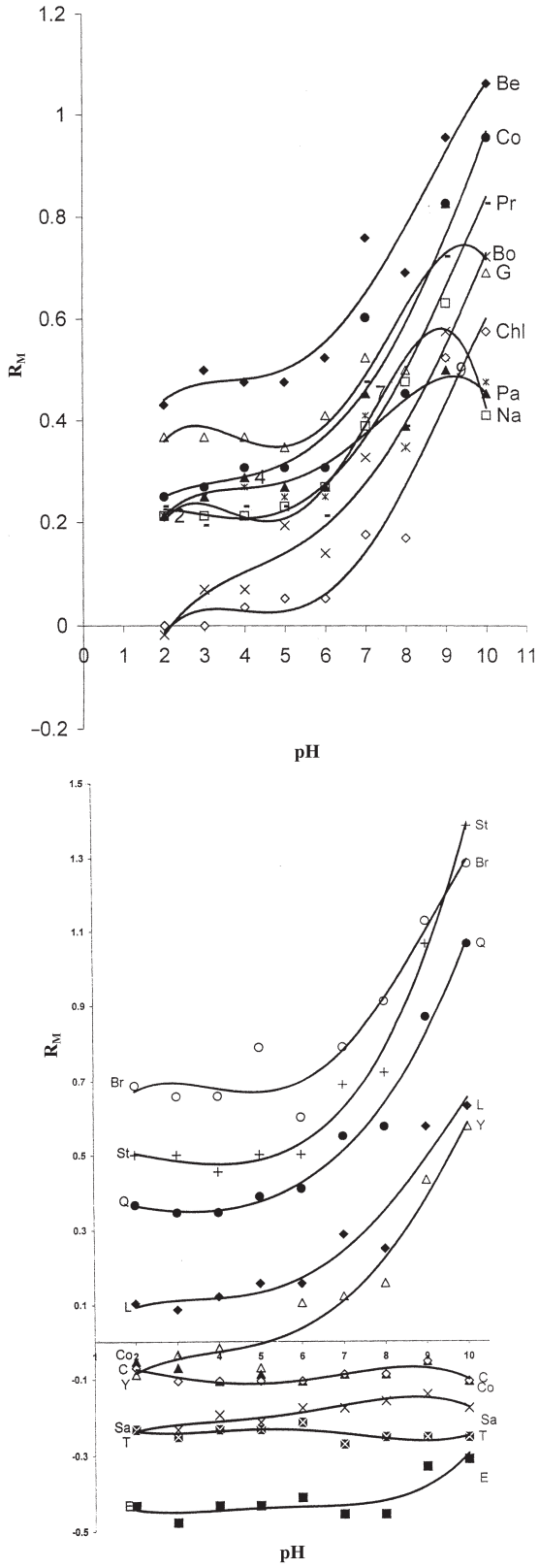


Figure 1. Dependence of R_M versus pH of a mobile phase for investigated alkaloids (A, B). System: C18 W, MeOH–H_2O (80:20) buffered with phosphate buffer 0.01M. (See Table I for abbreviations.)

Effect of amine kind and concentration

Further improvement of efficiency, peak symmetry, and separation selectivity for alkaloids in RP systems was obtained when different amines such as DEA, TEA, trioctylamine, triizopenthylamine, and TBA-Cl were added to the mobile phase.

The retention (Figure 2) as a function of DEA concentration (0.005–0.1M) and of TBA-Cl (0.01–0.05M) was investigated. The retention of alkaloids decreases with the increase of DEA concentration in the mobile phase and markedly decreases with the increase in TBA-Cl concentration because of the blockage of ionized silanols by the amine. Changes in amine concentration can

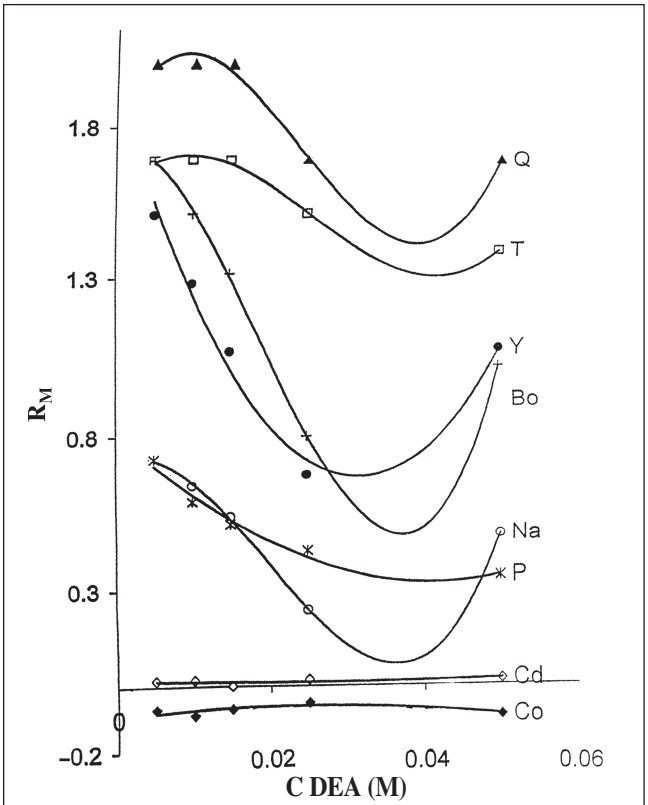


Figure 2. Relationships between R_M and DEA concentration in mobile phase for selected alkaloids. System: C18 W MeOH–H_2O (90:10). (See Table I for abbreviations.)

