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# Retention and separation studies of cholesterol and bile acids using thermostated thin-layer chromatography

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**Abstract**

The influence of temperature on retention and separation of cholesterol and bile acids, using reversed-phase thin-layer chromatography, was studied. As mobile phases methanol–water mixtures of various compositions were used. Chromatographic experiments were performed using vapor-saturated chambers at temperatures ranging from 5 to 60°C. A linear relationship between $R_M$ values and temperature $(1/T)$ as well as mobile phase composition was observed. The elution order of steroids under the conditions investigated was discussed. Each chromatogram was evaluated using simple optimization parameters and the best chromatographic conditions for the separation of multicomponent samples were chosen. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Mobile phase composition; Temperature effects; Optimization; Cholesterol; Bile acids

## 1. Introduction

Bile acids are well-known metabolites of cholesterol. This class of compounds can form micellar structures with various organic substances [1,2]. As size, charge and shape of structures formed are strongly dependent on the physicochemical properties of bile steroids, these supramolecular complexes have been studied extensively [3,4]. In living organisms bile steroids play a main role in the cholesterol balance and fat digestion or absorption. Therefore, determination of bile acids and their metabolites in biological samples is becoming increasingly important for the diagnosis of several diseases and disorders [5–9]. Although various separation techniques such as gas chromatography [10], liquid chromatography [11] and capillary electrophoresis [12] are commonly applied for the determination of bile steroids, thin-layer chromatography (TLC) still offers practical advantages [13]. The main advantage of the TLC technique results from its simplicity, inexpensive equipment needed and ease of operation. In addition, a large series of samples can be analyzed simultaneously and within short periods of time. Due to low absorption of bile acids in the UV region, TLC is a very attractive method for quantification of these compounds in biological samples [14–16]. It is noteworthy that separation and quantification can be directly performed in biological fluids without earlier sample purification [17–19].

From a chromatographic point of view, bile steroids form a non-homogeneous group of solutes and therefore many problems for a good separation of multicomponent mixtures were observed [19]. Numerous papers have reported the influence of

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several conditions on the separation and retention of bile steroids, e.g., type of stationary phase, mobile phase compositions, eluent pH and ionic strength as well as concentration of non-chiral or chiral modifiers [13,19,20]. Nevertheless, the influence of temperature has been studied rarely [6,12,21], especially using TLC [22]. In general, the investigation of temperature optimization is still in progress and usually it is not known how temperature selectivity varies with sample type and how other separation conditions affect temperature selectivity [23–27].

The aim of this work is a systematic temperature–retention and temperature–separation study of cholesterol and bile steroids using a wide range of methanol–water mobile phases and water-wettable RP-18 plates.

## 2. Experimental

### 2.1. Reagents

Steroids (Table 1) as well as methanol and 2-propanol (99.9%, HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Phosphomolybdic acid hydrate was a product of Merck (Darmstadt, Germany). All reagents were used as obtained.

### 2.2. Chromatography

Chromatographic experiments were performed on wettable with water RP-18W HPTLC plates from Merck. The chromatographic chambers (110 mm× 60 mm wide×15 mm deep) were saturated with the vapor of the mobile phase under 1 atm pressure (1 atm=101 325 Pa). Chromatographic experiments were performed at the temperatures 5, 10, 20, 30, 40, 50 and 60°C, controlled by circulating water from a thermostat with an accuracy of ±0.5°C.

Table 1
List of solutes investigated

| No. | Steroid | Abbreviation |
|---|---|---|
| 1 | Taurodeoxycholic acid, sodium salt | NaTDC |
| 2 | Glycocholic acid | GCA |
| 3 | Glycocholic acid, sodium salt | NaGC |
| 4 | Glycodeoxycholic acid | GDCA |
| 5 | Glycodeoxycholic acid, sodium salt | NaGDC |
| 6 | Cholic acid | CA |
| 7 | Cholic acid, sodium salt | NaC |
| 8 | Deoxycholic acid | DCA |
| 9 | Deoxycholic acid, sodium salt | NaDC |
| 10 | Chenodeoxycholic acid | CDCA |
| 11 | Chenodeoxycholic acid, sodium salt | NaCDC |
| 12 | Lithocholic acid | LCA |
| 13 | Cholesterol | |

Stock solutions of steroids were prepared in pure methanol at a concentration of 1 mg $ml^{-1}$. From stock solutions 1.5-μl samples were transferred to the plate start points with a micropipette (1.5 μg of each steroid was chromatographed). After drying, plates were placed in chambers and thermostated 25 min before development in order to obtain proper temperature and saturation equilibrium. When a new temperature was chosen, the chromatographic device was thermostated at least 30 min. The $R_F$ and $R_M$ values were calculated in the usual manner and are based on the average of at least five independent determinations of each solute.

