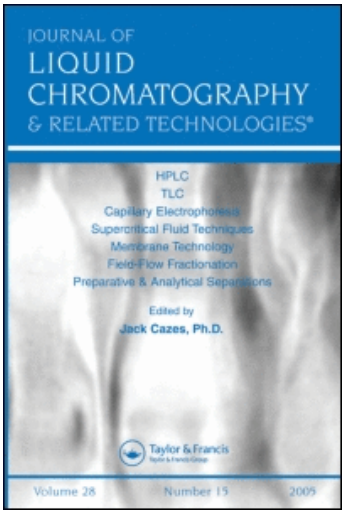

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TLC of Selected Bile Acids: Detection and Separation

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Abstract: The selected bile acids such as: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), lithocholic acid (LC) were separated by using thin layer chromatography on glass plates precoated with silica gel 60 with a concentrating zone. A robust and sensitive detection procedure for selected bile acids using the sulphuric acid as a visualizing reagent was described. Phosphomolybdic acid (10%) in ethanol was used as the comparative visualizing reagent. Spot intensities on the plates were quantified after dipping in sulphuric acid solutions and heating at temperatures from 60°C to 120°C for times ranging from 2 to 45 min. The best detection conditions for high signal intensity [AU] were determined. Particularly robust and sensitive detection of investigated bile acids separated was observed using the solution of sulphuric acid in methanol in the volume composition 1:19, and for temperature equal to 90°C and for heating for 20 min. Comparison and characterization of chromatographic spots of examined compounds on the basis of resolution (R_S), separation factor (α), constant of the pair separation (R_F^{α}), and ΔR_F values were discussed. The R_S parameter, serving to evaluate the substance separation, was determined by visual and densitometric methods. It was proven that the R_S parameter determined by the visual method for two adjacent substances is always larger than determined by the densitometric method. It was stated that the densitometric method is correct, objective, and assures standard conditions to the parameter R_S determination. All bile acids have been best separated only by developing with *n*-hexane-ethyl acetate –methanol-acetic acid in volume composition 20:20:5:2 as the mobile phase.

Keywords: Bile acids, Adsorption TLC, Spot visualization, Sulphuric acid, Separation, Densitometric analysis, Separation factors

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INTRODUCTION

Separation and quantification of bile acids (free, glycine and taurine-conjugated acids) from biological materials are very important diagnostic indicators of liver and gastro-intestinal diseases in humans.[1] Because of their structural similarities, separation of bile acids and their metabolites is difficult. Routinely, a diagnostic enzymatic method is used, because it is easy and quick to perform. Besides enzymatic methods, bile acids are analyzed mainly with the use of electrochemical and chromatographic techniques.[1] Chromatography represents the method of choice for detailed analyses of the bile acid profiles in biological materials.[2]

Separation evaluation of compounds by different chromatographic techniques is a subject of many scientific papers. Separation of a mixture in liquid chromatography depends on the selective retention of the migrating components in the column by the stationary phase. Retention, arising from selective interactions of each component with stationary phase, is effected by simultaneous migration with the flowing mobile phase.[3–7] The quality of obtained TLC separation is described by the values of retention parameters (R_F and R_M) and separation parameters (ΔR_F, R_S, α, R_F^{α}) in TLC.[8–14] The separation factor (α), constant of the pair separation (R_F^{α}) and ΔR_F, are calculated on the basis of the R_F values of the separated compounds. Only the resolution (R_S) takes into account the chromatographic spot broadening. Now and again, the R_S values are calculated by use of the visual method of a measurement.[9–11]