Table III. R_f, A_S, and N/m Values for Investigated Alkaloids Obtained on RP18 W Plates in Eluent Systems*

	0.05M DEA + 80% MeOH			0.05M TEA + 80% MeOH			0.05M TBA-Cl + 80% MeCN		
Alkaloid	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m
Berberine	†0.02			†0.02			0.38	0.89	8800
Boldine	0.11	1.29	200	0.18	0.92	600	0.55	0.7	5600
Chelidonine	0.22	0.36	600	0.31	0.56	2000	0.56	0.69	5900
Emetine	0.05	1.4	500	0.1	0.67	1700	0.52	2	11400
Glaucine	0.07	3	100	0.19	0.83	1100	0.52	0.5	25200
Codeine	0.11	0.63	200	0.16	0.5	600	0.52	0.13	16100
Narceine	0.38	1.73	2800	0.42	1.38	2100	0.53	2.5	20600
Narcotine	0.24	0.36	1400	0.38	0.73	1400	0.53	1.75	25600
Papaverine	0.32	0.63	900	0.47	0.69	1900	0.52	0.56	25200
Paracodine	0.29	0.64	900	0.42	0.75	2100	0.52	0.5	25200
Protopine	0.03	1.33	300	0.07	1	2000	0.54	2	34600
Brucine	†0.03			0.08	1.17	500	†		
Quinine	†0.03			0.09	1	1400	0.33	0.4	300
Ephedrine	†0.02			†0.06			0.59	0.36	14700
Yohimbine	†0.08			0.17	2.38	300	0.55	1.18	5600
Caffeine	0.57	1	8800	0.59	0.92	9000	0.49	1	9700
Colchicine	0.58	0.94	6000	0.6	0.86	6400	0.36	0.53	3500
Lobeline	0.08	2	100	0.12	2.29	1100	0.52	1.25	45600
Santonine	0.64	1.2	12500	0.65	1.17	12500	0.54	1.11	12400
Strychnine	†0.02			0.08	1	2500	0.49	0.5	6300
Theophylline	0.65	0.94	12800	0.67	0.83	4400	0.55	0.78	7200

* Systems: 1, MeOH–H_2O (80:20) + 0.05M DEA; 2, MeOH–H_2O H_2O(80:20) + 0.05M TEA; and 3, MeOH–H_2O (80:20) + 0.05M TBA-Cl.
† Very wide peak.

Table IV. R_f, A_S, and N/m Values for Investigated Alkaloids Obtained on RP18 W Plates in Eluent Systems*

	0.001M TBA-Cl + 80% MeCN			0.005M TBA-Cl + 80% MeCN			0.01M TBA-Cl + 80% MeCN			0.025 MTBA-Cl + 80% MeCN			0.05M TBA-Cl + 80% MeCN		
Alkaloid	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m
Berberine	0.09	1	200	0.3	2.8	8900	0.41	0.57	14200	0.36	1.14	7600	0.38	0.89	8800
Boldine	0.02			two spots			two spots			0.37	0.33	400	0.55	0.7	5600
Chelidonine	†0.17			0.56	0.09	600	0.49	1.53	1100	0.42	1.6	2600	0.56	0.69	5900
Emetine	†0.22			0.35	1.63	800	0.44	2	2500	†0.05			0.52	2	11400
Glaucine	0.19	1.1	200	0.38	0.82	1300	0.43	1.5	6600	0.33	0.72	1000	0.52	0.5	25200
Codeine	0			two spots			†0.01			0.22	0.44	100	0.52	0.13	16100
Narceine	0.16	1.5	400	0.38	1.42	200	0.47	1.1	9000	0.22	1.4	2000	0.53	2.5	20600
Narcotine	0.12	1.56	500	0.34	1.7	1400	0.42	1.88	4900	0.42	0.56	6000	0.53	1.75	26000
Papaverine	0.17	1.12	200	0.36	3.83	1700	0.46	1.43	9900	0.38	0.89	7100	0.52	0.56	25200
Paracodine	0.17	0.65	300	0.35	2	3700	0.44	1	20200	0.38	0.7	5800	0.52	0.5	25200
Protopine	†0.09			0.33	0.81	1500	0.43	1.22	8700	0.44	0.42	13900	0.54	2	34600
Brucine	0			two spots			two spots			two spots			two spots		
Quinine	0.13	0.38	100	two spots			two spots			0.28	0.42	300	two spots	0.4	300
Ephedrine	0.57	0.88	18700	0.58	0.8	21500	†0.6			0.39	0.78	500	0.59	0.36	14700
Yohimbine	†0.14			0.41	2.44	600	0.48	2.31	1400	0.44	0.88	4300	0.55	1.18	5600
Caffeine	0.46	1	7500	0.45	1	6900	0.46	0.92	8500	0.47	1	6500	0.49	1	9700
Colchicine	0.34	0.8	2600	0.33	0.85	2700	0.33	0.64	3500	0.34	0.75	2300	0.36	0.53	3500
Lobeline	0.18	0.82	400	0.36	3.17	2500	0.44	1.4	27400	0.42	1.17	16100	0.52	1.25	45600
Santonine	0.54	1	11800	0.53	1	13300	0.55	1.13	20600	0.54	3.69	9000	0.54	1.11	12400
Strychnine	0.12	0.82	200	0.34	1.06	1700	0.42	0.25	6200	0.29	0.86	2400	0.49	0.5	6300
Theophylline	0.56	0.9	12400	0.56	0.92	14300	0.57	0.83	13000	0.59	1	14300	0.55	0.78	7200

* System: MeOH–H_2O (80:20) + TBA-Cl.
† Very wide peak.

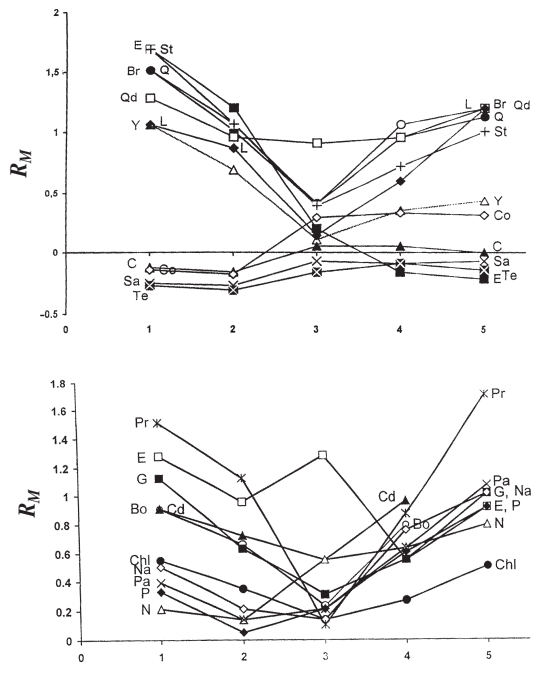


Figure 3. Graphical comparison of R_M values obtained for alkaloids (A, B) in following chromatographic systems of C18 W: 1, DEA 0.05 M MeOH–H_2O (80:20); 2, TEA 0.05M MeOH–H_2O (80:20); 3, TBA-Cl 0.025M MeCN–H_2O (80:20); 4, TOA 0.025M MeCN–H_2O (80:20); and 5, TIPA 0.025M MeCN–H_2O (80:20). (See Table I for abbreviations.)

