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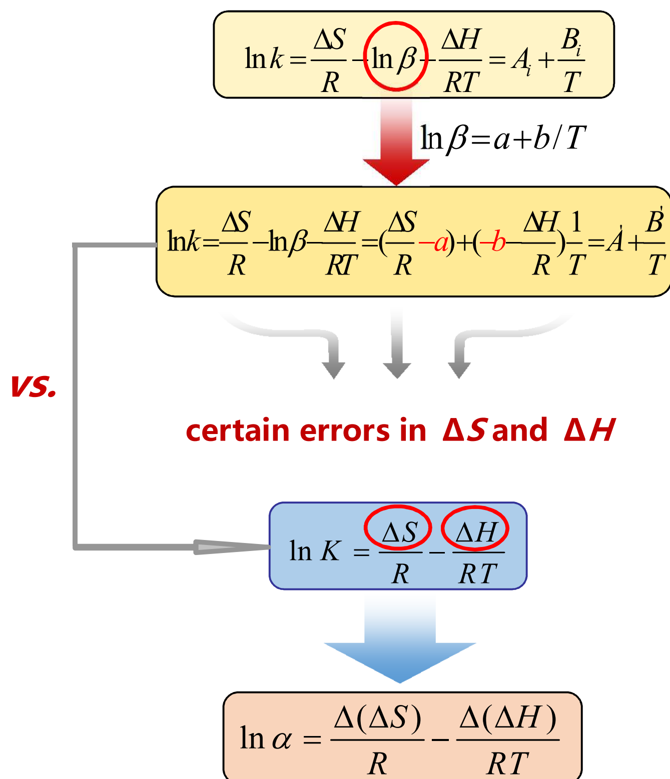

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1 **Selectivity-column temperature relationship as a new strategy in**
2 **predicting separation of structurally analogues on HPLC by using**
3 **different stationary phases**

4

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12

13 **Abstract**

14 The effect of change in column temperature on van't Hoff equation, as well as
15 the relationship between separation efficiency and column temperature on high
16 performance liquid chromatography (HPLC) by using different stationary phases,
17 have been discussed and compared in this paper. For this purpose, six flavonoid
18 glycosides were selected to establish van't Hoff equations on C_{18}, cholesterol, C_8 and
19 porous polymer octadecyl bonded (ODP) stationary phases. The results indicated that,
20 for all the columns, the changes in phase ratio arising from the varying column
21 temperatures had no significant impact on the linearity of van't Hoff equation,
22 however, it indeed led to certain errors in the intercept (change in entropy, ΔS^0) and
23 slope (change in enthalpy, ΔH^0) of the equation, which are considered as important
24 parameters in illuminating chromatographic mechanism on HPLC. Thereby, a new

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protocol has been proposed in this paper to correct these errors, with the aim of offering solid data of ΔS^0 and ΔH^0. Furthermore, a relationship relating selectivity and column temperature was deduced in theory, and then validated by using the six flavonoid glycosides in this work. This relationship has been applied to predict the separation of six steroid hormones on HPLC with high consistency between experimental and predicted selectivity factors (average relative errors less than 2.2%, 1.0%, 6.1% and 5.1% for C_{18}, cholesterol, C_8 and ODP columns, respectively). The proposed new strategy in predicting selectivity greatly facilitates optimization processes for HPLC by avoiding tedious condition experiments, and furthermore, column temperature is proposed to involve in the optimization processes as an important parameter, making separation of structural analogues effectively. In this temperature-involved optimization method, stationary phase sensitive to temperature change, i.e. cholesterol, is recommended.

Keywords: High performance liquid chromatography (HPLC); van't Hoff equation; Cholesterol bonded stationary phase; porous polymer octadecyl bonded stationary phase (ODP); Flavonoid glycosides; Estrogens

1. Introduction

Good separation has been always a prerequisite for high performance liquid chromatography (HPLC),[1] especially in recent years since the ion-suppression or ion-enhancement effect resulting from the co-eluted analytes in mass spectrometry (MS) has been recognized, which recalls the requirement for good separation in LC-MS. However, separation of structural analogues, such as isomers and