Spots of bile acids can be visualized by spraying the chromatographic plate with an anisaldehyde-sulphuric acid reagent (solution of 8 mL sulphuric acid, 0.5 mL anisaldehyde, 10 mL acetic acid, and 85 mL methanol),[15] a solution of antimony (III) chloride (Carr Price reagent) in chloroform (1:5, w/v),[15] a manganese (II) chloride-sulphuric acid reagent (solution of 0.2 g manganese (II) chloride, 30 mL water, 30 mL methanol, and 2 mL sulphuric acid)[20] or a 10% or 50% water solution of sulphuric acid and then heating until the spots became visible.[16,17] Bile acids can be also detected by dipping plates into 10% of phosphomolybdic acid in ethanol and then heating for 20 min at 120°C[15,17,18] or by spraying the plates with a 1% solution of phosphomolybdic acid in 2-propanol and then heating at 120°C for 5–10 min.[19] Unconjugates and conjugates of chenodeoxycholic acid and deoxycholic acid were detected by spraying with 20% phosphomolybdic acid in methanol and heating at 110°C.[20] Wardas and Jedrzejczak[21] separated selected free and conjugated bile acids by NP-TLC. Eleven visualizing agents were used for detection of these investigated bile acids. The best results of detection of bile acids were obtained with bromocresol blue.[21] Sulphuric acid is used as a general visualizing reagent in TLC, in particular for sterols, steroids, bile acids, and gibberellins.[22] Selected bile acids were detected by spraying with 30% sulphuric acid in ethanol and heating at 110°C[23] or at 100°C.[24]

The separation evaluation of different groups of organic compounds has been the subject of our numerous investigations.[9–11,25–32] The aim of this work was:

- the development a method of optimal detection conditions of selected bile acids using methanolic solutions of sulphuric acid;
- the comparison of separation effects of selected bile acids on thin layer chromatography by the use of the visual and densitometric method.

The subjects of our study were the selected bile acids: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC).

EXPERIMENTAL

Chemicals

The following components of the mobile phase n-hexane (Merck, Germany), ethyl acetate (POCh, Gliwice, Poland), acetic acid 99.5% (POCh, Gliwice, Poland), and methanol (Merck, Germany) were used for the adsorption TLC analysis. The commercial samples of C (lot: 94F-0213), DC (lot: 51H0458), CDC (lot: 68F0868), LC (lot: 24F-0325), GLC (lot: 52H7821), GDC (lot: 70H505015), and GC (lot: 20H5011) (St. Louis, Sigma Company, USA) were used as test solutes. Ethanol (96%, POCh, Gliwice, Poland; pure p. a.) was used for the preparation of bile acids solutions. Sulphuric acid, 95% (Chempur, Piekary Śląskie, Poland) and phosphomolybdic acid (POCh, Gliwice, Poland) were used to prepare the visualizing reagents.

Sample Preparation

The ethanolic solution of the above mentioned bile acids in the concentration 0.7 mg/1 mL of each acid was prepared.

Thin Layer Chromatography

Adsorption TLC was performed on 20 × 20 cm glass plates precoated with silica gel 60 with concentrating zone (E. Merck, #1.11845, lot: 9615964). The plates were prewashed with methanol and dried for 24 h at room temperature (18 ± 1°C). Before use, the plates were activated at 120°C for 30 min. Micropipettes (Camag, Switzerland) were used to apply the standard solutions to the plates. The solution of the standard acids were manually

spotted on a chromatographic plate in the quantity 0.7 μg of each standard in 1 μL ethanol. The plates were developed at room temperature (18 ± 1°C) in a classical chamber (Camag, Switzerland) previously saturated with mobile phase. A mixture of *n*-hexane-ethyl acetate-acetic acid and *n*-hexane-ethyl acetate-methanol-acetic acid in various volume compositions were used as the mobile phases. Of the mobile phases, 50 mL were used in all cases. The development distance was 14 cm. The plates were dried at room temperature using a fume cupboard.

Visualizing Reagents

Sulphuric Acid

The investigated bile acids were evaluated on the plates using the solution of: sulphuric acid in methanol in different volume compositions (1:10, 1:11, 1:12, 1:13, 1:14, 1:15, 1:16, 1:17, 1:18, 1:19, and 1:20) as visualizing reagents. The dried plate was dipped in particular sulphuric acid solutions for 15 s. It was then heated at temperatures from 60 to 120°C for times ranging from 2 to 45 min.