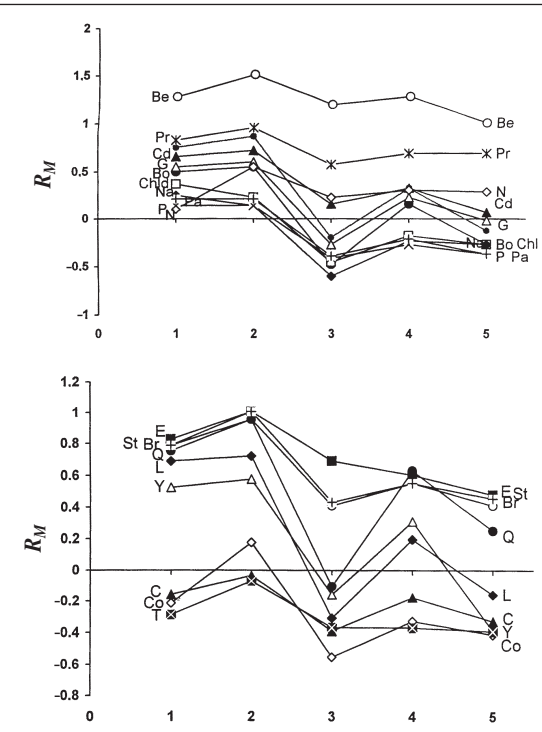


Figure 4. Graphical comparison of R_M values obtained for alkaloids (A, B) in chromatographic systems of C18 W modifier–H_2O (80:20) with 0.01 mL of DEA. Modifiers: 1, MeOH; 2, MeCN; 3, THF; 4, iPrOH; and 5, DX. (See Table I for abbreviations.)

also lead to changes in selectivity. Amines, as strong bases, interact with ionized silanols, thus blocking the interactions of these groups with the compounds analyzed. This explains why considerable improvement in peak symmetry, system efficiency, and separation selectivity was observed in all systems containing amines (besides changes in retention) (Tables III and IV). In Table III, values of theoretical plate number for chromatographed alkaloids in systems with an increasing diethanolamine (DEA) concentration are presented; in Table IV, those with increasing concentrations of TBA-Cl are shown. The highest theoretical plate number was obtained when eluent systems containing the highest concentration of amines were applied. In most cases, that symmetry of peaks also improves with the increase of DEA concentration. However, in the case of TBA-Cl, asymmetry factors do not change for the better with the concentration of that amine. In most cases, the best peak symmetry was obtained when TBA-Cl concentration of 0.01M was applied in the mobile phase. TBA-Cl, as a substance with large molecules, adsorbs on residual surface silanols and more effectively covers the surface of adsorbent, even at a lower concentration by blocking residual silanols.

The influence of the amine type in the mobile phase on the retention of alkaloids is shown as graphic comparisons of R_M values in Figure 3. The change of amine causes differences in separation selectivity, especially when the eluent with the quaternary amine was used. The best separation for isoquinoline alkaloids was obtained in a system containing 0.05M DEA in methanol–H_2O (80:20). However, for other alkaloids, a system with 0.025M tri-

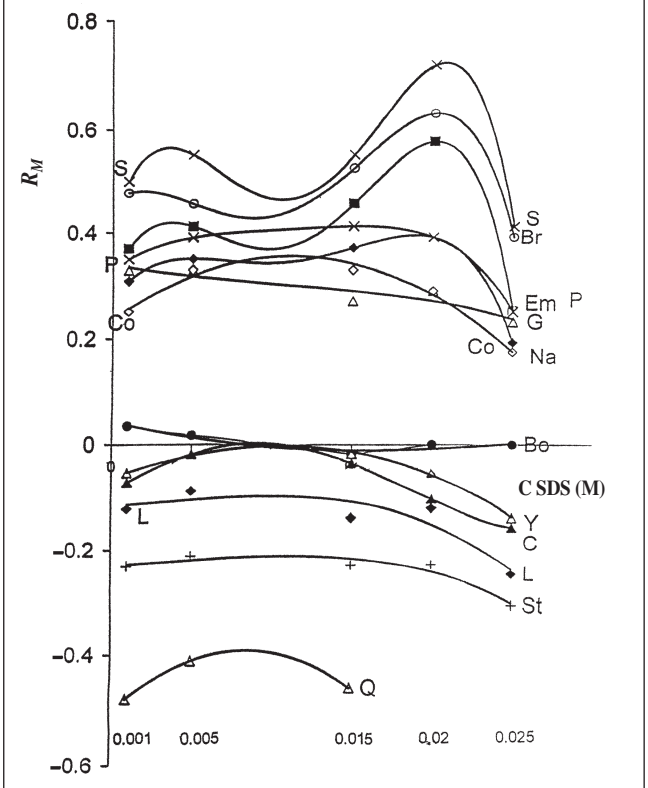


Figure 5. Relationships between R_M and SDS concentration in mobile phase for selected alkaloids. System: C18 W MeOH–H_2O (80:20) buffered with phosphate buffer 0.01 mL at pH 3. (See Table I for abbreviations.)

Table V. R_f, A_S, and N/m Values for Investigated Alkaloids Obtained on RP18 W Plates in Eluent Systems*