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50 stereoisomers, is always a thorny problem for chromatographic analysis up to date. In
51 developing and validating an HPLC method, the most common approach is to
52 optimize the mobile phase composition after an appropriate column has been
53 selected.[2-3] It is worth noting that column temperature is also a potential variable
54 with many virtues[4-9]. First, a change in temperature can have a pronounce effect on
55 analysis speed and separation efficiency because the mass transfer rate relating with
56 mobile phase viscosity and solute diffusion is affected largely by temperature. Second,
57 temperature can have a marked effect on the selectivity of chromatographic separation
58 as the change in retention with temperature is often different for various analytes.
59 More importantly, the regulation of temperature is very convenient and simple, as it
60 requires only a column thermostat allowing fast enough equilibration over the
61 working temperature range. In spite of the strong role of temperature in HPLC, the
62 correlational studies have been rarely reported, which may be mainly due to the fact
63 that the temperature generally has only a narrow regulating range in the practical work
64 because the thermal stability of traditionally used silica-based stationary phase has
65 rigorous conditions (often less than 60°C), which limits the application of temperature
66 for optimization.[10]

67 This situation, however, is changing gradually because novel stationary phases
68 have been emerging in recent years. Some thermally stable phase materials, such as
69 graphitized carbon types, zirconium oxide based phases and
70 polystyrene/divinylbenzene copolymers have been used in HPLC,[11-12] which can
71 greatly expand the available temperature range even up to over 100°C. But these
72 stationary phases usually show lower separation efficiency than the silica-based
73 column materials.[11] In consequence, column materials sensitive to the temperature
74 changes, for instance, recently developed cholesterol bonded-silica stationary phase,

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75 is another promising alternative, as tiny temperature changes may have appreciable
76 impact on retention, efficiency, and selectivity on HPLC.[3,14]
77 In an HPLC separation, the mechanism can be described as the increasing
78 temperature alters analyte retention by changing the free energy between the analyte
79 and the stationary phase, which can be depicted using van't Hoff equation, commonly
80 presented as the dependence of retention factor (k) on temperature (T). In van't Hoff
81 equation, the phase ratio (β) is always regarded as a constant over the experimental
82 temperature range.[15-16] In fact, the influence of change in temperature on the mobile
83 phase volume is obviously greater than that on the stationary phase volume, which
84 may give rise to variation in β under different temperatures. Nevertheless, no
85 researches involved in van't Hoff equation took this change into careful consideration
86 as far as our knowledge goes. Moreover, almost all the published studies only focused
87 on using van't Hoff equation to interpret retention behavior and mechanism of small
88 molecules on various columns at different temperatures,[17-23] but few report was
89 aimed to improve selectivity of temperature-programmed HPLC method, let alone
90 making reliable prediction for separation tendency on the basis of van't Hoff equation.
91 Accordingly, it is the purpose of this paper to evaluate the effect of phase ratio
92 differing in various temperatures on van't Hoff equation, and proposed a new protocol
93 to correct the errors in enthalpy and entropy resulting from this effected van't Hoff
94 equation. Furthermore, a novel relationship based on the selectivity and column
95 temperature was deduced theoretically and validated by using homologous flavonoid
96 glycosides. This relationship was then applied to predict the selectivity of C_{18},
97 cholesterol, C_8 and porous polymer octadecyl bonded (ODP) stationary phases for
98 structurally analogous estrogens under different temperatures.
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101 **2. Theoretical Section**

102 In HPLC, the thermodynamic partition coefficient (K) between the stationary and 103 the mobile phases is proportional to the free energy of transfer (ΔG^0) involving the 104 change in enthalpy (ΔH^0) and entropy (ΔS^0)[24]

105
$$\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT\ln K \quad (1)$$

106 where ΔH^0 and ΔS^0 represent the standard partial molar enthalpy change and the 107 standard partial molar entropy change associated with the transfer of a solute from the 108 mobile to the stationary phase, respectively. T is the thermodynamic temperature in 109 Kelvin, and R is the gas constant. With values for ΔH^0 and ΔS^0 at one particular 110 temperature, it is possible to calculate K using

111
$$\ln K = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT} \quad (2)$$

112 Since K equals $k\times\beta$, Eq. 2 becomes

113
$$\ln K = \ln(k\times\beta) = \ln k + \ln\frac{V_M}{V_S} \quad (3)$$

114 where β ($\beta=\frac{V_M}{V_S}$) is known as the phase ratio of a column (V_S is the volume of the 115 stationary phase, and V_M is the volume of the mobile phase, respectively). V_S can be 116 understood as a part of the total column volume, V_C, into which non-retained 117 compounds cannot penetrate.[25-26] With this simplified convention, β can be expressed 118 as

119
$$\beta = \frac{V_M}{V_C - V_M} \quad (4)$$

120 Therefore, the dependence of k on column temperature called van’t Hoff 121 equation is given

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122
$$\ln k = \frac{\Delta S^0}{R} - \ln\frac{V_M}{V_S} - \frac{\Delta H^0}{RT} = \frac{\Delta S^0}{R} - \ln\beta - \frac{\Delta H^0}{RT} = A_i + \frac{B_i}{T} \quad (5)$$

