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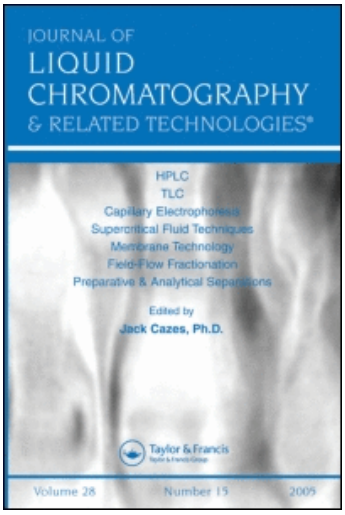

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Effect of Chromatographic Conditions on the Separation of Selected Alkaloids on Phenyl Stationary Phase by an HPLC Method

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Abstract: Some alkaloid standards and synthetic or natural alkaloid mixtures were chromatographed on phenyl-silica stationary phases using various aqueous eluents: aqueous methanol or acetonitrile mixture, buffered aqueous mobile phases at pH 3 to suppress silanols' ionisation, or at pH 8 to suppress alkaloids' ionisation. Strong silanophilic interactions that can occur with these analytes and the adsorbent surface can lead to poor peak shape and resolution. Mobile phases with anionic ion-pairs such as sodium dodecyl sulphate, octanesulfonic acid sodium salt, or HDEHP were used to improve efficiency, peak shape, and selectivity. The effect of silanol blockers – different amines on the retention, efficiency, and peak shape for RP systems, was analysed.

Keywords: HPLC, Phenyl stationary phase, Alkaloids, IP-reagents, Silanol blockers, Peak shape, System efficiency

INTRODUCTION

Alkaloids are pharmacologically active compounds which are widely used as pharmaceuticals and synthesised as secondary methabolites in plants. Many of these compounds are strongly toxic. Therefore, they are often the subject of scientific interest and analysis. Since alkaloids appear in solutions as ionized and unionized forms, they are difficult to separate chromatographically. Mobile phase pH is a major factor for the separation of analytes with

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acid-base properties.[1–5] Retention models of ionizable solutes in liquid chromatography with a function of pH and solvent composition, were reviewed by Schoenmakers and co-workers.[6,7] The theory of RP retention for ionic compounds with a function of pH assumes that a given solute exists in ionized and non-ionized forms, and interacts by two various modes with chromatographic system components.

For example, due to their basic nature, alkaloids can interact with silanol groups of silica matrix, which remain underivatized on bonded RP stationary phases.[8,9] Silica is also the most widely used material in chromatography. Silica supports are still superior to other supports in terms of efficiency, rigidity, and performance. However, there are several problems with silica based materials: such as peak tailing in the chromatography of basic compounds, irreproducibility for the same chemistry columns, and limited pH stability. Protonated basic compounds can interact with residual silanol groups of the stationary phase, as shown in the equation:

$$XH^+ + SiO^-Na^+ \Longleftrightarrow Na^+ + SiO^-XH^+ \quad (1)$$

Thus, besides the reversed phase retention mechanism, an ion-exchange retention mechanism also occurs, which often results in symmetry of peaks, irreproducible retention, and poor separation. Asymmetric peaks can be explained in terms of kinetic phenomena, i.e., when the kinetics of mass transfer of one type of column site is slower than the other.[10] For basic solutes, the kinetics of the ion-exchange interaction with silanol groups may be slower than those with the alkyl ligands, giving rise to peak tailing.[11]

Silanol interaction can be reduced by:

- use of a low pH mobile phase (2.0–3.5) because, at these pH values, the ionisation of silanol groups is largely suppressed;[12]
- use of a high pH mobile phase (>7.0) where ionisation of some alkaloids is suppressed;[13]
- addition of an ion-pairing reagent to the mobile phase.[14,15] The surface of a nonpolar adsorbent is covered by sorbed molecules of a negative ion-pairing reagent. The ion-pairing reagent is attracted to the stationary phase because of its hydrophobic alkyl group, and the charge carried by the reagent thereby attaches itself to the stationary phase. This negative charge on the stationary phase is balanced by positive ions (Na^+, K^+) from the reagent or buffer. A positively charged sample ion–protonated alkaloid can now exchange with a Na^+ (K^+) ion, resulting in the retention of the sample ion by an ion-exchange process;[16]
- addition of a silanol blocker to the eluent, i.e., $pK_{a\ \text{silanol blocker}} > pK_{a\ \text{basic analyte}}$. The more basic compound will interact with the residual silanols more strongly, allowing the less basic compound to interact solely with the alkyl ligand of the stationary phase;[11,17]
- selecting a stationary phase.[9,18–20]