	0.1M DEA + 80% MeOH			0.1M DEA + 80% MeCN			0.1M DEA + 80%THF			0.1M DEA + 80% izoprop.			0.1M DEA + 80% dioksanu		
Alkaloid	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m
Berberine	0.05	0.6	200	0.03	1.33	100	0.06	1.6	500	0.05	0.86	300	0.09	0.88	700
Boldine	0.24	1.5	1800	0.22	2.7	800	0.75	1	8200	0.41	1.4	600	0.65	0.56	3400
Chelidonine	0.3	0.5	2200	0.37	0.81	1700	0.74	1	13200	0.6	0.43	3300	0.65	1.57	8200
Emetine	0.15	0.83	4900	0.12	1.67	900	0.61	0.08	5100	0.32	1.1	4600	0.57	0.68	4300
Glaucine	0.22	0.86	1700	0.2	1.08	1400	0.65	0.81	10000	0.37	1.05	1800	0.51	0.8	3500
Codeine	0.18	1	900	0.16	0.75	700	0.41	0.5	1500	0.32	1	2800	0.46	0.56	5100
Narceine	0.44	1.33	2200	0.22	1.3	2000	0.37	2.75	2600	0.33	1.23	3400	0.34	0.5	4100
Narcotine	0.36	0.95	1900	0.42	0.84	3100	0.8	0.34	6400	0.63	0.88	8400	0.65	1.13	8100
Papaverine	0.42	0.88	1700	0.42	1	2600	0.72	1.25	33400	0.65	0.93	9100	0.7	1	13100
Paracodine	0.38	1.3	1400	0.38	1	2000	0.71	1.33	61000	0.62	0.82	4800	0.7	0.85	17000
Protopine	0.13	1	3500	0.1	1.38	400	0.21	1.24	700	0.17	1.5	1900	0.17	1.22	400
Brucine	0.14	1.5	3100	0.1	2.25	1000	0.28	1.27	770	0.22	1	900	two spots		
Quinine	0.15	1.2	4600	0.1	1.8	800	0.56	0.21	2700	two spots			0.36	1.05	1200
Ephedrine	0.13	2.75	2100	0.09	6.67	800	0.17	0.91	800	two spots			†0.25		
Yohimbine	0.23	1.36	1300	0.21	1	600	0.59	0.17	4000	0.33	3.83	100	0.71	0.24	9900
Caffeine	0.59	1	6800	0.52	1	7300	0.71	0.83	22900	0.6	0.88	7600	0.68	0.8	12500
Colchicine	0.62	0.89	5700	0.4	0.68	2574	0.78	0.88	47800	0.68	1	8100	0.72	0.75	10000
Lobeline	0.17	2.14	1700	0.16	1.8	1100	0.67	0.52	3100	0.39	1.5	3600	0.59	1.24	5500
Santonine	0.67	0.94	1344 2	two spots	2	200	0.72	1	28000	0.68	2.83	18400	0.7	1	22600
Strychnine	0.14	1	9300	0.09	1.25	2800	0.27	1.33	2900	0.2	0.5	2300	0.26	0.8	2000
Theophylline	0.66	1.44	8500	0.54	1.1	10400	0.7	0.67	38200	0.7	0.88	13200	0.71	1	23400

* System: 0.1M DEA, organic modifier–water (80:20).
† Very wide peak.

Table VI. R_f, A_S, and N/m Values for Investigated Alkaloids Obtained on RP18 W Plates in Eluent Systems*

	0.025M HDEHP			0.025M SDS			0.025M OSA-Na		
Alkaloid	R_f	A_S	N/m	R_f	A_S	N/m	R_f	A_S	N/m
Berberine	0.23	0.86	1500	0.23	1	3500	0.25	0.71	2500
Boldine	0.5	0.6	4400	0.48	0.52	3700	0.55	0.5	7200
Chelidonine	0.5	0.83	1900	0.5	0.86	1500	0.52	0.71	2000
Emetine	†0.34			†			0.42	0.28	400
Glaucine	0.32	0.39	1300	0.36	0.59	700	0.35	0.57	900
Codeine	0.39	0.31	3000	0.37	0.73	1200	0.46	0.24	1400
Narceine	two spots			two spots			two spots		
Narcotine	0.39	0.39	5200	0.39	0.71	3600	0.43	0.39	3200
Papaverine	0.38	0.28	1400	0.39	0.78	1300	0.39	0.61	1700
Paracodine	0.37	0.28	1500	0.36	0.78	2300	0.37	0.6	2200
Protopine	0.42	0.36	3400	0.41	0.6	3600	0.44	0.43	3800
Brucine	0.21	1.25	200	0.23	0.5	200	0.27	0.73	100
Quinine	0.34	0.15	400	0.29	0.75	1000	0.35	0.5	1100
Ephedrine	two spots			†0.74			two spots		
Yohimbine	0.53	0.63	2600	0.56	0.31	1100	0.55	0.87	2600
Caffeine	0.53	1	9600	0.58	0.92	10000	0.52	1	7300
Colchicine	0.54	0.93	7100	0.59	0.93	9200	0.54	0.87	6200
Lobeline	0.4	0.48	2400	0.4	1.13	2400	0.41	0.45	3300
Santonine	0.59	1.5	14000	0.64	1.67	16300	0.56	2	8500
Strychnine	0.23	0.82	200	0.28	0.76	1100	0.31	0.65	1000
Theophylline	0.63	0.89	10800	0.67	1	12100	0.62	1	13000

* System: MeOH–phosphate buffer pH 3 (80:20) and 0.025M of IP reagent.
† Very wide peak.

octylamine in methanol–H_2O (80:20) has better selectivity of separation.

In further studies, the influence of an organic modifier on retention and system efficiency was investigated. Figure 4 depicts the selectivity of a system with RP 18 W eluents containing 0.1M DEA and different organic modifiers as a graphic comparisons of R_M values. Changing the organic modifier causes large differences in separation selectivity for all alkaloids investigated. Papaverine and boldine, for example, are not separated in systems with methanol and tetrahydrofuran, but they are well separated in systems with acetonitrile and isopropanol as organic modifiers in an aqueous mobile phase containing DEA. Codeine, glaucine, and protopine, which are not separated when methanol or acetonitrile as eluent modifiers were used, are well separated in other eluent systems. Yohimbine, lobeline, and quinine are not separated in the THF system but are sufficiently separated when dioxane as the eluent modifier was used. The use of various modifiers also influence peak symmetry and system efficiency. Large differences of N and A_S values were observed for systems containing different modifiers in a mobile phase with 0.01M of DEA (see Table V). For paracodine in a system containing dioxane, a 17,000 theoretical plate number was obtained, whereas in a system with methanol, only a 1400 theoretical plate number was calculated. For santonine, the efficiency was as follows: N = 28,000 in system with tetrahydrofuran and N = 13,400 with methanol. The asymmetry factor for yohimbine is 3.83 in the system containing 2-propanol, and 1.0 in system with acetonitrile, whereas for emetine it is 1.1 in the system containing 2-propanol and only 0.83 in the system with methanol, respectively. It can be noted that the highest efficiency was obtained in systems with tetrahydrofuran or dioxane as modifiers. However, when methanol was used as the modifier, the majority of peaks have acceptable asymmetry factors ($0.9 < A_s < 1.5$).