123 In general, if a single mechanism controls the retention over the experimental
124 temperature range, ln$k$ *versus* $1/T$ plots should be linear.[27] By plotting ln$k$ with $1/T$,
125 the enthalpic and entropic contributions to the chromatographic retention can be
126 calculated: -ΔH^0 from the slope, and ΔS^0 from the intercept of the plot. However, as
127 mentioned above, the intercept of Eq. 5, A_i, is not a definite constant due to the
128 uncertainty of β invariable over the experimental temperature range, which may exert
129 an influence on the ln$k$ *versus* $1/T$ plot. On the contrary, it is observed that ln$K$ is
130 explicitly linear with $1/T$ as described in Eq. 2, and K can be calculated through Eq. 3
131 by k and β measured at different temperatures, which means establishment of ln$K$
132 *versus* $1/T$ linear plot is more rational because it can effectively avoid the possible
133 errors arising from changed β existing in ln$k$ *versus* $1/T$ plot.

134 In HPLC, the selectivity factor (α) is usually represented as

135
$$\alpha = \frac{K_n}{K_{n-1}} = \frac{k_n}{k_{n-1}} \quad (6)$$

136 The change in α between two adjacent solutes with temperature can be derived
137 by substituting Eq. 2 into Eq. 6

138
$$\begin{aligned}\ln\alpha &= \ln\frac{K_n}{K_{n-1}} = \ln\frac{k_n}{k_{n-1}} \\ &= \frac{\Delta S_n^0 - \Delta S_{n-1}^0}{R} - \frac{\Delta H_n^0 - \Delta H_{n-1}^0}{RT} \\ &= \frac{\Delta(\Delta S^0)}{R} - \frac{\Delta(\Delta H^0)}{RT}\end{aligned} \quad (7)$$

139 Based on Eq. 7, the selectivity of column at arbitrary temperature can be
140 predicted by the fitted parameters, ΔH^0 and ΔS^0 obtained from the linear ln$K$ *versus*
141 $1/T$ plot of Eq. 2.

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143

144 ## 3. Experimental

145 ### 3.1 Materials and reagents

146 Methanol and acetonitrile of HPLC grade were obtained from Merck (Darmstadt,
147 Germany). Acetic acid of analytical grade (36%) was provided by Sinopharm
148 Chemical Reagent Beijing Co., Ltd. (Beijing, China). Distilled water was purified
149 using a Milli-Q water purification equipment (Millipore Intertech., MA, USA).

150 Flavonoid glycosides such as flavonoid glycosides I, II, III, IV, V and VI used as
151 the training set were all laboratory-made. Estrogens, i.e. estriol (E3), 17β-estradiol
152 (17β-E2), 17α-estradiol (17α-E2), ethinyl estradiol (EE), estrone (E1) and
153 progesterone (P4) used as the test set were purchased from the National Institute for
154 the Control of Pharmaceutical and Biological Products (Beijing, China).

155

156 ### 3.2 Apparatus

157 All chromatographic experiments were carried out on a fully automated
158 ThermoFisher LC-U3000 liquid chromatography (ThermoFisher Scientific,
159 Massachusetts, USA) equipped with a double gradient pump, an auto-sampler with a
160 20 μL loop and a diode array detector. All the data were collected and processed by
161 Chromeleon 7.20 work station (TheromFisher Scientific, California, USA). The
162 chromatographic columns used were Sepax GP-C_{18} ,150 mm × 4.6 mm i.d., 5 μm
163 (Sepax Technologies Inc., SuZhou, China), Cosmosil cholesterol column, 5 μm, 150
164 mm × 4.6 mm i.d. (Nacalai Tesque Inc., Kyoto, Japan), Sepax GP-C_8 ,150 mm × 4.6
165 mm i.d., 5 μm (Sepax), and Shodex Asahipak ODP-50 4D, 150 mm × 4.6 mm i.d., 5
166 μm (Showa Denko K. K., Tokyo, Japan).