Methanol–water mobile phases were prepared with 0 to 100% methanol (10%, v/v, steps) using twice distilled water. Prior to use mobile phases were filtered through a 1.5-μm membrane.

Steroids were visualized by spraying the plates with a 1% solution of phosphomolybdic acid in 2-propanol and then heating at 120°C for 5–10 min. After this time the solutes were visualized as navy blue spots on a yellow background.

## 3. Results and discussion

### 3.1. Retention study

As model compounds for chromatographic investigations cholesterol and bile acids series were chosen (Fig. 1). Bile acids were studied both in the form of acid and salt with the exception of taurodeoxycholic and lithocholic acids (Table 1). The influence of two factors changed simultaneously, i.e., temperature and mobile phase composition, on the chromatographic behavior of steroids was examined. Generally, as can be seen from the data presented in Table 2, nearly linear relationships between the retention parameter ($R_M$) and the reciprocal of the temperature were observed. Slopes of Van 't Hoff plots for all investigated steroids are

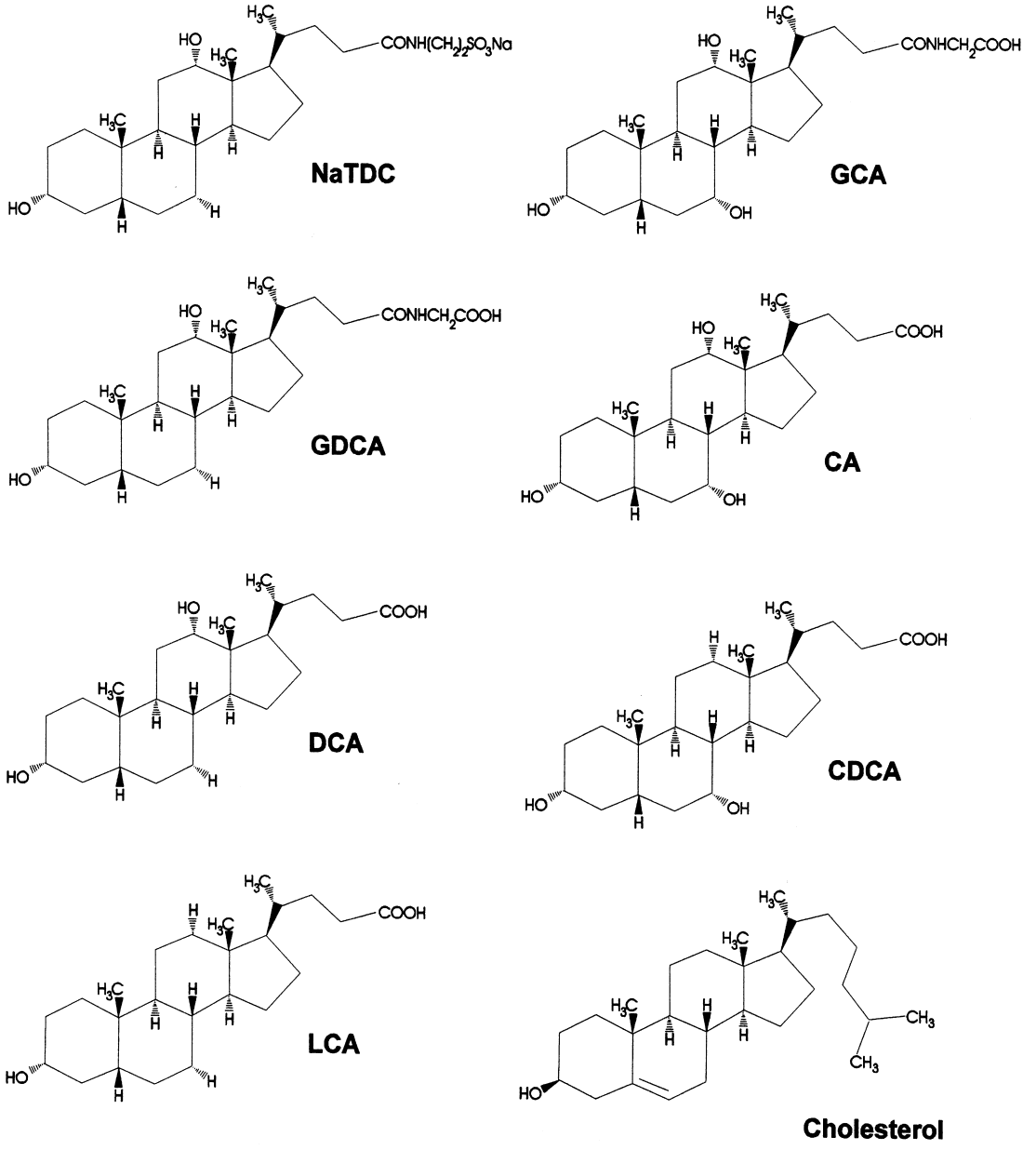


Fig. 1. Chemical structures of investigated steroids.

relatively small in comparison with macrocyclic compounds, chromatographed under similar conditions [28]. It is noteworthy, that under the conditions investigated, the chromatographic behavior of bile acids and their salts is similar.

In each case sharp spots without tailing tendency were obtained. Temperature changes, within the range investigated, do not influence the elution order of steroids. The sequence of solutes remain also unchanged, when the concentration of organic modifier in binary mobile varies from 0 to 100% (v/v) (Fig. 2). From a practical point of view it is always

Table 2
Regression coefficients (intercept, slope) and correlation coefficient ($r$) of the regression equation $R_M$=slope(1000/$T$)+intercept for studied steroids measured by RP-18W plates and methanol–water mixtures as eluents[a]

| | Steroid | Intercept | Slope | $r$ |
|---|---|---|---|---|
| *Mobile phase: 100% methanol* | | | | |
| 1 | NaTDC | −2.5 (0.2) | 0.63 (0.07) | 0.97 |