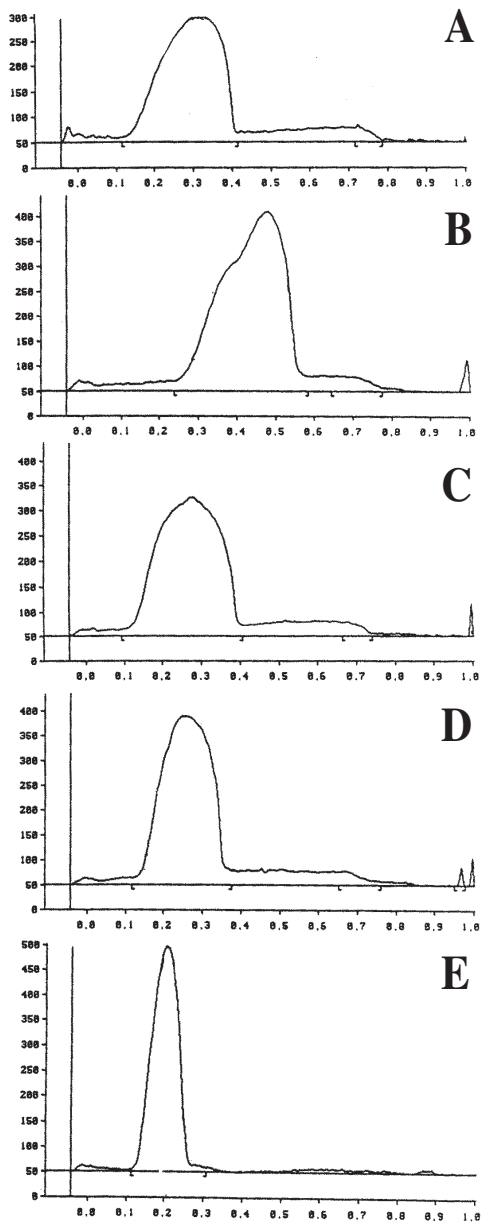


Figure 6. Densitograms of glaucine chromatographed in system C18 MeOH–H_2O (8:2) buffered with phosphate buffer 0.01M at pH 3 containing SDS in concentrations: A, 0.001M; B, 0.005M; C, 0.015M; D, 0.02M; and E, 0.025M.

Effect of IP reagent kind and concentration

In the search for systems of different separation selectivity and systems with high efficiency, anionic IP reagents, such as: SDS, pentane 1-sulfonic acid sodium salt (PSA-Na), octane 1-sulfonic acid sodium salt (OSA-NA), HDEHP, tri-*n*-butyl phosphate (TBP),

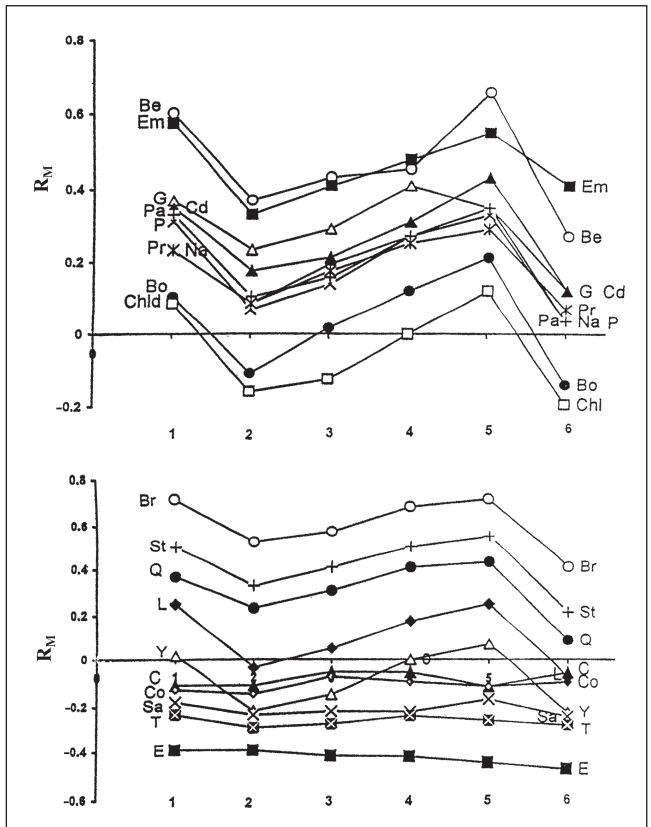


Figure 7. Graphical comparison of R_M values obtained for alkaloids (A, B) in chromatographic systems C18 W MeOH–H_2O (80:20) buffered with phosphate buffer 0.01M at pH 3 containing 0.01M of ion-pair reagent: 1, SDS; 2, PSA-Na; 3, OSA-Na; 4, HDEHP; 5, TBP; and 6, TFA. (See Table I for abbreviations.)

and trifluoroacetic acid (TFA) were added in various concentration to mobile phases. Figure 5 shows the dependency of R_M values on the concentration of SDS as an IP reagent. The retention of alkaloids initially increases with the increase of SDS concentration in the mobile phase (ranging from 0.001–0.02M SDS), after which a little decrease in retention was observed in most cases. The explanation is that, when the surface becomes saturated with the IP reagent, further increase in its concentration leads to the increase in the counterion (Na^+) concentration, which competes with the retention of the sample ion on the