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The bonded stationary phase with a range of functionalities made possible the rapid growth of RPC for separating a wide range of compound types and also ionic compounds. Stationary phases containing phenyl functionality are rather popular as packing materials for reversed phase HPLC.[21–25]

The influence of phenyl functionality of the bonded phase on the retention of aromatic analytes has been studied on a structure retention relationship.[24,26,27] The phenyl bonded phases have been used successfully to resolve many compounds e.g., phenolic acids,[28,29] hormones,[30,31] alkaloids,[32,33] flavonoids,[34,35] and different pharmaceuticals.[36,37] Phenyl type phases with their hydrophobic $\pi-\pi$ active aromatic moieties may introduce an additional component to the retention of aromatic solutes.[38–40]

Therefore, analytes with π systems will display different retention behaviour on π containing stationary phases, compared to widely used alkyl bonded phases.[24] In a chromatographic system, these interactions can occur between π-electrons of the stationary phases and the analytes.[28]

Selectivity of reversed phase columns can be described quantitatively by parameters of hydrophobicity, steric resistance, hydrogen bond acidity and basicity, and column cation exchange capacity.[28] In the case of the phenyl stationary phase, it appears that additional solute–column interactions, especially $\pi-\pi$ interactions, contribute to retention and special selectivity of the column. Additionally, the retention and selectivity in IP-RP systems can be controlled by the change of type and concentration of the organic modifier in the aqueous mobile phase.[29]

The aim of this paper was to investigate the various parameters influencing the retention, peak shape, efficiency, and separation selectivity of selected alkaloids from different classes on phenyl stationary phases. The effects of buffer pH, amine concentration in mobile phase, as well as ammonia and kind and concentration of anionic IP reagents, were examined. Systems with the best parameters and highest selectivity were used for the separation of alkaloid standards mixtures as well as plant extracts.

EXPERIMENTAL

Analysis was performed using a liquid chromatograph LC-10 AT_{VP} Shimadzu equipped with a Phenyl HYPERSIL 150 × 4.6 mm column, Shimadzu detector SPD–10 AV_{VP} and Rheodyne 20 μL injector. Detection was at wavelength 254 nm. All chromatographic measurements were carried out at 22°C with an eluent flow rate of 1.0 mL/min. Acetonitrile and methanol of chromatographic quality, octane-1-sulfonic acid sodium salt (OSA-Na), and diethylamine (DEA) were from Merck (Darmstadt, Germany). Bis-(2-ethylhexy0l) phosphate (HDEHP), sodium dodecyl sulphate (SDS) were from Fluka (Buchs, Switzerland). Ammonia was from Polish Reagents (Gliwice, Poland). The pH of phosphate buffers used in experiments in 0.01 M L^{-1} concentrations were measured in aqueous solutions.

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The plant extract was obtained from *Fumaria officinalis* L. herb by percolation with 2% acetic acid in water. The extract was evaporated to dryness in a vacuum evaporator and dissolved in methanol.

Alkaloid standards are listed in Table 1.

RESULTS AND DISCUSSION

Alkaloid standards (Table 1) were chromatographed on phenyl-silica column in various eluents. Experiments were performed on a phenyl type column because of the higher efficiency and enhanced resolution. Silica with aromatic ligands on the stationary phase surface can give significant differences in retention and separation in comparison to widely used alkyl bonded phases. Because of their smaller shape, their planar structure, polarisable character, and consequently, better retention mechanism, phenyl bonded phases are known to be more retentive for aromatic substances such as alkaloids.[18,30,33]

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In a system containing the mixture of acetonitrile and water, tailing peaks and low efficiency of this system was obtained. Under these conditions, alkaloids are present as ionised and neutral forms. Silanol groups are also dissociated. Ionised forms of alkaloids strongly interacts with free silanols, which causes peaks' tailing, and low efficiency of the column. In eluent systems containing 70% methanol in water the asymmetry factor was acceptable only for 6 alkaloids, and only for lobeline, narcotine, scopolamine, and strychnine was the number of theoretical plates pro meter higher than 10000 (Table 1).

