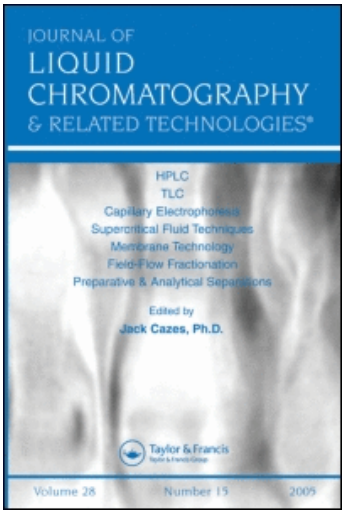

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Separation of Selected Bile Acids by TLC. I

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ABSTRACT

Seven of the selected bile acids were investigated: cholic acid (C), chenodeoxycholic acid (CDC), deoxycholic acid (DC), lithocholic acid (LC), glycocholic acid (GC), glycodeoxycholic acid (GDC), and glycolithocholic acid (GLC). The retardation factor (R_F), ΔR_F values, the separation factors (α), resolution factors (R_S), as well as the constants of the pair separation ($R_F{}^{\alpha}$), indicate that the mobile phase *n*-heptane–ethyl acetate–acetic acid about volume composition 25 : 20 : 8, is the best for separations of investigated bile acids on aluminum plates precoated silica gel 60 F_{254}.

Key Words: Bile acids; TLC.

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INTRODUCTION

The end products of cholesterol utilization are the bile acids. Bile acids are synthesized in the liver. The most abundant bile acids in human bile are chenodeoxycholic acid (CDC) and cholic acid (C). They are referred to as the primary bile acids. The primary bile acids are acted upon by bacteria and converted to the secondary bile acids. These secondary bile acids are identified as deoxycholic acid (DC) and lithocholic acid (LC). The primary and secondary bile acids are conjugated at the carboxylic group with either taurine or glycine. The conjugation of taurine and glycine increases polarity and solubility of the primary and secondary bile acids.[1–4] Bile acid synthesis has clinical significance. Bile acids perform important physiologically significant functions. In living organisms bile steroids play a role in the cholesterol balance and fat digestion or absorption. Therefore, the separation and the determination of bile acids in biological samples is very important for the diagnosis of many diseases.[1,5–22]

The aim of our study was to work out the optimum conditions of the separations of selected biological active substances, being bile acids, which were investigated by thin-layer chromatography (TLC) on aluminum plates of precoated silica gel 60 F_{254}.

EXPERIMENTAL

Chemicals

The components of mobile phases: *n*-heptane (Reachim, Russia), ethyl acetate (Chempur, Piekary Sląskie, Poland), and acetic acid (POCh, Gliwice, Poland) were for TLC analysis. The commercial samples of C, CDC, DC, LC, glycocholic acid (GC), glycodeoxycholic acid (GDC), and glycolithocholic acid (GLC) (St. Louis, Sigma Chemical Company, USA) were used as test solutes. Methanol (POCh, Gliwice, Poland; pure p.a.) was used for the preparation of solutions of bile acids. Sulfuric acid (Chempur, Piekary Sląskie, Poland) was used to prepare the visualizing reagent.

Sample Preparation

The methanolic solutions of the above-mentioned bile acids, concentration 5 mg/mL of each, were prepared.

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Thin Layer Chromatography

Adsorption TLC was performed on 20 × 20 cm aluminum plates precoated with 0.2 mm layers of silica gel 60 F_{254} (#5554, E.Merck). Before use the plates were activated at 120°C for 30 min. Microsynergy (10 μL, Hamilton) was used to apply the standard solutions to the plates. Solutions of the standard acids were spotted on a chromatographic plate in quantities of 50 μg of each standard in 10 μL methanol. The chromatograms were developed at room temperature in a 20 cm × 20 cm horizontal chamber (Camag, Switzerland) using *n*-heptane–ethyl acetate–acetic acid in various volume compositions of 20 : 25 : 5, 20 : 20 : 5, 22 : 20 : 5, 22 : 21 : 5, 22 : 22 : 5, 25 : 20 : 2, 25 : 20 : 5, and 25 : 20 : 8 as the mobile phases. Fifty milliliter of mobile phases were used in all cases. The development distance was 14 cm. The plates were dried at room temperature using a fume cupboard. The investigated bile acids were evaluated on plates using 10% solution of sulfuric acid in water as a visualizing reagent. The spots were developed by heating the sprayed plates at 120°C for 20 min.