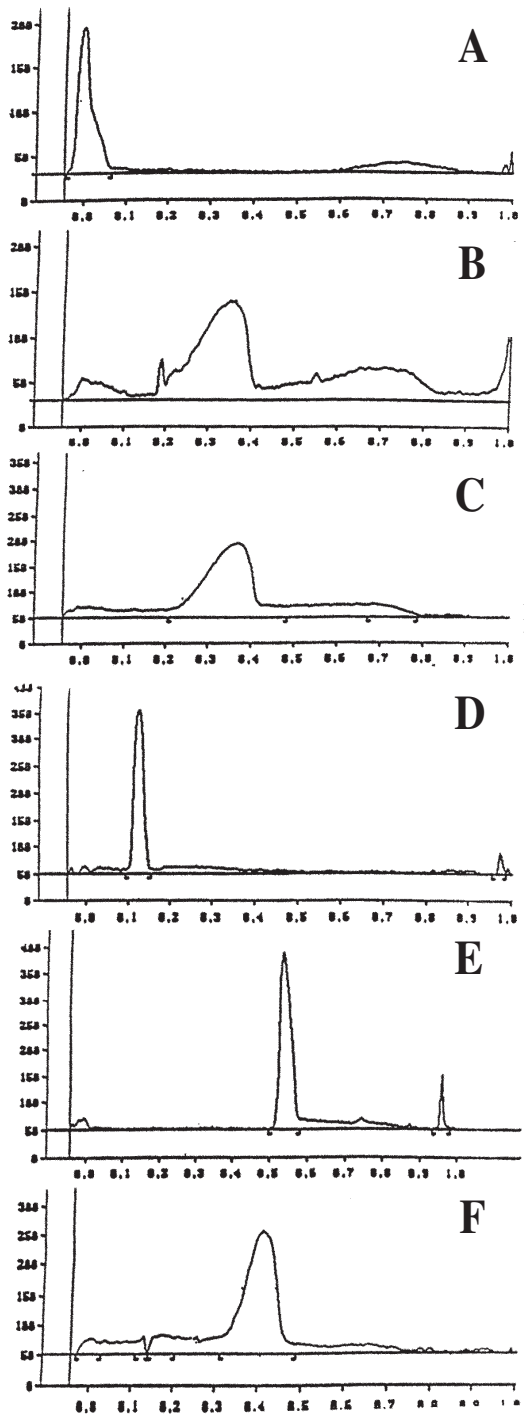


Figure 8. Densitograms of protopine chromatographed on C18 W layer in different eluent systems: A, MeOH–H_2O (80:20); B, MeOH–H_2O (80:20) buffered with phosphate buffer at pH 3; C, MeOH–H_2O (80:20) buffered with phosphate buffer at pH 8; D, MeOH–H_2O (80:20) containing 0.1M DEA; E, MeCN–H_2O (80:20) containing 0.05M TBA-Cl; F, MeOH–H_2O (80:20) buffered with phosphate buffer at pH 3 containing 0.025M SDS.

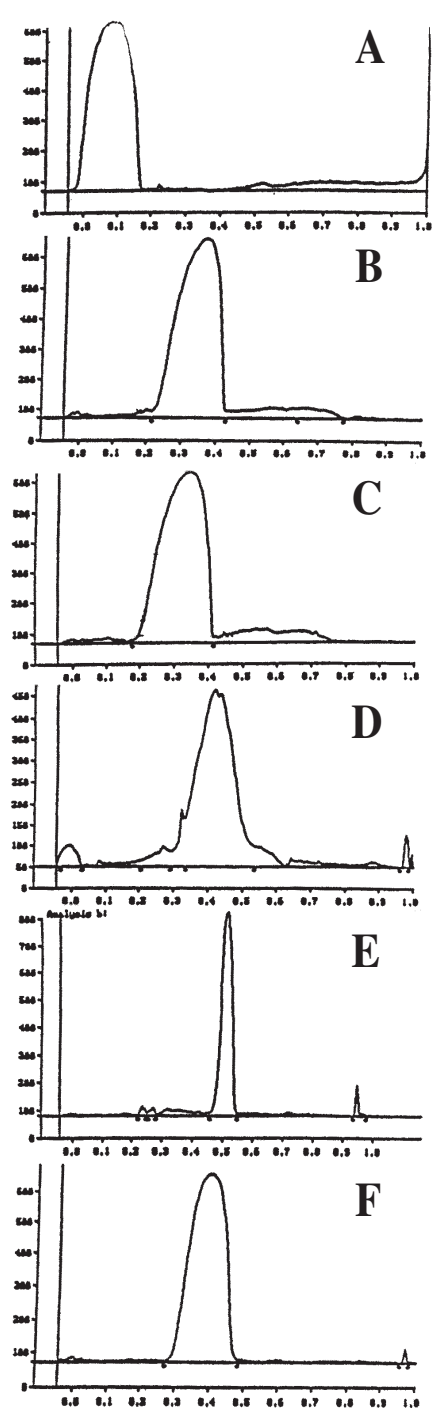


Figure 9. Densitograms of papaverine chromatographed on C18W layer in different eluent systems: A, MeOH–H_2O (80:20); B, MeOH–H_2O (80:20) buffered with phosphate buffer at pH 3; C, MeOH–H_2O (80:20) buffered with phosphate buffer at pH 8; D, MeOH–H_2O (80:20) containing 0.1M DEA; E, MeCN–H_2O (80:20) containing 0.05M TBA-Cl; and F, MeOH–H_2O (80:20) buffered with phosphate buffer at pH 3 containing 0.025M SDS.

Table VII. R_f, A_S, and N/m Values for Investigated Alkaloids Obtained on RP18 W Plates in Eluent Systems*

	0,001 M SDS + 80% MeOH			0,005 M SDS + 80% MeOH			0,015 M SDS + 80% MeOH			0,02 M SDS + 80% MeOH			0,025 M SDS + 80% MeOH		
Alkaloid	**RF**	**AS**	**N/m**	**RF**	**AS**	**N/m**	**RF**	**AS**	**N/m**	**RF**	**AS**	**N/m**	**RF**	**AS**	**N/m**
Berberine	0.17	1.13	900	0.17	2	1300	0.16	2.5	1000	†0.18			0.23	1	3500
Boldine	0.45	0.47	1100	0.38	1.08	800	0.4	0.95	1800	0.28	1.89	2300	0.48	0.52	3700
Chelidonine	0.48	0.37	800	0.49	0.45	600	0.51	0.24	800	0.5	0.23	900	0.5	0.86	1500
Emetine	†			†			†			†			†		
Glaucine	0.3	0.68	400	0.28	0.81	200	0.26	0.92	300	0.21	0.72	800	0.36	0.59	700
Codeine	0.32	0.35	400	0.32	0.58	400	0.35	0.41	1100	0.34	0.37	1100	0.37	0.73	1200
Narceine	two spots			two spots			two spots			two spots			two spots		
Narcotine	0.34	0.57	900	0.3	0.95	700	0.3	0.93	2000	0.27	1.17	1800	0.39	0.71	3600
Papaverine	0.33	0.67	600	0.31	0.86	500	0.3	1.09	500	0.29	1	800	0.39	0.78	1300
Paracodine	0.31	0.5	900	0.29	0.68	800	0.28	0.89	1000	0.29	0.79	1600	0.36	0.78	2300
Protopine	0.35	0.65	1100	0.28	1.55	400	0.29	1	1400	0.28	0.88	1500	0.41	0.6	3600
Brucine	†0.11			†0.12			0.19	0.64	100	0.15	0.47	200	0.23	0.5	200
Quinine	0.25	1.15	200	0.26	0.91	300	0.23	1.35	400	0.19	1	500	0.29	0.75	1000
Ephedrine	0.75	0.09	1400	0.72	0.1	20600	0.74	0.09	1300	†			†0.74		
Yohimbine	0.53	0.47	2100	0.5	0.35	2300	0.53	0.3	800	0.33	1.93	1800	0.56	0.31	1100
Caffeine	0.53	0.92	8600	0.51	0.93	6900	0.51	0.92	9000	0.53	0.86	7600	0.58	0.92	10000
Colchicine	0.54	0.93	7100	0.51	0.87	6300	0.52	1.4	6600	0.56	0.93	7600	0.59	0.93	9200
Lobeline	0.36	0.56	1400	0.32	0.87	900	0.32	0.85	1000	0.34	0.42	1200	0.4	1.13	2400
Santonine	0.57	2.13	9900	0.55	1.67	8100	0.58	0.44	6400	0.57	1.82	13000	0.64	1.67	16300
Strychnine	0.24	0.88	300	0.22	1	300	0.22	0.77	500	0.16	0.79	800	0.28	0.76	1100
Theophylline	0.63	1	13700	0.62	0.93	11600	0.63	1	16000	0.63	1	600	0.67	1	12100