To improve the peaks' shape and efficiency buffered mobile phases were applied. Mobile phase pH is an important consideration, both in terms of optimising peak shape and altering the efficiency of the system and the separation selectivity. Retention, efficiency and peak shape were examined in eluent systems containing phosphate buffers at pH 3.5 and 7.8. At pH 3.5 most silanol groups are undissociated even though alkaloids are protonated. Thus, there is a limited possibility for ion-exchange effects. In the system, for most of the investigated alkaloids, asymmetric peaks and poor efficiency were obtained. Only for narceine, colchicine, and santonine were the peaks' symmetry acceptable. At high pH values the dissociation of most alkaloids is suppressed, which leads to the decrease of interactions between neutral alkaloid molecules and residue surface silanols. Additionally, neutral molecules strongly interact with hydrophobic surface ligands. In the system containing buffer at pH 3.5 on a phenyl column, peaks were asymmetric and were tailing for most alkaloids. However, in the system containing buffer at pH 7.8 – for 13 alkaloids on the phenyl column, asymmetry factors were in the range of acceptable values (0.8 – 1.5). The number of theoretical plates was higher for most alkaloids in the system containing buffer at pH 7.8 in eluent, in comparison to the system at pH 3.5. For 15 investigated compounds on the phenyl stationary phase $N/m > 10000$ was obtained.

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Table 1. Retention times (t_r), assymetry factors (A_S) and theoretical plate number (N/m) values for investigated alkaloids obtained on phenyl column

		70 MeOH + buffer pH 3.5			60 MeOH + buffer pH 7.8			30 MeCN + buffer pH 3.5 + 0.025 M OS			30 MeCN + buffer pH 3.5 + 0.005 M SDS			40 MeCN + 1% NH_3			40 MeCN + buffer pH 3.5 + 0.05 M DEA		
Symbol	Alkaloid	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N
Be	Berberine	71.77	*	*	14.41	0.94	27960	21.68	2.01	9600	42.25	2.00	40220				11.10	1.12	24300
Bo	Boldine	11.93	1.77	1670	3.90	1.78	9340	4.47	2.05	6150	8.03	2.88	13340	1.89	0.72	2510	2.95	0.97	3940
Chl	Chelidonine	26.78	*	*	14.66	0.69	22640	11.70	3.58	2110	21.33	14.00	4340	9.01	0.79	12170	11.10	0.75	14060
D	Dionine	11.34	2.11	760	5.75	3.54	8010										3.77	1.15	21470
Em	Emetine	*			11.69	2.05	17750	16.20	3.33	2170				8.33	2.74	10080	7.33	1.12	27960
G	Glaucine	*			8.83	2.56	21070	9.73	2.14	5240				6.59	2.11	10660	6.14	1.22	24940
Nc	Narceine	9.48	1.42	2860	3.50	0.87	6180	7.43	*	*	7.32	2.80	14290	1.37	1.02	2210	10.96	0.87	29360
P	Papaverine	18.00	4.63	1567	5.38	1.20	17640	9.09	3.10	650	15.68	6.20	300	4.58	1.31	11300	4.88	1.11	15650
Pa	Paracodine	21.27	2.63	380	5.29	1.15	22740	9.34	2.04	5610	16.58	4.35	5890	4.59	1.30	15990	4.98	1.02	20270
Pr	Protopine	26.43	*	*	8.51	1.17	30790	11.10	1.11	7540	20.94	3.00	9790	9.08	1.74	16230	8.44	0.88	34090
S	Sanquinarine	54.45	*	*	19.90	*	*				35.67	2.21	19860				18.20	3.74	9860
Br	Brucine	11.23	*	*	7.38	7.35	2810							9.03	3.35	3280	3.53	1.88	5870
Q	Quinnie	19.39	*	*	6.58	3.17	17940	11.04	2.67	3690				5.81	2.49	7420	4.68	1.07	16220
C	Cinchonine				7.49	3.96	14900	9.49	2.21	5060	16.75	8.05	10760	5.82	3.26	4050	4.53	1.12	25420

(*continued*)

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Table 1. Continued

Symbol	Alkaloid	70 MeOH + buffer pH 3.5			60 MeOH + buffer pH 7.8			30 MeCN + buffer pH 3.5 + 0.025 M OS			30 MeCN + buffer pH 3.5 + 0.005 M SDS			40 MeCN + 1% NH_3			40 MeCN + buffer pH 3.5 + 0.05 M DEA		
		t_R	A_S	N	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N	t_R	A_S	N
E	Ephedrine	3.82	*	*	2.68	1.87	1320							6.15	3.01	1500	2.98	2.60	2900
H	Hioscyamine																		
Ho	Homatropine	8.18	*	*	5.76	*	*							8.68	1.26	25700			
Y	Yochimbine	14.38	2.42	330	5.31	0.99	21530	7.77	1.75	4720	14.37	*	*						
Caff	Caffeine	*			2.41	1.78	7070	2.39	1.26	8330									
Co	Colchicine	2.88	0.82	3720	3.88	1.33	6020	4.19	3.12	2980	2.77	0.90	6220	2.80	1.02	4020	2.81	0.85	4370
L	Lobeline	62.94	*	*	10.68	1.10	25610	24.10	1.67	4640							12.03	0.87	7360
N	Novocaine	5.61	2.14	410	2.86	2.17	4330	3.35	2.34	5430				7.68	4.67	260	3.24	1.57	4310
Pl	Pilocarpine				5.38	1.07	26580	9.72	1.16	10470									
Sa	Santonine	4.99	1.16	13670	3.60	1.15	25560	6.35	1.31	15080	4.70	1.21	29370	4.18	1.34	13750	4.69	1.02	11460
Sc	Scopolamine	1.84	*	*	3.03	1.46	9310				2.77	0.89	26540	2.91	1.20	8250			
St	Strychnine	11.38	3.63	480	3.95	1.08	14260	6.40	1.10	3530	11.49	2.73	29430	12.24	2.67	1800	4.81	3.25	10540
T	Tubocuratrine	*			5.38	1.20	17640				2.21	1.78	1300	1.30	0.84	2700	1.73	1.18	1600