| 2 | GCA | −1.8 (0.2) | 0.47 (0.07) | 0.95 |
| 3 | NaGC | −1.8 (0.3) | 0.45 (0.08) | 0.93 |
| 4 | GDCA | −2.9 (0.5) | 0.8 (0.1) | 0.93 |
| 5 | NaGDC | −2.9 (0.5) | 0.8 (0.1) | 0.93 |
| 6 | CA | −2.7 (0.5) | 0.8 (0.2) | 0.91 |
| 7 | NaC | −2.7 (0.5) | 0.8 (0.2) | 0.91 |
| 8 | DCA | −1.9 (0.6) | 0.5 (0.2) | 0.81 |
| 9 | NaDC | −2.0 (0.6) | 0.6 (0.2) | 0.80 |
| 10 | CDCA | −2.0 (0.6) | 0.6 (0.2) | 0.83 |
| 11 | NaCDC | −2.1 (0.4) | 0.6 (0.1) | 0.90 |
| 12 | LCA | −2.7 (0.5) | 0.8 (0.2) | 0.92 |
| 13 | Cholesterol | −2.1 (0.5) | 0.7 (0.2) | 0.90 |
| *Mobile phase: methanol–water (90:10)* | | | | |
| 1 | NaTDC | −3.0 (0.2) | 0.78 (0.07) | 0.98 |
| 2 | GCA | −1.6 (0.3) | 0.41 (0.09) | 0.93 |
| 3 | NaGC | −1.7 (0.3) | 0.45 (0.09) | 0.91 |
| 4 | GDCA | −1.9 (0.4) | 0.5 (0.1) | 0.91 |
| 5 | NaGDC | −1.9 (0.3) | 0.5 (0.1) | 0.91 |
| 6 | CA | −1.5 (0.3) | 0.43 (0.08) | 0.92 |
| 7 | NaC | −1.6 (0.2) | 0.47 (0.06) | 0.96 |
| 8 | DCA | −2.0 (0.6) | 0.6 (0.2) | 0.82 |
| 9 | NaDC | −2.1 (0.6) | 0.6 (0.2) | 0.83 |
| 10 | CDCA | −2.1 (0.5) | 0.6 (0.2) | 0.87 |
| 11 | NaCDC | −2.1 (0.5) | 0.6 (0.2) | 0.87 |
| 12 | LCA | −2.0 (0.4) | 0.7 (0.1) | 0.94 |
| 13 | Cholesterol | −3.4 (0.5) | 1.3 (0.1) | 0.97 |
| *Mobile phase: methanol–water (80:20)* | | | | |
| 1 | NaTDC | −2.6 (0.3) | 0.68 (0.08) | 0.97 |
| 2 | GCA | −2.2 (0.2) | 0.63 (0.07) | 0.97 |
| 3 | NaGC | −2.2 (0.2) | 0.63 (0.07) | 0.97 |
| 4 | GDCA | −2.3 (0.3) | 0.69 (0.09) | 0.96 |
| 5 | NaGDC | −2.3 (0.3) | 0.69 (0.09) | 0.96 |
| 6 | CA | −2.0 (0.3) | 0.64 (0.09) | 0.95 |
| 7 | NaC | −2.0 (0.3) | 0.64 (0.09) | 0.95 |
| 8 | DCA | −1.6 (0.3) | 0.57 (0.09) | 0.94 |
| 9 | NaDC | −1.6 (0.3) | 0.56 (0.08) | 0.95 |
| 10 | CDCA | −1.7 (0.4) | 0.6 (0.1) | 0.93 |
| 11 | NaCDC | −1.7 (0.4) | 0.6 (0.1) | 0.92 |
| 12 | LCA | −2.7 (0.4) | 1.0 (0.1) | 0.97 |
| 13 | Cholesterol (B) | −6.0 (1.0) | 2.3 (0.3) | 0.97 |
| *Mobile phase: methanol–water (70:30)* | | | | |
| 1 | NaTDC | −2.3 (0.3) | 0.65 (0.08) | 0.96 |
| 2 | GCA | −2.4 (0.3) | 0.75 (0.09) | 0.96 |
| 3 | NaGC | −2.4 (0.3) | 0.75 (0.09) | 0.96 |
| 4 | GDCA | −2.9 (0.3) | 0.94 (0.08) | 0.98 |
| 5 | NaGDC | −2.8 (0.2) | 0.93 (0.07) | 0.98 |
| 6 | CA | −2.3 (0.2) | 0.84 (0.07) | 0.98 |