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168 **3.3 Methods**

169 For C_{18} column: solutes were eluted by the mobile phase consisting of methanol
170 and 0.1% acetic acid (30:70, v/v) with the flow rate at 1.0 mL/min. For cholesterol
171 column: solutes were eluted by the mobile phase consisting of methanol and 0.1%
172 acetic acid (25:75, v/v) with the flow rate at 0.4 mL/min. For C_8 column: solutes were
173 eluted by the mobile phase consisting of methanol and 0.1% acetic acid (30:75, v/v)
174 with the flow rate at 1.0 mL/min. For ODP column: solutes were eluted by the mobile
175 phase consisting of methanol and 0.1% acetic acid (30:75, v/v) with the flow rate at
176 0.8 mL/min. The type and composition of mobile phases were individually the
177 optimized conditions for each stationary phase. For all the stationary phases,
178 temperatures were ranged from 25°C to 55°C. The wavelength was set at 277 nm for
179 flavonoid glycosides and 220 nm for estrogens, respectively. The solutes were
180 dissolved in the mobile phase used for the cholesterol column and the injected volume
181 was 10 µL in each experiment. All the experimental retention times (t_R) were obtained
182 by averaging the results of at least three independent injections. The retention factors
183 k was calculated according to the equation $k = (t_R - t_M)/t_M$, where t_M was the column
184 hold-up time, determined by using sulfourea as the non-retained marker.[14] The
185 column hold-up volume, V_M, was calculated by t_M and F_c ($V_M = F_c \times t_M$, where F_c is
186 the volumetric flow rate), and then corrected by subtracting extra-column volume (V_0),
187 which was determined through substituting the column with a zero-volume union.[28-29]
188 The volume of the stationary phase (V_S) was calculated as a difference between V_C
189 and the corrected V_M.

190

191

192 **4. Results and Discussion**

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193 **4.1 Effect of temperature on the phase ratio**

194 To investigate the effect of temperature on phase ratio, values of β were
195 experimentally measured by V_C and V_M via Eq. 4 with temperatures ranged from 25°C
196 to 55°C with 10°C increments on C_{18}, cholesterol, C_8, and ODP columns, respectively.
197 Figure 1 shows the regression of the experimental phase ratios and column
198 temperatures on four columns. It was obvious that, β increased as temperature
199 increased, which confirmed the suspicion we proposed that β varies during
200 temperature changes. However, the good linear relationship of $\ln\beta = a + b/T$ can be
201 obtained with the correlation coefficients R^2 better than 0.99. By substituting this
202 linear fitting into Eq. 5, van't Hoff equation can be re-written as:

203 $$\ln k = \frac{\Delta S^0}{R} - \ln\beta - \frac{\Delta H^0}{RT} = (\frac{\Delta S^0}{R} - a) + (-b - \frac{\Delta H^0}{R})\frac{1}{T} = A' + \frac{B'}{T} \quad (8)$$

204 Although the form of Eq. 8 is just the same as van't Hoff equation, the physical
205 significance of the slope and intercept are both totally different, that is, the intercept
206 and slope of the classical van't Hoff equation cannot truly express ΔH^0 and ΔS^0 in a
207 chromatographic process. On the contrary, since it makes no assumptions about the
208 fixed quantity of β at different temperatures, the slope and intercept of Eq. 2 truly
209 reflect ΔH^0 and ΔS^0 values in the chromatographic process because $\ln K$ was calculated
210 by k and β measured at each temperature through Eq. 3. The experimental $\ln k$, as well
211 as $\ln K$ of six flavonoid glycosides, were respectively plotted against $1/T$ on C_{18},
212 cholesterol, C_8 and ODP columns, and the fitting parameters are shown in Table 1. It
213 can be seen from Table 1 that, although the straight line seemed to fit the data well
214 when $\ln k$ was plotted against $1/T$, the slope and intercept were markedly different
215 from those of $\ln K$-$1/T$ plot due to the additional items a and b respectively involved in
216 intercept and slope in Eq. 8. The relative errors of k-related and K-related ΔH^0
217 (2.8%~3.3% for C_{18}, about 0.9% for cholesterol, 4.4%~5.5% for C_8 and 11.5%~17.4%

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218 for ODP, respectively), as well as the errors of ΔS^0 (2.8%~3.4% for C_{18}, 3.6%~4.1%
219 for cholesterol, 2.7%~3.6% for C_8 and 21.5%~28.3% for ODP, respectively) are listed
220 in Table 1, revealing the inaccuracy of conventional van't Hoff equation in acquiring
221 ΔH^0 and ΔS^0, while it is suggested that the solid and accurate ΔH^0 and ΔS^0 data should
222 be obtained through Eqs. 2 and 3. For ODP column, the errors of ΔH^0 and ΔS^0 were
223 more significant than others, which may be attributed to the large values of intercept
224 and slope of the lnβ-1/T plot shown in Figure 1 (d), which means temperature has
225 much more influence on β for ODP stationary phase.