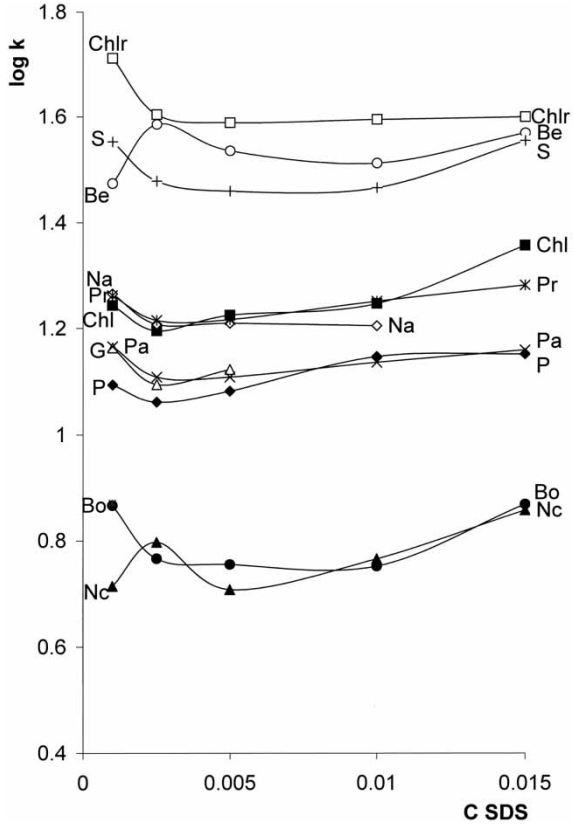

Figure 1. Dependence of log k values against SDS concentration for alkaloids chromatographed on phenyl column in eluent system 40% MeCN + acetate buffer at pH 3.5 + SDS.

Afterwards, the effect of ion-pairing reagents in eluents on peaks symmetry and system efficiency was examined. In Table 1, values of asymmetry factor and theoretical plate number for the investigated alkaloids chromatographed on the phenyl column in the eluent system containing aqueous methanol and addition of octane sulfonic acid sodium salt (OSA-Na) or sodium dodecyl sulphate (SDS) as ion-pairing reagents are presented. In the system containing OSA-Na, symmetric peaks were obtained for 5 alkaloids; in the system containing 0.01 ML^{-1} SDS in eluent for only 3 investigated alkaloids. In systems with addition of both examined ion-pairing reagents the obtained results were unsatisfied. Peaks' symmetry and system efficiency were worse in comparison to the systems with buffered aqueous mobile phases for most chromatographed alkaloids. Theoretical plate numbers were low in systems with OSA-Na, only for 2 alkaloids

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was N/m higher than 10,000 in the system with SDS N/m > 10,000 in 8 cases. However, we can apply a higher concentration of IP reagent. Increase of SDS concentration does not cause marked changes in alkaloids' retention (see Figure 1). The theoretical plate number as a function of SDS concentration in the range 0.001–0.015 mL^{-1} is presented in Figure 2. The increase of SDS concentration initially (in range 0.001–0.0025 mol L^{-1}) causes the increase in theoretical plate numbers of investigated alkaloids in all cases. Further increase of SDS concentration results in an increase of theoretical plate number in most cases, but N/m for some alkaloids decrease. However, in an eluent system containing 0.015 mL^{-1} SDS, peaks for most of investigated alkaloids were tailing, asymmetric, and wide.