Table 2 (*continued*)

| | Steroid | Intercept | Slope | *r* |
|---|---|---|---|---|
| 7 | NaC | −2.3 (0.3) | 0.83 (0.08) | 0.98 |
| 8 | DCA | −2.2 (0.3) | 0.86 (0.09) | 0.97 |
| 9 | NaDC | −2.0 (0.3) | 0.81 (0.09) | 0.97 |
| 10 | CDCA | −2.0 (0.3) | 0.81 (0.09) | 0.97 |
| 11 | NaCDC | −1.9 (0.4) | 0.8 (0.1) | 0.96 |
| 12 | LCA | −3.7 (0.3) | 1.44 (0.09) | 0.99 |
| *Mobile phase: methanol–water (60:40)* | | | | |
| 1 | NaTDC | −2.5 (0.3) | 0.8 (0.1) | 0.96 |
| 2 | GCA | −2.6 (0.3) | 0.9 (0.1) | 0.97 |
| 3 | NaGC | −2.6 (0.3) | 0.9 (0.1) | 0.97 |
| 4 | GDCA | −3.5 (0.4) | 1.2 (0.1) | 0.98 |
| 5 | NaGDC | −3.6 (0.4) | 1.2 (0.1) | 0.98 |
| 6 | CA | −3.1 (0.4) | 1.2 (0.1) | 0.98 |
| 7 | NaC | −3.0 (0.4) | 1.2 (0.1) | 0.98 |
| 8 | DCA (A) | −5.2 (0.4) | 2.0 (0.1) | 0.99 |
| 9 | NaDC (A) | −4.9 (0.2) | 1.86 (0.07) | 0.99 |
| 10 | CDCA (A) | −3.3 (0.8) | 1.4(0.2) | 0.95 |
| 11 | NaCDC (A) | −3.2 (0.8) | 1.3 (0.2) | 0.94 |
| 12 | LCA | −3.1 (0.9) | 1.4 (0.3) | 0.91 |
| *Mobile phase: methanol–water (50:50)* | | | | |
| 1 | NaTDC | −2.0 (0.4) | 0.7 (0.1) | 0.93 |
| 2 | GCA | −2.4 (0.4) | 0.9 (0.1) | 0.96 |
| 3 | NaGC | −2.2 (0.3) | 0.84 (0.08) | 0.98 |
| 4 | GDCA | −4.1 (0.4) | 1.5 (0.1) | 0.99 |
| 5 | NaGDC | −4.0 (0.3) | 1.49 (0.09) | 0.99 |
| 6 | CA | −4.1 (0.8) | 1.7 (0.2) | 0.95 |
| 7 | NaC | −4.3 (0.7) | 1.7 (0.2) | 0.96 |
| *Mobile phase: methanol–water (40:60)* | | | | |
| 1 | NaTDC | −2.3 (0.4) | 1.0 (0.1) | 0.97 |
| 2 | GCA | −2.6 (0.2) | 1.09 (0.05) | 0.99 |
| 3 | NaGC | −2.9 (0.3) | 1.18 (0.09) | 0.99 |
| 4 | GDCA (B) | −7.4 (1.8) | 2.8 (0.6) | 0.94 |
| 5 | NaGDC (B) | −7.7 (1.3) | 2.9 (0.4) | 0.97 |
| *Mobile phase: methanol–water (30:70)* | | | | |
| 1 | NaTDC (A) | −1.6 (0.4) | 0.9 (0.1) | 0.96 |
| 2 | GCA (A) | −2.7 (0.8) | 1.3 (0.2) | 0.94 |
| 3 | NaGC (A) | −3.1 (0.6) | 1.4 (0.2) | 0.97 |