226 The retention mechanisms of flavonoid glycosides on C_{18}, cholesterol, C_8 and
227 ODP stationary phases also had been compared. The results showed that all the
228 ln$K$-1/T had good linear relationships ($R^2 > 0.99$), as well as the similar fitting
229 parameters ($\Delta H^0 < 0$, $\Delta S^0 < 0$), which implied that the retention mechanisms of these
230 analytes on four kinds of stationary phases were all dominated by hydrophobic
231 interaction, and all belonged to enthalpy-driving process [1,15]. On the other hand, the
232 calculated values of *a* and *b* for each flavonoid glycoside respectively obtained from
233 the differences between *k*-related and *K*-related ΔS^0 and ΔH^0 via Eq. 8 were evaluated.
234 Since lnβ = a + b/T, the parameters *a* and *b* should be always constants under the same
235 chromatographic conditions regardless of the analytes investigated. However, it was
236 observed that *a* and *b* values calculated by using various flavonoid glycosides had
237 more or less differences with each other. The cholesterol-bonded stationary phase
238 exhibited only slight discrepancy in obtaining *a* and *b* for the six flavonoid glycosides
239 with RSDs at 2.0% and 0.1%, respectively, while the RSDs of *a* and *b* obtained from
240 the six solutes on C_{18}, C_8 and ODP stationary phase were higher with values at 3.4%
241 and 1.4%, 7.0% and 7.0%, and 6.4% and 6.5% for *a* and *b*, respectively. These results
242 indicated that the property of the solutes might have influence on the mobile and

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243 stationary phases. The high consistency on the cholesterol-bonded stationary phase is
244 probably attributed to the decline in numbers of residual silanols on stationary phase
245 surface which are apt to cause secondary interactions such as hydrogen-bonding and
246 so on in comparison with C_{18}, C_8, and ODP stationary phases, and in consequence, the
247 minor structural differences among various solutes can be ignored. Moreover, as
248 shown in Table 1, the slopes of $\ln k$-$1/T$ and $\ln K$-$1/T$ plots obtained on the cholesterol
249 column were both obviously larger than those obtained on other columns, which
250 confirmed the fact that the cholesterol bonded stationary phase was indeed more
251 sensitive to temperature changes.
252
253 **Figure 1**
254 **Table 1**
255
256 **4. 2 Relationship between selectivity and column temperature on RPLC**
257 To evaluate the effect of column temperature on chromatographic selectivity
258 based on Eq. 7, as well as to compare the thermodynamic chromatographic properties
259 of four types of stationary phases for the separation of structural analogues,
260 experiments were conducted by separating a mix of six flavonoid glycosides with
261 temperatures ranged from 25°C to 55°C with 10°C increments on C_{18}, cholesterol, C_8
262 and ODP columns, respectively. The chromatograms of the six flavonoid glycosides
263 on C_{18} and cholesterol columns at different temperatures are shown in Figure 2. It can
264 be observed that, elevated temperature reduced the analysis times of analytes on both
265 of the two columns (the same tendency could be also seen on C_8 and ODP columns,
266 which were not shown in Figure 2), which might due to the exothermic enthalpy
267 change associated with transfer of solutes from the mobile to stationary phases

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268 dominated the retention process in all the four chromatographic systems.[16,17] The
269 shorter retention times and smaller relative retention values resulting from rising
270 temperature would go against good separation from the perspective of
271 chromatographic thermodynamics. However, it is obvious from Figure 2 that the
272 increasing temperature played a beneficial impact on separation efficiency, especially
273 for cholesterol-bonded stationary phase. Thus, it is assumed that on the cholesterol
274 column the improved and narrower peak shapes caused by elevated temperatures
275 according to chromatographic kinetics may dominate the separation process, that is,
276 the half peak width rapidly decreases on the cholesterol column as column
277 temperature rising, which compensates the negative influence on the separation effect
278 brought by the shortened retention factors.

279 Figure 3 illustrated the change in selectivity factor α, which was obtained by the
280 experimental t_R values of two adjacent solutes via Eq. 6, against the temperature. As
281 shown in Figure 3 that, α varied at different temperatures for all the investigated
282 compounds. For C_{18} column, as can be seen from Figure 3 (a), α_2 and α_4 decreased as
283 temperature increased, while α_1 and α_5 increased with the temperature increased.
284 However, these α values were all larger than 1.12 over the investigated temperature
285 interval, which means all the flavonoid glycosides exhibited acceptable separation
286 from 25°C to 55°C, except for compounds III and IV, the peaks of which were
287 completely overlapped ($\alpha_3 \approx 1$) on the C_{18} column within the experimental temperature
288 range (see Figure 2 (a-e)). For cholesterol column, as shown in Figure 3 (b), the
289 changes in selectivity with temperatures led to differences in resolution: the positive
290 slope indicated that α_2 and α_5 rapidly decreased as temperature increased, while α_1, α_3
291 and α_4 increased gradually with the temperature increased. In consequence, these
292 tendency resulted in the optimal separation for the six flavonoid glycosides at 50°C