The addition of ammonia or diethylamine as silanol blockers to mobile phase causes considerable improvement of peaks' shape and the increase of

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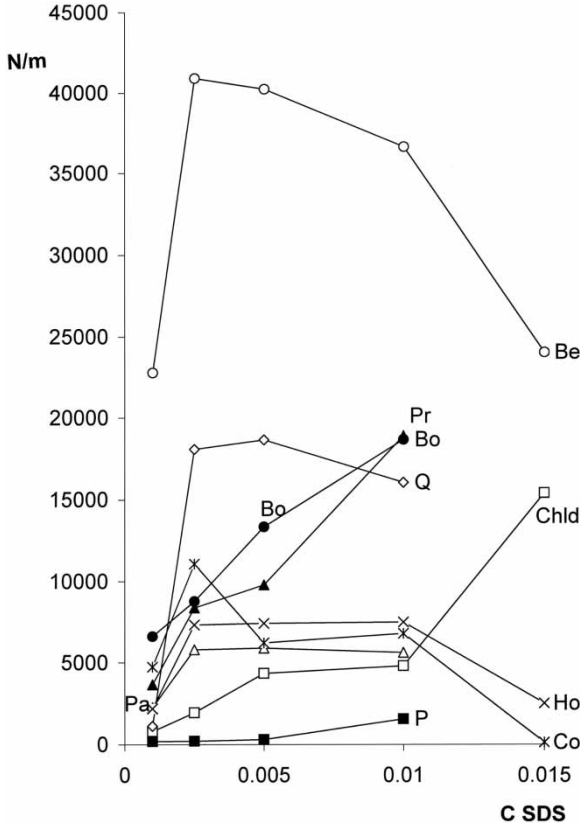

Figure 2. Dependence of N/m values against SDS concentration for alkaloids chromatographed on phenyl column in eluent system containing 40% MeCN + acetate buffer at pH 3.5 + SDS.

theoretical plate number. In the system with ammonia, good symmetry of peaks was obtained for 8 alkaloids, in the system with diethylamine, for 16 investigated alkaloids (see Table 1). In these systems, theoretical plate number was very high: in the first system for 8 compounds $N/m > 10{,}000$, in the system with addition of diethylamine, efficiency was higher than 10,000 for 13 alkaloids $N/m > 10{,}000$) (Table 1). The higher number of

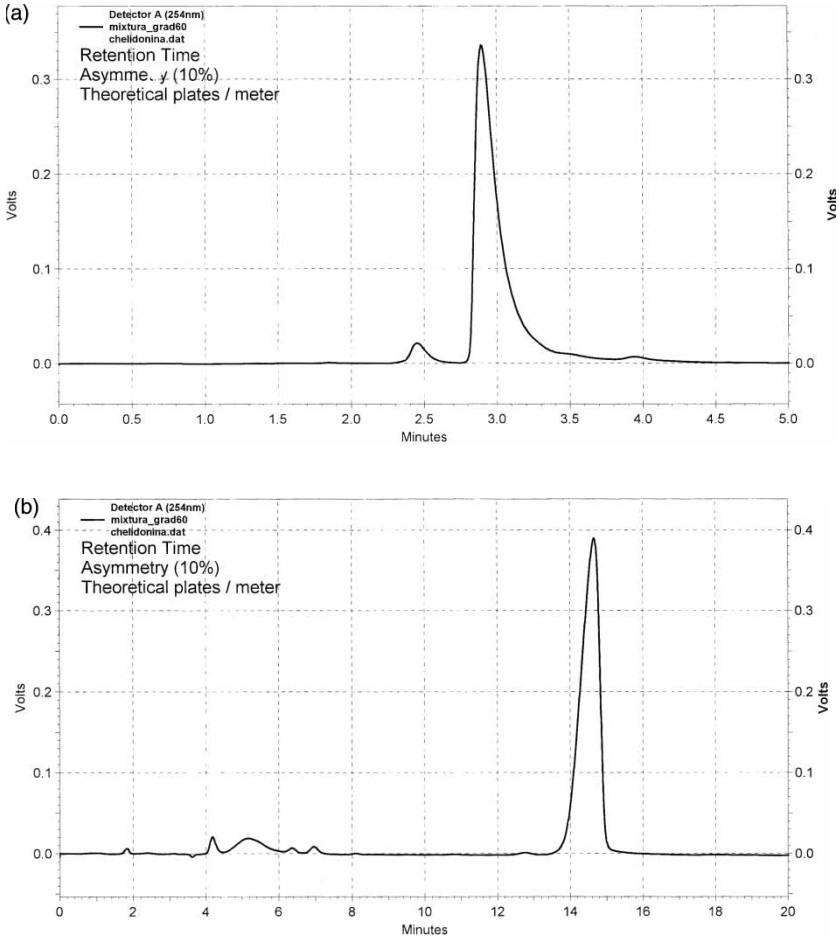

Figure 3. Comparison of peak profiles obtained on phenyl column in eluent systems containing: a) 70% MeCN + 20% phosphate buffer pH 3.5; b) 60% MeCN + 20% phosphate buffer pH 7.8; c) 30% MeCN + 20% phosphate buffer pH 3.5 + 0.025ML^{-1} OSA-Na; d) 40% MeCN + 20% phosphate buffer pH 3.5 + 0.021ML^{-1} SDS; e) 40% MeCN + 20% phosphate buffer pH 3.5 + 0.05ML^{-1} DEA; f) 40% MeCN + 1% NH_3.