[a] Measured temperature range was 5–60°C and number of samples was seven except: (A) temperature range 10–60°C, number of samples, six and (B) temperature range 20–60°C, number of samples, five. The values in parentheses indicate the standard error at 95% significance level.

important to predict the elution order on a given chromatographic system. The retention behavior of solutes in studied system is easy to predict on the basis of the quantitative polarity concept. As first are eluted the most polar conjugated bile acids, next the free bile acids and cholesterol. Within each group, the elution order depends primarily on the number of OH groups. Similar retention behavior has been observed by Lepri et al. [29] as well as in case of different compounds classes e.g., estrogenic steroids or polycyclic aromatic hydrocarbons metabolites, which have structures very similar to steroids [30–32].

Fig. 2 illustrates retention profiles of eight bile

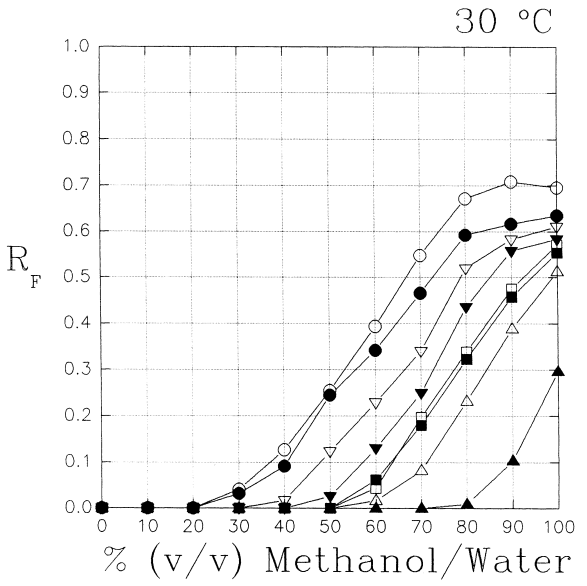


Fig. 2. Plots of $R_F$ values for steroids versus concentration of methanol in water (%, v/v) at constant temperature 30°C. NaTDC (○); GCA (●); GDCA (▽); CA (▼); DCA (□); CDCA (■); LCA (△); cholesterol (▲).

steroids using different methanol–water mobile phases and temperature set at 30°C. No steroids elute when the concentration of methanol in mobile phase is less than 30% (v/v). Generally, the investigated solutes are moderately and strongly adsorbed on the support material. Considering all temperatures investigated, $R_F$ values of steroids are always less than 0.8.

The shape of the curves presented in Fig. 2 are similar to those obtained for different classes of compounds studied by Soczewiński and Wachtmeister [33]. The curves in Fig. 2 can be easily linearized replacing $R_F$ by $R_M$. In this case the regression coefficient $r$ of regression equation, $R_M$ vs. mobile phase composition, usually varies from 0.950 to 0.998. A linear relationship between $R_M$ values and % (v/v) of methanol in water can be very useful in the optimization of chromatographic systems [34,35].