Phosphomolybdic Acid[15]

Ten percent phosphomolybdic acid in ethanol was used as the comparative visualizing reagent. After spraying by phosphomolybdic acid solution, the plate was heated at 120°C for 20 min.[15]

Spectrodensitometric and Densitometric Analysis

The spectrum was recorded using a Camag Scanner TLC 3, operated in absorbance mode and controlled by winCATS 1.4.2 software. The radiation sources were a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm and a tungsten lamp emitting a spectrum between 370 and 800 nm. Start wavelength was 200 nm and end wavelength was 700 nm. The slit dimensions were 12.00 × 0.90 mm, Macro; the optimized optical system was resolution; the scanning speed was 20 nm s^{-1}; the data resolution was 1 nm $step^{-1}$; the measurement type was remission; and the measurement mode was absorption; the optical filter was second order.

Densitometric scanning was then performed at multi wavelength in the range of 380 to 460 nm, at change of wavelength at every step 20 nm. The radiation sources were a deuterium lamp emitting a continuous UV spectrum between 190 and 450 nm and a tungsten lamp emitting a spectrum of between 370 to 800 nm. The slit dimensions were 12.00 × 0.90 mm, Macro; the optimized optical system was light; the scanning speed was

20 mm s^{-1}; the data resolution was 100 μm $step^{-1}$; the measurement type was remission; and the measurement mode was absorption; the optical filter was second order. Each track was scanned three times and baseline correction (lowest slope) was used.

Separation Factors

The chromatograms were done in triplicate and each track was scanned three times, and the mean of the R_F values were calculated. Next, the R_F values were recalculated on R_M values.

The separation factors, namely: ΔR_F values, selectivity (α),[8] and constant of the pair separation (R_F^{α})[12] were calculated for all the densitograms.

ΔR_F was calculated according to the formula:

$$\Delta R_{F(1,2)} = R_{F1^{-}RF2} \tag{1}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

The selectivity (α) was calculated using the equation:

$$\alpha = \frac{1/R_{F1} - 1}{1/R_{F2} - 1} \tag{2}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} < R_{F2}$.

The constant of the pair separation (R_F^{α}) was calculated for the investigated compounds as the ratio of the R_F values of the two adjacent peaks on the densitogram:

$$R^{\alpha}_{F(1,2)} = \frac{R_{F1}}{R_{F2}} \tag{3}$$

where R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram; and $R_{F1} > R_{F2}$.

Resolution Factors

Visual Method of R_S Calculation

The visual method of the R_S calculation was based on the chromatographic parameters obtained directly from the chromatogram. The resolution of two spots ($R_{S(c)}$) was calculated using the formula:[8]

$$R_{S(c)} = 2 \times \frac{d}{s} \tag{4}$$

where d is the distance between the centers of two adjacent spots on the chromatogram, and s is the sum of the widths of the two spots in the direction of flow of mobile phase.

Densitometric Method of R_S Calculation

The peak resolution ($R_{S(b)}$) was calculated using the equation:[13,14]

$$R_{S(b)} = \frac{2d}{w_{b1} + w_{b2}} \tag{5}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{b1} and w_{b2} are the peaks width at the base.

The peak resolution ($R_{S(h)}$) was also calculated using the equation:[13]

$$R_{S(h)} = \frac{d}{w_{h1} + w_{h2}} \sqrt{\ln 4} \tag{6}$$

where d is the distance between the centers of two adjacent peaks on the densitogram, whereas w_{h1} and w_{h2} are the peaks width at half height.

The average values of peak resolution ($R_{S(a)}$) were also calculated according to the formula:

$$R_{S(a)} = \frac{R_{S(b)} + R_{S(h)}}{2} \tag{7}$$

RESULTS AND DISCUSSION

The separations of bile acids: cholic acid (C), glycocholic acid (GC), glycolithocholic acid (GLC), deoxycholic acid (DC), chenodeoxycholic acid (CDC), glycodeoxycholic acid (GDC), and lithocholic acid (LC) on silica gel 60 with concentrating zone and by use of n-hexane-ethyl acetate-acetic acid and n-hexane-ethyl acetate-methanol-acetic acid in different volume compositions were investigated. It was affirmed that *n*-hexane-ethyl acetate-methanol-acetic acid mobile phase in the volume composition 20:20:5:2 is optimum for the separation of the investigated bile acids.