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293 with all the α values larger than 1.2. As the temperature further increased (up to 55°C),
294 the separation between compounds II and III (α_2=1.08 in Figure 3 (b)), as well as V
295 and VI (α_5=1.08 in Figure 3 (b)) were not complete on the cholesterol column. For C_8
296 column, as it can be seen in Figure 3 (c), α_2, α_3 and α_4 decreased as temperature
297 increased, while α_1 increased with the temperature increased. For compounds V and
298 VI, the peaks of which were completely overlapped on C_8 column at the low
299 temperature range (α_5≈1, 25°C and 35°C). Additionally, as shown in Figure 3 (c), the
300 smaller slope of the lnα-1/T plot suggested that the chromatographic behaviors of the
301 flavonoid glycosides were not sensitive to changes in temperature on C_8 stationary
302 phase. For ODP stationary phase, α_2, α_3 and α_5 gradually decreased as temperature
303 increased, while α_1 and α_4 increased with the temperature increased. According to the
304 tendency of selectivity with temperature changes, it can be speculated that the
305 appropriate column temperature for separating all the analytes is ranged between 25°C
306 and 35°C on ODP column.

307 On the other hand, the slope and intercept of each lnα-1/T plot in Figure 3
308 respectively implied the differences in the enthalpy and entropy change of two
309 adjacent solutes based on Eq. 7. Table 2 lists the best-fit values of the intercept, the
310 slope, as well as the correlation coefficient of each plot on C_{18}, cholesterol, C_8 and
311 ODP columns. It can be observed from Table 2 that, all the fittings had satisfactory
312 linearity with R^2 larger than 0.94, except for the lnα_3-1/T plot (R^2=0.3617) on C_{18}
313 column, lnα_5-1/T plot on C_8 (R^2=0.8299) and ODP (R^2=0.9260) columns, respectively,
314 which due to the inevitable error arising from the completely overlapped peaks of III
315 and IV on C_{18} column, as well as V and VI on C_8 and ODP columns. The fitted $\Delta(\Delta S)$
316 and $\Delta(\Delta H)$ were compared with the calculated ones obtained from relevant data from
317 Table 1, and the results were presented in Table 3. The high agreement of fitted and

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318 calculated data (with the *RE* values of $\Delta(\Delta S)$ 0.1%~2.5% for C_{18}, 0.0%~0.1% for
319 cholesterol, 0.2%~4.0% for C_8 and 0.3%~0.7% for ODP, and the *RE* values of $\Delta(\Delta H)$
320 0.1%~2.1% for C_{18}, 0.0% for cholesterol, 0.0%~1.6% for C_8 and 0.0% for ODP,
321 respectively) confirmed that availability of the deduced Eq. 7. With the new strategy
322 of prediction in resolution by Eq. 7 proposed in this study, one can conveniently
323 evaluate the separation tendency of the tested compounds on a column with the
324 change in temperature, thereby cleverly avoiding those time-consuming and laborious
325 condition experiments.
326
327 **Figure 2**
328 **Figure 3**
329 **Table 2**
330 **Table 3**
331
332 **4.3 Prediction of selectivity for estrogens**
333 As shown in Figure 2, all the flavonoid glycosides tested cannot reach to
334 acceptable separations at commonly used temperatures (25°C~35°C) on the four
335 columns even with the optimized type and proportion of the mobile phase. However,
336 after column temperature was introduced into the optimization process as the third
337 parameter, the satisfactory separation on cholesterol column would be realized by
338 regulating temperatures. However, this regulating measure would not be helpful in
339 separating the investigated compounds on C_{18} and C_8 columns. Therefore, setting up a
340 simple model incorporating retention with temperatures such as Figure 3 may allow
341 chromatographers to conveniently predict separation trend of structural analogues on
342 various chromatographic columns at different temperatures. To further demonstrate