### *3.2. Separation study*

Minimum $\Delta hR_F$ values for adjacent spots or peaks are often used for studies of solutes separation in TLC methods [36]. According to the data presented in Fig. 2, the investigated chromatographic system is non-selective for deoxycholic and chenodeoxycholic acids. Therefore, for evaluation of samples separation an optimization criterion constructed as $\Sigma\Delta hR_F$ was chosen. As can be seen in Fig. 3, the mobile phase composition as well as the temperature changes produces significant differences in the separation of an eight-component sample. The influence of temperature on retention and separation of bile steroids using TLC has been studied by Rivas-Nass et al. [22]. Authors reported that separation of bile acids can be improved by varying the temperature especially in the subambient region (temperatures to −20°C were studied). However, using up to six components of mobile phase might cause fundamental problems in component mixing and homogeneity of mobile phases during the whole chromatographic process. Therefore, for these chromatographic systems separation changes of bile acids at different temperatures can be interpreted mainly as changes of mobile phase compositions versus temperature.

For the chromatographic system investigated the best separation is observed when the concentration of methanol in the mobile phase varies from 70 to 80% (v/v). If the separation of bile acids without cholesterol is considered the curves' maximum in Fig. 3 are moved to low concentrations of methanol. In this

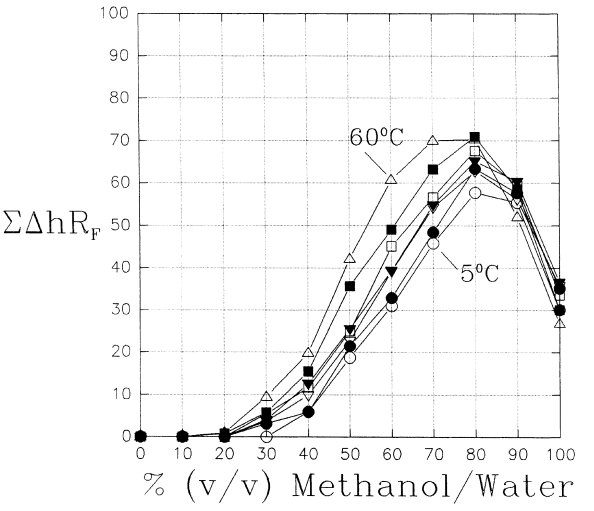


Fig. 3. Relationships between $\Sigma\Delta hR_F$ values and composition of mobile phases at different temperatures, obtained for mixtures consisting of eight steroids. 5°C (○), 10°C (●), 20°C (▽), 30°C (▼), 40°C (□), 50°C (■), 60°C (△).

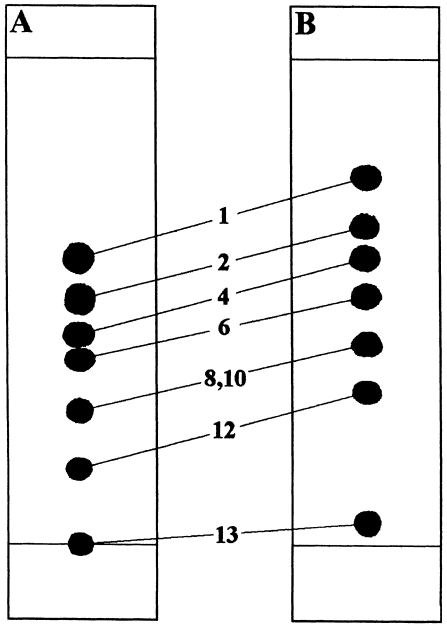


Fig. 4. Chromatographic separation of steroids at 5°C (A) and 50°C (B) using RP-18W plates and methanol–water (80:20, v/v) mobile phase. Spot numbers correspond to steroids numbers listed in Table 1.

case the best separation can be achieved using mobile phases containing methanol in a concentration from 60 to 70% (v/v). In the higher temperature region the separation can be significantly improved particularly for mobile phases containing methanol in concentrations from 40 to 80%. As can be seen from the chromatograms presented in Fig. 4 an excellent separation (excepting DCA and CDCA) was obtained at 50°C. From a practical point of view, higher temperatures provide faster analysis due to low viscosity of mobile phases. In addition, temperature increase will usually improve the efficiency of typical chromatographic systems [37].

## 4. Conclusions

Simultaneous changes of two critical parameters: temperature and mobile phase composition provides a useful tool for searching of the best chromatographic conditions for multiple separation of bile acids. Using a typical reversed-phase chromatographic system in which chiral or non-chiral agents do not modify binary mobile phases a relatively weak retention–temperature response for the studied steroids is observed. However, separation studies of the multicomponent mixtures shown that the degree of separation in the high temperature region can be increased due to an improvement of the efficiency of the chromatographic system.

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