Separation Factor α[23]

The separation factor (α) was calculated using equation:

$$\alpha = \frac{(1/R_{F1} - 1)}{(1/R_{F2} - 1)} \tag{1}$$

where R_{F1} and R_{F2} are R_F values of two adjacent spots; and $R_{F1} < R_{F2}$.

Resolution Factor R_S[23]

The resolution factor (R_S) was calculated using equation:

$$R_S = 2 \times \frac{a}{b} \tag{2}$$

where a is distance between the centres of two adjacent spots, and b is sum of the widths of the two spots in the direction of flow.

Constant of the Pair Separation $R_F{}^{\alpha}$[24,25]

The constant of the pair separation ($R_F{}^{\alpha}$) was calculated using the formula:

$$R_F{}^{\alpha} = \frac{R_{F1}}{R_{F2}} \tag{3}$$

where R_{F1} and R_{F2} are R_F values of two adjacent spots; and $R_{F1} > R_{F2}$.

RESULTS AND DISCUSSION

Chromatographic separations of studied bile acids ware characterized using R_F, ΔR_F values, as well as, separation factors α, separation factors R_S, and constants of the pair separation, R_F^{α}.

Dependence of R_F value of studied bile acids vs. various volume compositions of mobile phase: *n*-heptane–ethyl acetate–acetic acid was presented in Fig. 1. Different influences of volume composition of mobile phase (*n*-heptane–ethyl acetate–acetic acid) was observed in the change of R_F value of separated bile acids. The smallest influence of volume composition of applied mobile phase on the separation of GC and GDC acids was observed. A larger influence was in the case of C and GLC, and the largest influence of volume composition of mobile phase was observed for remaining acids, i.e., CDC, DC, and LC acids. Results presented in Fig. 1 indicate, that the acids: CDC, DC, and LC, do not separate from mobile phase in the volume

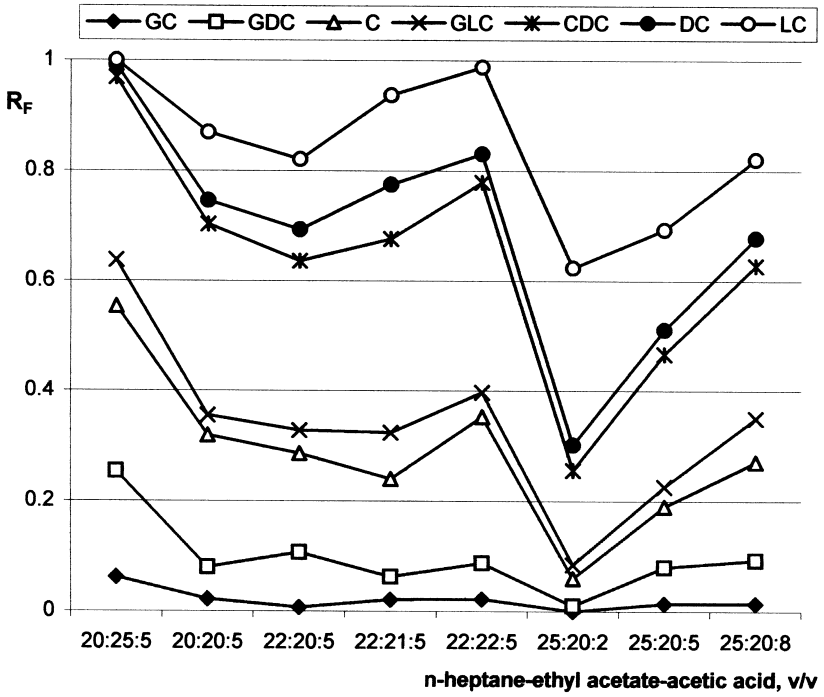

Figure 1. The dependence between R_F and the volume composition of the mobile phase: *n*-heptane : ethyl acetate : acetic acid.

composition of 20 : 25 : 5. Yet, the mobile phase in this volume composition best separates GC from GDC.

The order of elution of investigated bile acids at applied mobile phase: *n*-heptane–ethyl acetate–acetic acid (regardless of work volume composition of mobile phase) is following: LC, DC, CDC, GLC, C, GDC, and GC.