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343 the applicability of this selectivity prediction protocol at different temperatures, six
344 estrogens used as the test set were analyzed on C_{18}, cholesterol, C_8 and ODP columns,
345 respectively. For this purpose, the type and proportion of the mobile phase were
346 simply optimized at first. Acetonitrile (B)-water (A) with the gradient program
347 (30%-40% solvent B from 0 to 5 min; 40% solvent B at 15 min; 40%-55% solvent B
348 from 15 to 22 min; 55% solvent B at 35 min; 55%-30% solvent B from 35 to 36 min;
349 50% solvent B at 38 min) were selected for C_{18} column, and acetonitrile-water (40:60,
350 v/v) were selected for cholesterol, C_8, and ODP columns, respectively. On C_{18},
351 cholesterol and C_8 columns, the flow rate was 1.0 mL/min, while on ODP the flow
352 rate was 0.8 mL/min. The injection volume was 10 μL, and the wavelength was set at
353 220 nm. Every single sample of the six estrogens was injected and t_R was recorded
354 individually. Since the rigorous linearity of Eq. 7 has been deduced in theory and
355 further confirmed in section 4.2, in this section two temperature points (30°C and
356 55°C) were chosen as the reference temperatures to build the model of lnα-1/T, by
357 using which the selectivity between two adjacent solutes under other temperatures can
358 be predicted directly. The predicted and experimental values for α at 40°C and 50°C
359 are summarized in Table 4. As shown in Table 4, the predicted α values were
360 extremely close to the calculated ones with the relative errors of 0.0%~2.2% for C_{18}
361 column, 0.0%~1.0% for cholesterol column, 0.0%~6.1% for C_8 column and 0.0%~5.1%
362 for ODP column, respectively, which demonstrated that the proposed method can
363 effectively predict the resolution of estrogens over the experimental temperature range.
364 By comparing the predicted α values obtained on four columns, it can be observed
365 that these four stationary phases showed little difference in separating the investigated
366 steroid hormones, indicating that all of the four columns can be used in analysis of the
367 six hormones. As shown in Table 4, α_5 increased as temperature increased, while α_1,

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368 α_2, α_3 and α_4 decreased with the temperature increased on all the columns, which
369 suggested that the investigated steroid hormones should be separated at lower
370 temperature on the four stationary phases. Since the cholesterol bonded stationary
371 phase is more sensitive to temperature changes, a greater improvement in separation
372 should be observed on the cholesterol column at lower column temperature. The
373 chromatograms for a mix of the six steroid hormones on cholesterol column at
374 different column temperatures are shown in Figure 4, which confirmed our
375 speculation.
376
377 **Figure 4**
378 **Table 4**
379
380
381 **5. Conclusions**
382 The use of temperature programming in HPLC is gaining momentum in recent
383 years, and the explanation of separation mechanism from the view of
384 chromatographic thermodynamics by using van’t Hoff equation is commonly
385 available in the literatures. However, it is clear that changes in phase ratio at various
386 temperatures would result in certain errors of enthalpy and entropy obtained from
387 classical van't Hoff equation. In this paper, a new strategy for gaining accurate values
388 of enthalpy and entropy was proposed, providing solid data support for theoretical
389 research in separation mechanism. More importantly, by examining the role
390 temperature played in the selectivity of compounds, a new model relating the
391 separation tendency of analytes and column temperature on HPLC was deduced and
392 validated in this work. According to this promising method, the change in resolution

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393 between homologous analytes with column temperature can be predicted conveniently,
394 making HPLC optimization process greatly simplified, and furthermore, in this way
395 adjustable parameters that can be optimized in HPLC are expanded since temperature
396 can be considered as the third dimension besides mobile phase type and composition.
397 In addition, by comparing the separation characteristics of C_{18}, cholesterol, C_8 and
398 ODP bonded stationary phases on HPLC, it is suggested that stationary phase
399 sensitive to temperature, e.g. cholesterol is particularly suitable to this
400 temperature-involved optimization method, which points out a new direction for
401 developing column materials.

402

403 **Acknowledgments**

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409

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450 **6. Table legends**

451

452 **Table 1.** Effect of temperature on the partition coefficients (K) and retention factors (k)
453 of flavonoid glycosides on C_{18}, cholesterol, C_8 and ODP columns. (RE=Relative
454 Error)

455

456 **Table 2.** Best-fit parameters of intercept and slope, as well as correlation coefficients
457 (R^2) of Eq. 7 for C_{18}, cholesterol, C_8 and ODP columns.

458

459 **Table 3.** The comparison between fitted and calculated $\Delta(\Delta S)$ and $-\Delta(\Delta H)$ values
460 obtained on C_{18}, cholesterol, C_8 and ODP columns.

461

462 **Table 4.** Predicted and experimental values of selectivity factor on C_{18}, cholesterol, C_8
463 and ODP columns at different temperatures.