The solutions of sulphuric acid in methanol in different volume compositions were used to detect investigated bile acids. Chromatographic plates with separated bile acids were dipped in particular sulphuric acid solutions and then heated at temperatures from 60°C to 120°C for times ranging from 2 to 45 min. The best detection conditions for high signal intensity [AU] of the separated bile acid spots were determined. Particularly robust and sensitive detection of investigated bile acids separated was observed using the solution of sulphuric acid in methanol in the volume composition 1:19

and for temperature equal to 90°C and at heating for 20 min. However, phosphomolybdic acid was used as the comparative visualizing reagent for the detection of studied bile acids. The absorption maximum of separated bile acids on silica gel 60 with concentrating zone after application of methanolic solution of sulphuric acid in volume composition 1:19 and heating at 90°C for 20 min and of phosphomolybdic acid as visualizing reagents are presented in Table 1. Colours of chromatographic spots of separated bile acids are also presented in Table 1.

The $R_{S(c)}$ values of separated bile acids were calculated on the basis of chromatographic spots by the use of a visual method using Equation (4). The retention parameter R_M and the separation factors ΔR_F, and selectivity α were calculated from the R_F values. The retention parameters R_F and R_M, and separation parameters ΔR_F, α, R_F^{α} and $R_{S(c)}$ are presented in Table 2. The $R_{S(c)}$ values obtained by the visual method are better for separated bile acids after their optimal detection using sulphuric acid in relation to the detection by the use of phosphomolybdic acid. Moreover, it was affirmed, that the background of a chromatogram after detection with phosphomolybdic acid is heterogeneous, which is the cause of appearance of large noises on densitograms. Therefore, phosphomolybdic acid as visualizing reagent under these conditions can be used only for qualitative or semi-quantitative investigations of investigated bile acids. Particularly robust and sensitive detection of investigated bile acids separated was observed using the solution of sulphuric

Table 1. Absorption maximum of investigated bile acids after separation on silica gel 60 and after the application of sulphuric acid[a] and phosphomolybdic acid[b] as visualizing reagents

	λ_{max} (nm)	
	After application of visualizing reagent	
Symbol of bile acid	Sulphuric acid (colour of spot on light beige background)	Phosphomolybdic acid (colour of spot on yellow/green background)
GC	458 (grey/green)	346 (navy blue)
GDC	393 (grey/blue)	345 (navy blue)
GLC	397 (grey)	346 (navy blue)
C	457 (green)	346 (navy blue)
CDC	379 (grey)	347 (navy blue)
DC	386 (green)	350 (navy blue)
LC	380 (grey)	350 (navy blue)

[a]Sulphuric acid in methanol in volume composition 1:19; the plate was immersed in dipping solution of sulphuric acid for 15 s, and it was then heated to 90°C for 20 min.

[b]10% Phosphomolybdic acid in ethanol; after spraying by phosphomolybdic acid solution, the plate was heated at 120°C for 20 min.

Table 2. R_F, R_M, separation parameters (ΔR_F, R_F^{α}, α), and $R_{s(c)}$ values obtained by visual method after the separation using n-hexane-ethyl acetate-methanol-acetic acid, 20:20:5:2 (v/v/v/v) and using the sulphuric acid solution[a] and phosphomolybdic acid[b] solution as visualizing reagents

	Retention parameters		Separation parameters				
						$R_{S(c)}$ values obtained by visual method after detection by use of	
Symbol of bile acid	R_F	R_M	ΔR_F	R_F^{α}	α	Sulphuric acid	Phosphomolybdic acid
GC	0.06	1.195					
GDC	0.13	0.826	0.07	2.167	2.34	5.00	5.20
GLC	0.34	0.288	0.21	2.615	3.45	8.29	6.40
C	0.42	0.140	0.08	1.235	1.41	2.40	1.54
CDC	0.73	−0.432	0.31	1.738	3.66	9.78	6.40
DC	0.78	−0.550	0.05	1.068	1.34	2.00	1.33
LC	0.94	−1.195	0.16	1.205	4.42	7.50	3.71