In the aim of finding optimum mobile phases to separate studied bile acids, differences between R_F values of neighboring spots of studied substances on the chromatogram were counted. ΔR_F values for particular pairs of neighboring substances on individual chromatograms were presented in Fig. 2, which were separated from mobile phase: *n*-heptane–ethyl acetate–acetic acid at different volume compositions. Separation of substances is satisfactory in the case, when $\Delta R_F \geq 0.05$. $\Delta R_F \geq 0.05$ were achieved for acids:

1. Glycocholic and GDC with all mobile phases, except for the mobile phase of volume compositions: 22 : 21 : 5 and 25 : 20 : 2.
2. Glycodeoxycholic and C using all mobile phases.
3. Cholic and GLC using only mobile phases with volume compositions: 20 : 25 : 5; 22 : 21 : 5 and 25 : 20 : 8.
4. Glycolithocholic and CDC using all mobile phases.
5. Chenodeoxycholic and DC using mobile phases with volume compositions: 22 : 20 : 5; 22 : 21 : 5; 22 : 22 : 5, as well as, 25 : 20 : 8.
6. Deoxycholic and LC using all mobile phases, except mobile phases with volume composition: 20 : 25 : 5.

These results indicate, that $\Delta R_F \geq 0.05$ were obtained for all pairs of investigated bile acids only with the use of *n*-heptane–ethyl acetate–acetic acid with volume composition 25 : 20 : 8 as mobile phase.

Neighboring spots of studied bile acids on chromatograms were additionally characterized by means of separation factors (α), resolution factors (R_S), and constants of the pair separation ($R_F{}^{\alpha}$). Values of separation factors R_S, α, and $R_F{}^{\alpha}$ for neighboring pairs of separated bile acids on chromatograms were presented in Figs. 3–5, respectively. Data in Fig. 3 indicate, that the values $R_S \geq 1$ for all pairs of studied bile acids were obtained using mobile phase with volume compositions 22 : 21 : 5, 22 : 22 : 5, as well as, 25 : 20 : 8. However, with the use of mobile phase of volume composition 22 : 22 : 5, a difference between R_F values of C and GLC is smaller than 0.05 ($\Delta R_{F(C/GLC)} = 0.044$). Similarly, with the use of mobile phase of volume composition 22 : 21 : 5, a difference between R_F values of GC and GLC is equal to 0.042. Therefore, R_S and ΔR_F values show, that optimum mobile phase for separation of studied bile acids is mobile phase: *n*-heptane–ethyl acetate–acetic acid with a volume composition of $25 + 20 + 8$. Moreover, results presented in Figs. 4 and 5 indicate, that the separation factors α and

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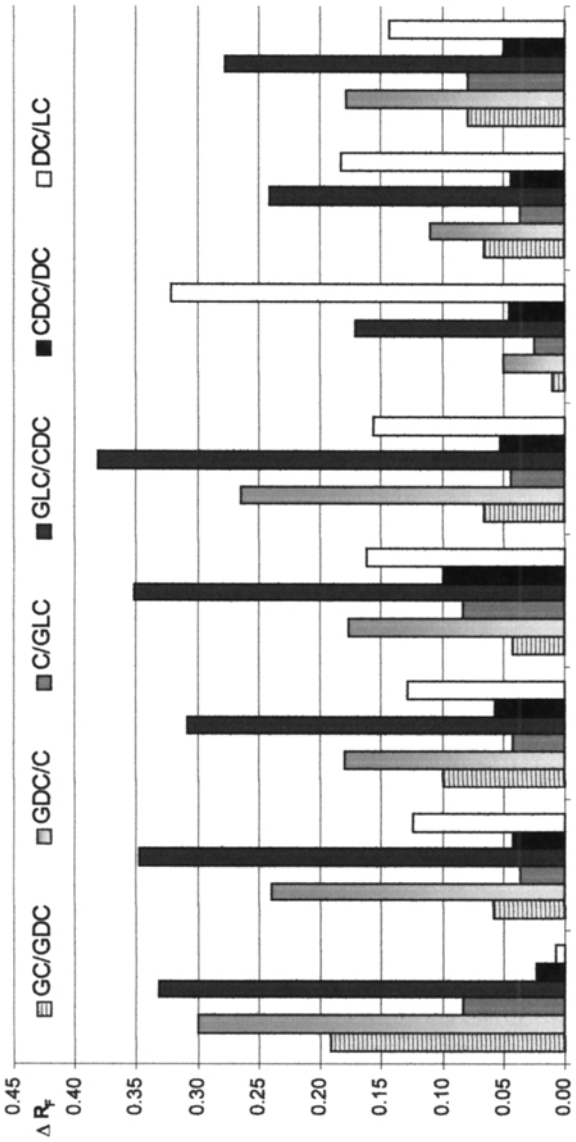