* Containing MeOH–phosphate buffer pH 3 (80:20) and SDS.
† Very wide peak.

adsorbent surface (9). The influence of SDS concentration on the peak symmetry and system efficiency can be compared through the analysis of appropriate values of N and A_s given in Table VI. It is seen that the increase of SDS concentration causes the improvement of chromatographic system efficiency. In most cases, the highest values of theoretical plate number were obtained when the eluent containing 0.025M of SDS was used. Simultaneously, the most symmetric peaks were obtained in systems with lower SDS concentrations (0.015M). In order to compare the efficiency of the systems, the densitograms obtained for boldine in eluent systems with an increasing concentration of IP reagent are presented in Figure 6.

To show the effect of different counterions on retention and selectivity, R_M values for alkaloids chromatographed in systems with various IP reagents are presented in Figure 7. The most selective system for the separation of quinoline alkaloids is the system with tributylphosphate, for other alkaloids that with octanesulfonic acid. Variation of the IP reagent type causes differences in retention, separation selectivity, and often changes the sequence of elution, especially visible for isoquinoline alkaloids. Chelidonine and boldine were not separated in a system containing SDS as the IP reagent (although peak symmetry, especially for boldine, was good). They are sufficiently well separated in systems with HDEHP or OSA-Na, although peak symmetry of each deteriorates. Table VII presents R_f values, asymmetry factors, and theoretical plate number values for investigated alkaloids in eluent systems containing 0.025 mL of various IP reagents. However, the most effective system with the highest values of theoretical plate number, with OSA-Na, gives asymmetric peaks. The most symmetric peaks in most cases are obtained when HDEHP was used as the IP reagent.

In Figures 8 and 9, densitograms obtained for protopine and papaverine in six different chromatographic systems are presented. In a system containing methanol and water, both alkaloids are strongly retained on RP18W adsorbent; the spots are on the chromatogram start line. After adding phosphate buffer to the mobile phases at pH 3 to suppress the silanol ionization, and at pH 8 to suppress the alkaloid ionisation, a decrease of retention is observed, but the peaks are still wide and asymmetric. Systems containing amines in eluents were more effective. Thus, the improvement of peak symmetry and of efficiency is noticed with narrow and very symmetric peaks. For example, A_S for protopine was 1.0, and A_S was 0.9 for papaverine in the system with DEA. Similar observations can be obtained for systems with TBA-Cl. In the system containing SDS as an IP reagent in a mobile phase, the efficiency gets worse in comparison with systems with amines. However, peak shape and symmetry in IP systems are better in comparison with those of buffered systems.

Systems containing different concentrations of DEA in aqueous mobile phases are the most efficient for the 17 investigated alkaloids. Videoscan of an RP18 W plate triple developed (27) with 80% MeOH in water + 0.05M DEA and an adequate densitogram

for the separation of isoquinoline alkaloid standards and a mixture are presented in Figure 10.

Conclusion

The majority of investigated alkaloids (without purine alkaloids and colchicine) are strongly retained in systems containing organic modifier in water on RP18 W plates. Selectivity of separation is very poor and spots are wide and tailing.

Variation of the mobile phase pH in the range 2–6 does not cause clear differences in retention, but at a lower pH spots (peaks) are more symmetric. The decrease of retention was observed at the highest pH values, but system efficiency was still poor.

Adding IP reagents leads to the improvement of peak symmetry and the increase of theoretical plate number in all cases, with better separation selectivity. The best efficiency and separation selectivity for the investigated alkaloids was obtained in systems containing amines in mobile phases.

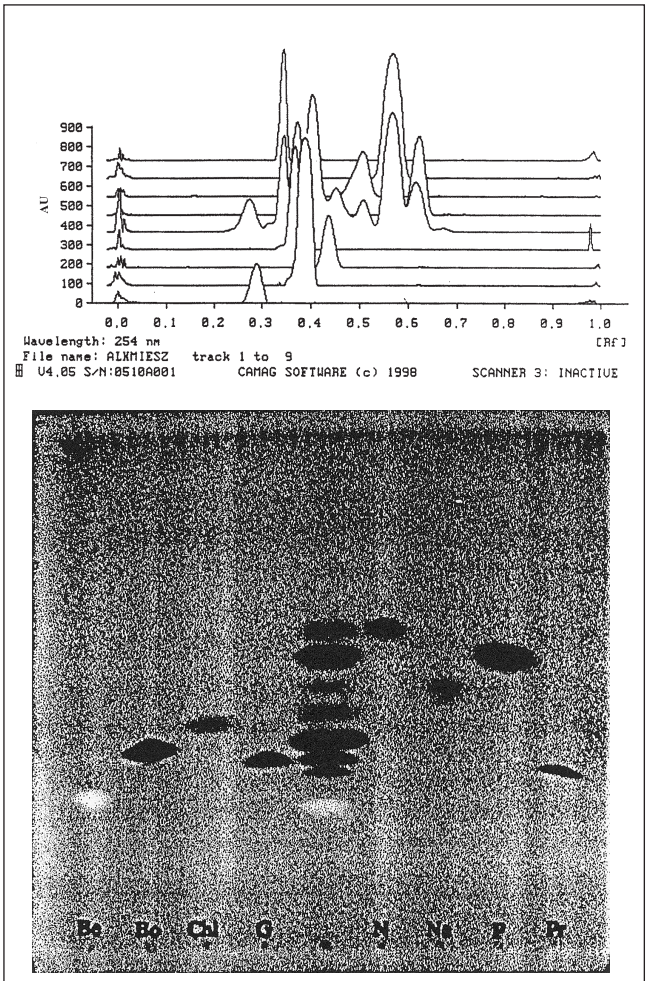


Figure 10. Videoscan (A) and densitogram (B) of an RP18 W plate with the separated mixture and isoquinoline alkaloid standards. Plate triple developed with the eluent: MeOH–$H_2$0 (80:20) containing 0.05 mL DEA. (See Table I for abbreviations.)