[a]Sulphuric acid in methanol in volume composition 1:19; the plate was immersed in dipping solution of sulphuric acid for 15 s, and it was then heated to 90°C for 20 min.
[b]10% Phosphomolybdic acid in ethanol; after spraying by phosphomolybdic acid solution, the plate was heated at 120°C for 20 min.

acid in methanol in the volume composition 1:19 and for temperature equal to 90°C and at heating for 20 min. In a further part of this work, densitometric analysis of the examined bile acids after this detection condition were performed. Densitometric scanning was then performed at multi wavelength in the range of 380 to 460 nm, with wavelength change at every step 20 nm. A three dimensional densitogram of investigated substances at different wavelengths (380, 400, 420, 440, and 460 nm) is presented in Figure 1. The resolution of peaks $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ were calculated from the Equations (5), (6), and (7) by the use of the obtained densitometric bands for the studied pairs of compounds at particular wavelengths. Those values are presented in Table 3. It was affirmed that $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ values calculated on the basis of the densitograms are considerably lower than the $R_{S(c)}$ values calculated using the visual method on the basis of the chromatograms. This shows that R_S values can be correctly marked exclusively on the basis of the densitograms. The scientific literature data indicate that at R_S values higher than 1.5 we can expect the complete separation of the neighboring compounds on the densitograms. It was shown that resolutions $R_{S(b)}$, $R_{S(h)}$, and $R_{S(a)}$ value larger than 1.5 were obtained at all analyzed wavelengths for the studied pairs of substances GC/GDC, GDC/GLC, C/CDC, and DC/LC. These conditions did not provide for the complete separation of

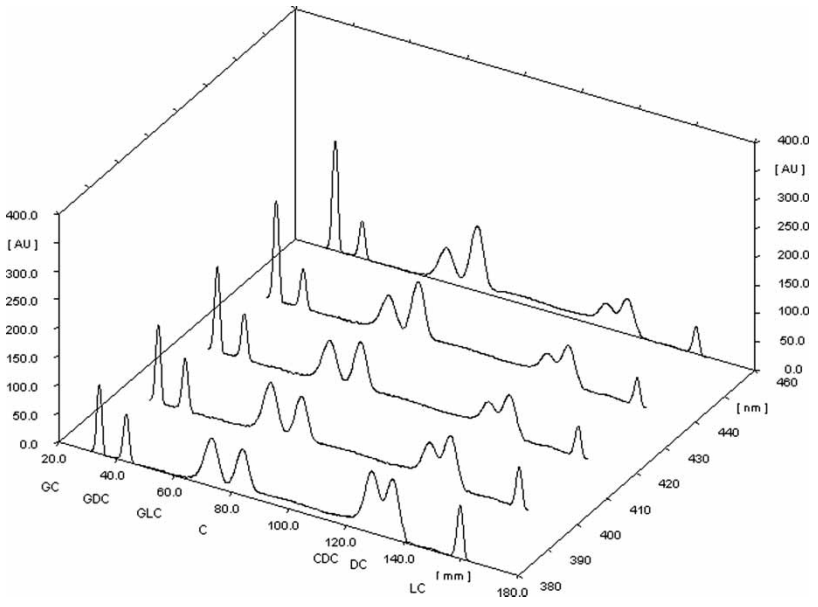

Figure 1. The densitograms of bile acids investigated (C–cholic acid, GC-glycocholic acid, GLC-glycolithocholic acid, DC-deoxycholic acid, CDC-chenodeoxycholic acid, GDC-glycodeoxycholic acid, LC-lithocholic acid) at wavelengths 380, 400, 420, 440 and 460 nm after their separation using a *n*-hexane–ethyl acetate-methanol–acetic acid, 20:20:5:2 (v/v/v/v) as mobile phase and after the application of sulphuric acid in methanol (1:19, v/v; the plate was immersed in dipping the solution of sulphuric acid for 15 s, and it was then heated to 90°C for 20 min) as visualizing reagent.

the pair of substances GLC/C and CDC/DC. The characteristics of the obtained densitometric bands are also presented in Table 3. The characteristic of the chromatographic band was realized using the densitometric method by determination of peak height, peak area, and the angle (β) between the tangents at the inflection points to the curves of the densitometric peak. From the obtained data, it is apparent that the band of glycocholic acid (GC) has the lowest numerical value of angle β. This shows that the band of glycocholic acid (GC) is compact in spite of its large area. However, the heights and areas of chromatographic band obtained at different wavelengths have differentiated values. The densitometric bands with the largest area were obtained at wavelengths in the neighborhood of absorption maximum (λ_{max}) for particular substances investigated.