(*continued*)

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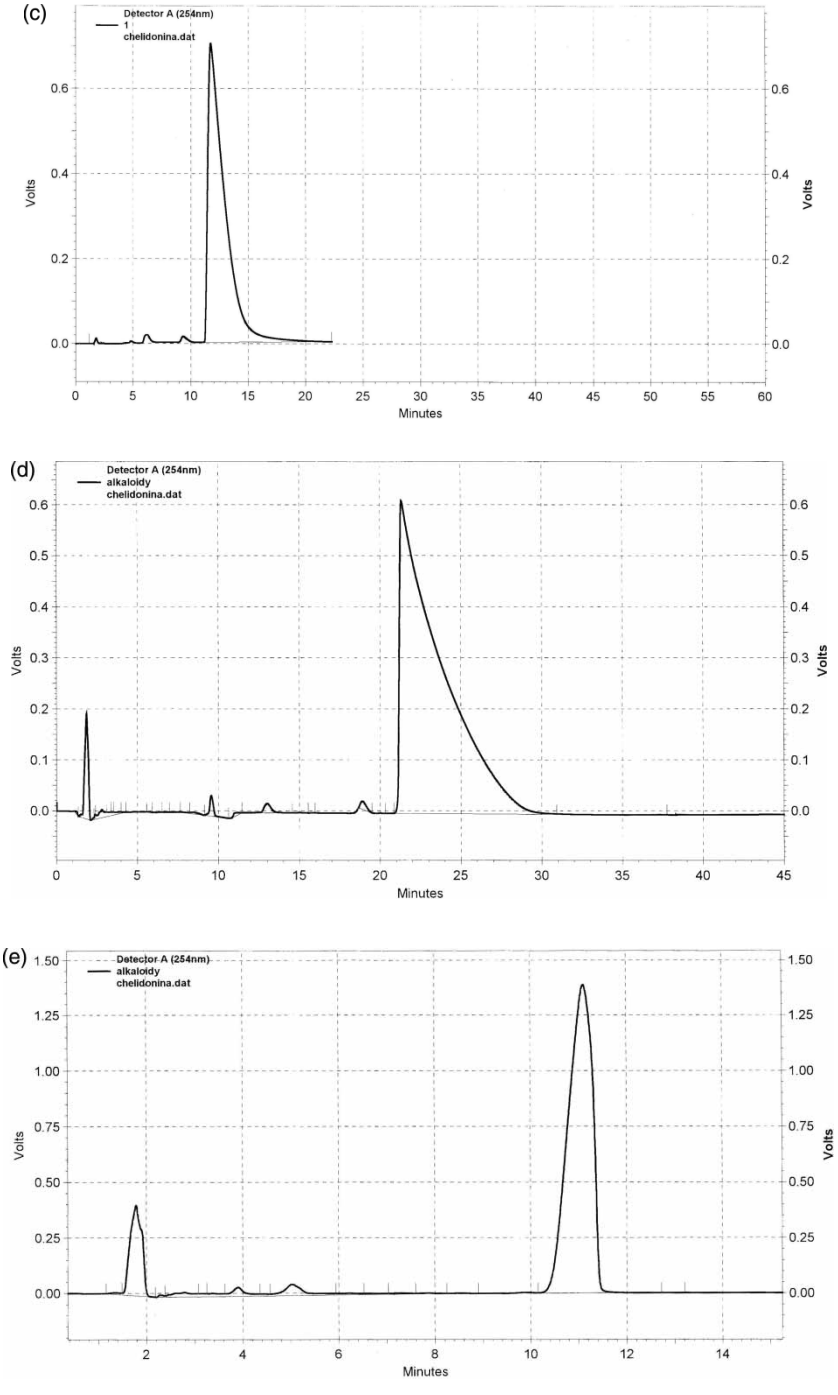