Figure 2. The ΔR_F values for particular pairs of neighboring bile acids on chromatograms separated from mobile phase: *n*-heptane : ethyl acetate : acetic acid different volume compositions.

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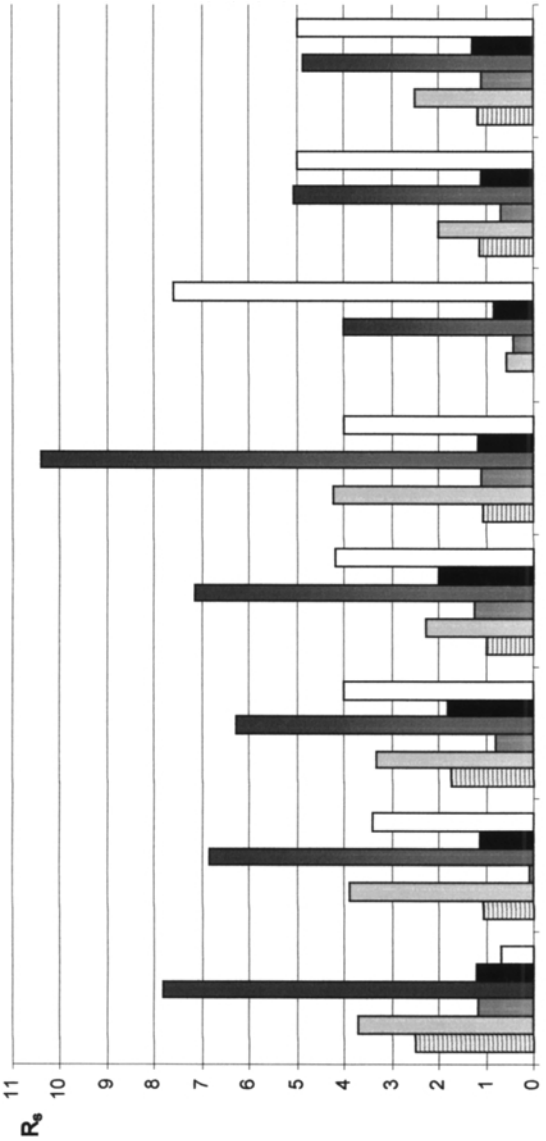

Figure 3. The resolutions factors (R_S) of bile acids separated from mobile phase: *n*-heptane : ethyl acetate : acetic acid with different volume compositions.

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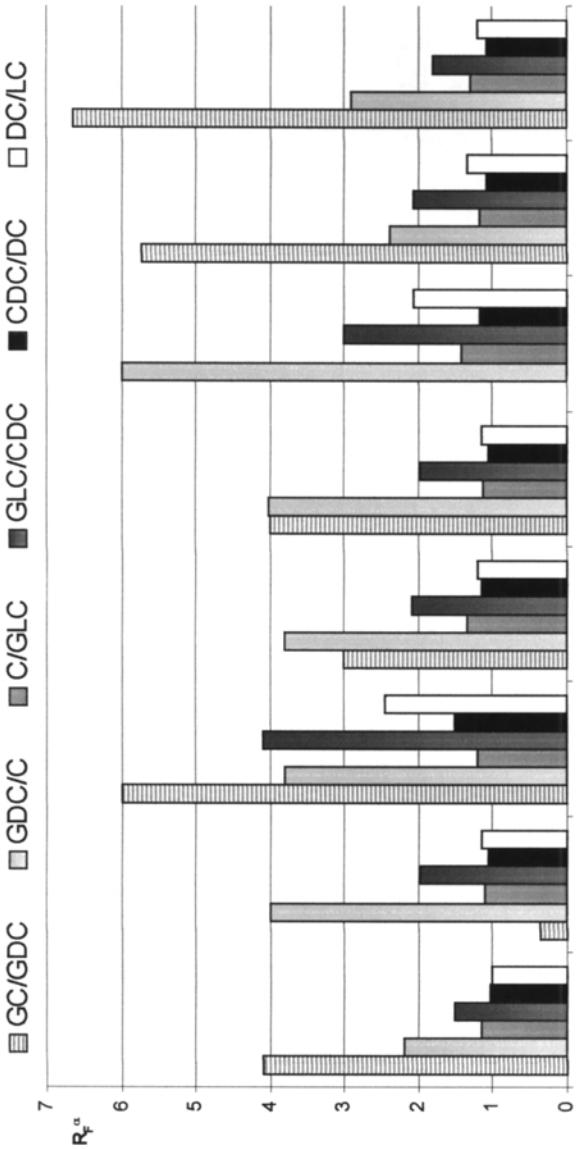