References

1. N. Fabre, C. Claparols, S. Richelme, M-L. Angelin, I. Fouraste, and C. Moulis. Direct characterization of isoquinoline alkaloids in crude plant extract by ion-pair liquid chromatography–electrospray ionization tandem mass spectrometry: example of *Eischoltzia California*. *J. Chromatogr. A* **904:** 35–46 (2000).
2. C. Tikhomiroff and M. Jolicoeur. Screening of *Catharanthus roseus* secondary metabolites by high-performance liquid chromatography. *J. Chromatogr. A* **955:** 87–93 (2002).
3. S.-L. Li, G. Lin, S.-W. Chan, and P. Li. Determination of the major isosteroidal alkaloids in bulbs of *Fritillaria* by high performance liquid chromatography coupled with evaporative light scattering detection. *J. Chromatogr. A* **909:** 207–14 (2001).
4. P.A. Steenkamp, F.R. van Heerden, and B-E. van Wyk. Accidental fatal poisoning by *Nicotiana glauca*: identification of anabasine by high performance liquid chromatography/photodiode array/mass spectrometry. *Forensic Sci. Int.* **127:** 208–17 (2002).
5. S-W. Sun, C.-H. Kuo, S.-S. Lee, and C.-K. Chen. Determination of bis-benzylisoquinoline alkaloids by high performance liquid chromatography (II). *J. Chromatogr. A* **891:** 189–94 (2000).
6. D.V. McCalley. Analysis of Cinchona alkaloids by high performance liquid chromatography and other separation techniques. *J. Chromatogr. A* **967:** 1–19 (2002).
7. R.J. Molyneux, D.R. Gardner, L.F. James, and S.M. Colegate. Polyhydroxy alkaloids: chromatography analysis. *J. Chromatogr. A* **967:** 57–74 (2002).
8. L.A. Ciolino, J.A. Turner, H.A. McCauley, A.W. Smallwood, and T.Y. Yi. Optimization study for the reversed phase ion pair liquid chromatography determination of nicotine in commercial tobacco products. *J. Chromatogr. A* **852:** 451–63 (1999).
9. L.R. Snyder, J.J. Kirkland, and J.L. Glajch. *Practical HPLC Method Development*, Wiley, New York, NY, 1997.
10. M.L. Bieganowska and A. Petruczynik. Ion pair chromatography and its application in analysis of pharmaceuticals. *Chem. Anal. (Warsaw)* **39:** 525–41 (1994).
11. J.H. Knox and G.B. Laird. Soap chromatography–a new high-performance liquid chromatographic technique for separation of ionizable materials. *J. Chromatogr.* **122:** 17–34 (1976).
12. K.G.C. Low, A. Bartha, H.A.H. Billiet, and L. de Galan. Systematic procedure for the determination of the nature of the solute prior to the selection of the mobile phase parameters for optimization of reversed-phase ion-pair chromatographic separations. *J. Chromatogr.* **478:** 21–38 (1989).
13. R.S. Deelder and J.H.M. van den Berg. Study on the retention of amines in reversed-phase ion pair chromatography on bonded phases. *J. Chromatogr.* **218:** 327–40 (1981).
14. A. Bartha, G. Vigh, H.A.H. Billiet, and L. de Galan. Studies in reversed-phase ion-pair chromatography. IV. The role of chain length of the pairing ion. *J. Chromatogr.* **303:** 29–38 (1984).
15. M.L. Bieganowska, A. Petruczynik, and M. Gadzikowska. Retention of some organic electrolytes in ion-pair reversed-phase high performance liquid and reversed-phase high-performance thin-layer chromatographic systems. *J. Chromatogr.* **520:** 403–10 (1990).
16. M.L. Bieganowska and A. Petruczynik. Retention parameters of some isomeric 2-benzoylbenzoic acids in reversed-phase ion-pair high performance thin-layer and column chromatography. *Chromatographia* **40:** 453–57 (1995).
17. P. Jandera, J. Chura**c**ek, and B. Taraba. Comparison of retention behaviour of aromatic sulphonic acids in reversed phase systems with mobile phases containing ion-pair ions and in systems with solutions of inorganic salts as the mobile phases. *J. Chromatogr.* **262:** 121–40 (1983).
18. A. Bartha, G. Vigh, H.A.H. Billiet, and L. de Galan. Effect of the type of ion-pairing reagent in reversed-phase ion-pair chromatography. *Chromatographia* **20:** 587–90 (1985).
19. C.B. Castells, C. Rrfols, M. Rosés, and E. Bosch. Effect of temperature on pH measurements and acid–base equilibria in methanol–water

mixtures**.** *J. Chromatogr. A* **1002:** 41–53 (2003).
20. K. Mazak, J. Vamos, A. Nemes, A. Racz, and B. Noszal. Lipopholicity of vicopocetine and related compounds characterized by reversed-phase thin layer chromatography. *J. Chromatogr. A* **996:** 195–203 (2003).
21. P.J. Houghton, A. Latiff, and I.M. Said. Alkaloids from *Mitragyna speciosa*. *Phytochemistry* **30:** 347–50 (1991).
22. A. Shalaby and H. Khalil. Reversed phase ion-pair chromatography of some alkaloids. *J. Liq. Chromatogr.* **22:** 2345–52 (1999).
23. D. Volkmann. Ion-pair chromatography with UV absorbing counterions on reversed-ohase layers. Part I. Chromatography with hydroxynaphthalenesulfonic acids. *J. High Resol. Chromatogr.* **6:** 378–80 (1983).
24. P. J. Houghton and I.M. Osibogun. The use of silica gel coated with liquid paraffin as a stationary phase for chromatographic analysis of phytochemicals. *Phytochem. Anal*. **1:** 22–25 (1990).
25. R.J. Petrovski, S.G. Yated, D. Weisleder, and R.G. Powell. Isolation, semi-synthesis and NMR spectral studies of loline alkaloids. *J. Nat. Prod.* **52:** 810–17 (1989).
26. J.D. Duncan and D.W. Armstrong. Chiral mobile phase additives in reversed phase TLC. *J. Planar Chromatogr.* **3:** 65–67 (1990).
27. Sz. Nyiredy, Ed. *Planar Chromatography*. Springer Scientific Publisher, Budapest, Hungary, 2001, p. 103.

Manuscript received January 21, 2004;
revision received January 3, 2005.