Analysis of chromatographic bands was performed not by visual but by their densitometric characteristic, which is a supplementary element of the separation effect evaluation. Each visual evaluation is subjective and not too precise in relation to the densitometric method. Only the densitometric

Table 3. Values of resolutions (R_S) obtained by densitometric method and characteristic of densitometric bands at various wavelength of investigated bile acids by NP-TLC technique using *n*-hexane – ethyl acetate-methanol – acetic acid, 20:20:5:2 (v/v/v/v) as mobile phase and after application of sulphuric acid[a] as visualizing reagent

Symbol of compound	R_S values calculated by use of Eqs.			Characteristic of densitometric band		
	$R_{S(b)}$ (5)	$R_{S(h)}$ (6)	$R_{S(a)}$ (7)	Height (AU)	Area (AU)	$\beta(^\circ)$
$\lambda = 380$ nm						
GC				120	2507	4.0
GDC	2.31	2.35	2.33	82	2002	6.0
GLC	4.31	4.41	4.36	84	5127	16.0
C	1.18	1.22	1.20	80	4581	17.0
CDC	4.42	4.45	4.44	107	6837	16.0
DC	0.68	0.67	0.68	102	5618	15.0
LC	3.17	3.26	3.22	93	2353	7.0
$\lambda = 400$ nm						
GC				136	2929	3.0
GDC	2.44	2.35	2.40	92	2254	6.0
GLC	4.24	4.12	4.18	93	5752	15.0
C	1.16	1.13	1.15	84	4854	15.0
CDC	4.42	4.45	4.44	69	4397	25.0
DC	0.69	0.66	0.68	92	5162	15.0
LC	2.70	2.46	2.58	70	1750	8.0
$\lambda = 420$ nm						
GC				149	3286	3.0
GDC	2.20	2.16	2.18	78	1952	6.5
GLC	4.12	4.12	4.12	76	4745	20.0
C	1.13	1.13	1.13	88	5200	15.0
CDC	4.42	4.30	4.36	49	3207	30.0
DC	0.64	0.63	0.64	71	4036	20.0
LC	2.82	2.82	2.82	52	1283	16.0
$\lambda = 440$ nm						
GC				171	3909	2.0
GDC	2.22	2.14	2.18	69	1704	8.0
GLC	4.12	4.12	4.12	64	4153	23.5
C	1.13	1.13	1.13	104	6218	18.5
CDC	4.42	4.30	4.36	44	2951	35.0
DC	0.64	0.61	0.63	68	3854	20.0
LC	3.29	3.30	3.30	46	1155	12.0
$\lambda = 460$ nm						
GC				194	4528	3.0
GDC	2.32	3.54	2.93	67	1671	11.0
GLC	4.00	3.92	3.96	64	4096	32.0

(*continued*)

Table 3. Continued

Symbol of compound	R_S values calculated by use of Eqs.			Characteristic of densitometric band		
	$R_{S(b)}$ (5)	$R_{S(h)}$ (6)	$R_{S(a)}$ (7)	Height (AU)	Area (AU)	$\beta(^\circ)$
C	1.11	1.06	1.09	118	7095	15.0
CDC	4.24	4.16	4.20	44	2958	48.0
DC	0.57	0.59	0.58	64	3603	37.0
LC	2.95	3.14	3.05	49	1223	17.0

[a]Sulphuric acid in methanol in volume composition 1:19; the plate was immersed in dipping solution of sulphuric acid for 15 s, and it was then heated to 90°C for 20 min.

method can be used for the objective evaluation of the separation effect and characteristic of particular chromatographic bands.

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