Figure 4. The constants of the pair separation (R_F^{α}) of bile acids separated from mobile phase: *n*-heptane : ethyl acetate : acetic acid and different volume compositions.

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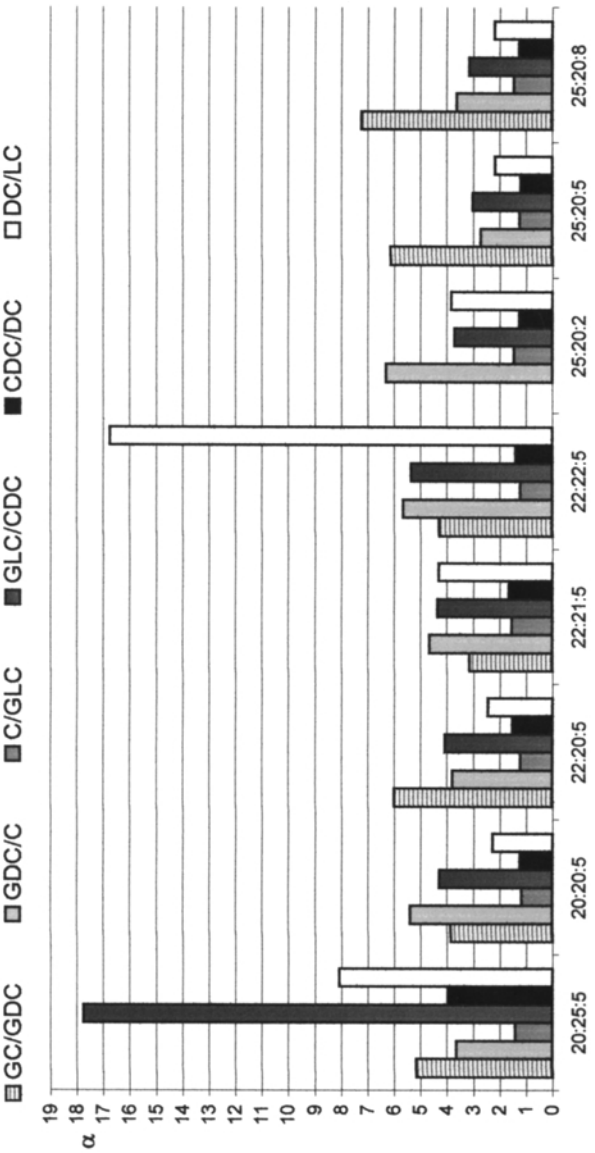

Figure 5. The separation factors (α) of bile acids separated from mobile phase: *n*-heptane : ethyl acetate : acetic acid in different volume compositions.

resolution factors R_F^{α} are larger than 1 for the chosen best mobile phase: *n*-heptane–ethyl acetate–acetic acid and volume composition 25 : 20 : 8 for all pairs of investigated bile acids.

Thus, these values of separation factors α, R_S, and R_F^{α} confirm that the choice of optimum mobile phase was correct. Moreover, Figs. 4 and 5 show, that on basis of values of separation factors, α and R_F^{α} there is little information of correct choices on the optimization of chromatographic separation. Therefore, the R_S and ΔR_F are parameters, which are most useful for evaluation of chromatographic separation of studied bile acids. Scanned chromatogram of separated bile acids using *n*-heptane–ethyl acetate–acetic acid of volume composition 25 : 20 : 8 as a mobile phase was presented in Fig. 6. However, R_F, ΔR_F values, as well as separation factors (α), resolution factors (R_S), and the constant pair separations (R_F^{α}), obtained using optimum mobile phases are listed in Table 1.