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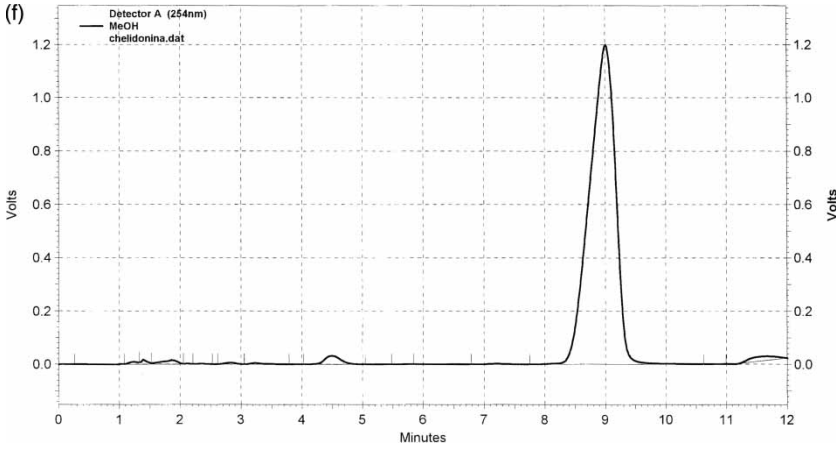

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symmetrical peaks was obtained in the eluent system with diethylamine for 16 investigated compounds, but the most efficient system was with buffer at pH 7.8, for 15 alkaloids N/m was higher than 10,000. The system with diethylamine in the eluent was more suitable for analysis of the group of isoquinoline alkaloids.

In order to compare the efficiency of different chromatographic systems, the chromatograms obtained for chelidonine on a phenyl-silica column in various eluents are presented in Figure 3. In the system containing phosphate buffer at pH 3.5 in mobile phase the peak is asymmetric and tailing. A more symmetric peak was obtained when a buffer at pH 7.8 in mobile phase was used. The most symmetric peak and highest theoretical plate number was obtained in the eluent system with diethylamine.

The influence of buffer at pH 3.5 and 7.8 on the retention and selectivity of separation of ion-pairing reagents, ammonia and diethylamine is shown as a graphic spectrum of log k values in Figure 4. The selectivity of separation of solutes and the sequence of the eluted compounds are different in systems containing various reagents in mobile phase. For example: santonine and strychnine are not separated in systems with OS-Na and with ammonia, but well separated in systems with SDS and DEA in eluents. Colchicine and boldine are well separated in systems with buffer at pH 3.5, SDS and DEA, but poorly separated in other systems. Satisfied selectivity of separation for most investigated compounds was obtained in systems with sodium dodecyl sulphate, ammonia, and, especially, with diethylamine.

In Figure 5 the chromatogram obtained for the mixture of 18 isoquinoline alkaloid standards on phenyl stationary phases is presented (eluent containing 0.05 m/L DEA in acetonitrile–buffered mobile phase). To

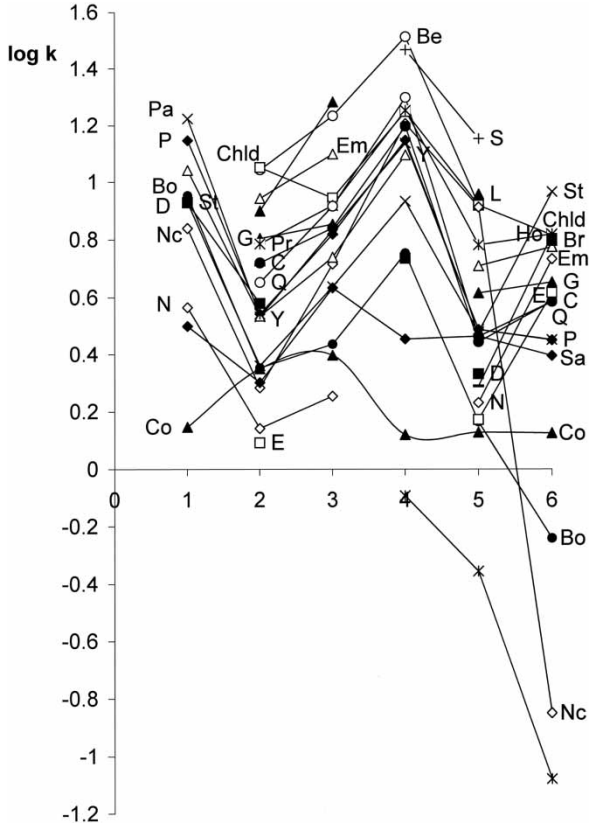

Figure 4. Comparison of log k values obtained on phenyl column in eluent systems containing: 1) 70% MeCN + 20% phosphate buffer pH 3.5; 2) 60% MeCN + 20% phosphate buffer pH 7.8; 3) 30% MeCN + 20% phosphate buffer pH 3.5 + $0.025 ML^{-1}$ OSA-Na; 4) 40% MeCN + 20% phosphate buffer pH 3.5 + $0.021 ML^{-1}$ SDS; 5) 40% MeCN + 20% phosphate buffer pH 3.5 + $0.05 ML^{-1}$ DEA; 6) 40% MeCN + 1% NH_3.

shorten analysis time, gradient elution was applied. It is seen that a good separation of the mixture was obtained. Therefore, this chromatographic system was used for the separation of alkaloids from plant extracts. In Figure 6, the chromatogram of *Fumaria officinalis* plant extract in the same system is presented, in which protopine and sanguinarine were intensified.