Szepesi et al.,[26] studied the separation of free and conjugated bile acids on silica gel, applying the one or two chromatographic developments in this same direction. Among these acids, were also ones, which were studied in the present work. They applied to single development of the mobile phase: isooctane–ethyl acetate–glacial acetic acid 25 : 20 : 5 (v/v). In these conditions, of the acids LC, DC, CDC, C, GLC, GDC, and GC, only GDC and GC were poorly separated ($\Delta R_{F_{(GC/GDC)}} = 0.05$). They were a small distance from the starting point. Thus, *n*-heptane–ethyl acetate–acetic acid 25 : 20 : 8 (v/v) is

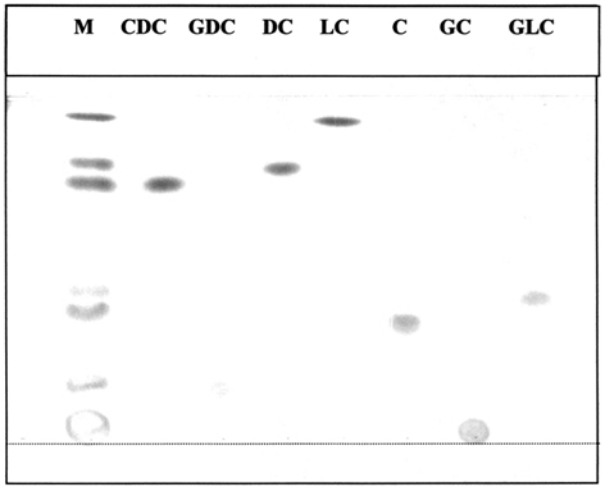

Figure 6. Scanned chromatogram of separated bile acids with use of *n*-heptane–ethyl acetate–acetic acid with volume composition 25 : 20 : 8.

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Table 1. R_F, ΔR_F, and separation factors values of selected bile acids separated with *n*-heptane–ethyl acetate–acetic acid, 25 : 20 : 8, v/v.

				Separation Factors		
Acid	Code	R_F	ΔR_F	α	R_S	R_F^{α}
Glycocholic	GC	0.014				
Glycodeoxycholic	GDC	0.093	$\Delta R_{F_{(GC/GDC)}} = 0.079$	$\alpha_{GC/GDC} = 7.228$	$R_{S_{GC/GDC}} = 1.158$	$R_F^{\alpha}{}_{GC/GDC} = 6.643$
Cholic	C	0.271	$\Delta R_{F_{(GDC/C)}} = 0.178$	$\alpha_{GDC/C} = 3.622$	$R_{S_{GDC/C}} = 2.500$	$R_F^{\alpha}{}_{GDC/C} = 2.914$
Glycolithocholic	GLC	0.350	$\Delta R_{F_{(C/GLC)}} = 0.079$	$\alpha_{C/GLC} = 1.449$	$R_{S_{C/GLC}} = 1.100$	$R_F^{\alpha}{}_{C/GLC} = 1.292$
Chenodeoxycholic	CDC	0.628	$\Delta R_{F_{(GLC/CDC)}} = 0.278$	$\alpha_{GLC/CDC} = 3.133$	$R_{S_{GLC/CDC}} = 4.875$	$R_F^{\alpha}{}_{GLC/CDC} = 1.794$
Deoxycholic	DC	0.678	$\Delta R_{F_{(CDC/DC)}} = 0.050$	$\alpha_{CDC/DC} = 1.247$	$R_{S_{CDC/DC}} = 1.273$	$R_F^{\alpha}{}_{CDC/DC} = 1.080$
Lithocholic	LC	0.821	$\Delta R_{F_{(DC/LC)}} = 0.143$	$\alpha_{DC/LC} = 2.178$	$R_{S_{DC/LC}} = 5.000$	$R_F^{\alpha}{}_{DC/LC} = 1.211$

best mobile phase chosen for the separation, because using it one obtained the complete separation of all of seven studied bile acids and $\Delta R_{F(GC/GDC)} = 0.079$.

Further investigations continue concerning separation of investigated bile acids on different chromatographic supports.

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