CONCLUSIONS

Unbuffered aqueous mobile phases are not useful for the analysis of alkaloids on phenyl columns. Selectivity of separation is very poor and peaks are asymmetric.

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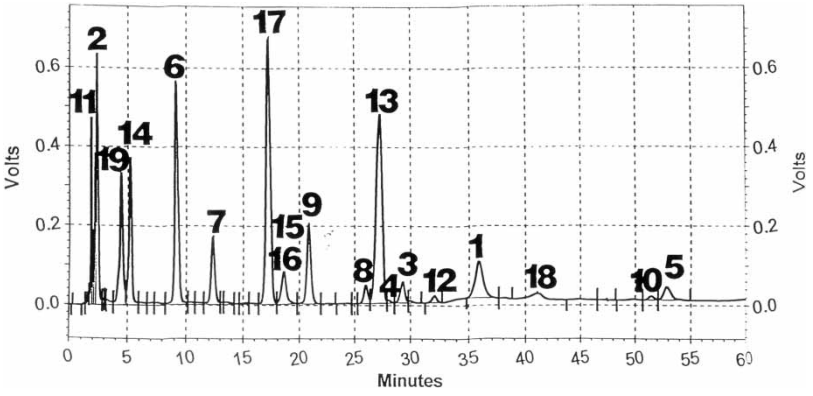

Figure 5. Chromatogram obtained in gradient elution system: 0–35 min 15–35% MeCN, 40–50 min 35–50% MeCN, 50–60 min 50% MeCN. Eluent containing 20% phosphate buffer at pH 3.5 and 0.05 ML^{-1} DEA for 18 isoquinoline alkaloid standards. Numbers indicate the following alkaloids: 1 – Be, 2 – Bo, 3 – Chld, 4 – Cheliluthine, 5 – Chelerithrine, 6 – Codeine, 7 – D, 8 – Em, 9 – G, 10 – Homochelidonine, 11 – Laudanosine, 12 – unidentified peak, 13 – Noscapine, 14 – Nc, 15 – P, 16 – Pa, 17 – Pr, 18 – S, 19 – T. (Abbreviations see Table 1.)

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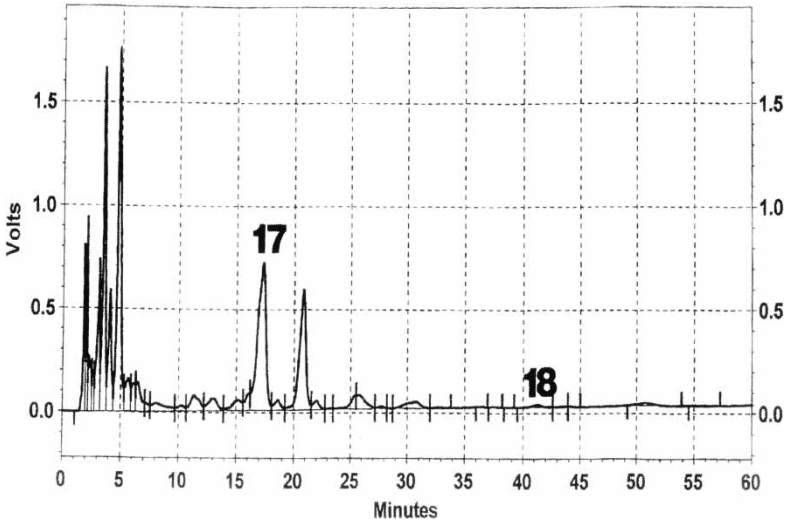

Figure 6. Chromatogram obtained in gradient elution system: 0–35 min 15–35% MeCN, 40–50 min 35–50% MeCN, 50–60 min 50% MeCN. Eluent containing 20% phosphate buffer at pH 3.5 and 0.05 ML^{-1} DEA for *Fumaria officinalis* extract. (Numbers as in Figure 5.)

The spread of asymmetry values for alkaloids on a given column is considerably reduced when buffered aqueous mobile phase at 7.8 was used.

Addition of ion-pair reagents to mobile phase does not lead to the improvement of peak symmetry, the selectivity of separation, and the increase of theoretical plate number.

The best efficiency and selectivity of separation for the investigated alkaloids was obtained in systems containing diethylamine as a silanol blocker in mobile phase.

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