464

465

466 **Figure Captions:**

467

468 **Figure 1.** Effect of temperature on the phase ratio on C_{18} column (a), cholesterol
469 column (b), C_8 column (c) and ODP column (d). Chromatographic conditions of C_{18}
470 column: methanol-0.02 mol/L acetic acid (30:70, v/v), flow rate 1.0 mL/min, injection
471 volume 10 μL; chromatographic conditions of cholesterol column: methanol-0.02
472 mol/L acetic acid (25:75, v/v), flow rate 0.4 mL/min, injection volume 10 μL;
473 chromatographic conditions of C_8 column: methanol-0.02 mol/L acetic acid (30:70,
474 v/v), flow rate 1.0 mL/min, injection volume 10 μL; chromatographic conditions of

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475 ODP column: methanol-0.02 mol/L acetic acid (30:70, v/v), flow rate 0.8 mL/min,
476 injection volume 10 μL.
477
478
479 **Figure 2.** Chromatograms of the six flavonoid glycosides on C_{18} column (a, b, c, d
480 and e) and cholesterol column (f, g, h, i and j) under different temperatures. See Fig. 1
481 for chromatographic conditions. Peaks: 1. flavonoid glycoside I (Schaftoside); 2.
482 flavonoid glycoside II; 3. flavonoid glycoside III (Isoschaftoside); 4. flavonoid
483 glycoside IV; 5. flavonoid glycoside V; 6. flavonoid glycoside VI.
484
485 **Figure 3.** Effect of temperature on the selectivity factor on C_{18} column (a), cholesterol
486 column (b), C_8 column (c) and ODP column (d). See Fig.1 for chromatographic
487 conditions. ▲ α_1 (selectivity factor of I and II); ▼ α_2 (selectivity factor of II and III); ♦
488 α_3 (selectivity factor of III and IV); ● α_4 (selectivity factor of IV and V; ■ α_5
489 (selectivity factor of V and VI).
490
491 **Figure 4.** Chromatograms of the six steroid hormones on cholesterol column at
492 different temperatures. (a) 20°C; (b) 30°C; (c) 40°C and (d) 50°C. Peaks: 1. Estriol
493 (E3); 2. 17α-Estradiol (17α-E2); 3. 17β-Estradiol (17β-E2); 4. Estrone (E1); 5. Ethinyl
494 estradiol (EE); 6. Progesterone (P4).

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495 **Tables**

496 **Table 1.** Effect of temperature on the partition coefficients (K) and retention factors (k) of flavonoid glycosides on on C_{18}, cholesterol, C_8 and

497 ODP columns. (RE=Relative Error)

	ln$K$-1/T					ln$k$-1/T					$RE_{(\Delta S)}$ (%)	$RE_{(\Delta H)}$ (%)
	Intercept	Slope	R^2	ΔS^0 (J)	$-\Delta H^0$ (KJ)	Intercept	Slope	R^2	ΔS^0 (J)	$-\Delta H^0$ (KJ)		
C_{18}												
I	-11.21±0.22	4340.4±70.0	0.9992	-93.22	36086.5	-11.39±0.22	4204.5±67.5	0.9992	-95.97	34956.0	3.0	3.1
II	-10.00±0.18	4026.1±55.4	0.9994	-83.18	33472.7	-10.18±0.15	3890.9±47.9	0.9996	-85.96	32348.8	3.3	3.4
III	-11.36±0.18	4495.3±55.2	0.9996	-94.48	37374.1	-11.54±0.15	4360.1±47.6	0.9996	-97.26	36250.2	2.9	3.0
IV	-11.12±0.30	4420.7±93.1	0.9987	-92.45	36754.0	-11.29±0.28	4282.7±88.1	0.9987	-95.15	35606.3	2.9	3.1
V	-10.75±0.25	4416.1±78.5	0.9991	-89.34	36715.5	-10.91±0.23	4278.1±72.3	0.9991	-92.04	35567.7	3.0	3.1
VI	-12.00±0.26	4768.1±80.5	0.9992	-99.73	39642.3	-12.18±0.24	4634.9±73.8	0.9992	-102.55	38534.7	2.8	2.8
Cholesterol												

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I	-12.90±0.04	4824.6±12.7	0.9999	-107.25	40111.9	-12.93±0.07	4638.8±21.5	0.9999	-108.23	38567.3	0.9	3.9
II	-11.81±0.02	4512.7±6.2	0.9999	-98.19	37518.3	-11.84±0.05	4326.7±15.2	0.9999	-99.16	35972.3	0.9	4.1
III	-12.98±0.04	4920.9±13.0	0.9999	-107.91	40912.1	-13.01±0.07	4734.7±21.9	0.9999	-108.87	39364.0	0.9	3.8
IV	-12.49±0.01	4793.1±1.9	1.0000	-103.87	39849.9	-12.52±0.04	4607.0±11.2	0.9999	-104.83	38302.4	0.9	3.9
V	-12.15±0.03	4743.4±7.9	0.9999	-101.03	39436.7	-12.18±0.05	4557.3±16.6	0.9999	-102.00	37889.5	0.9	3.9
VI	-13.51±0.01	5213.2±2.8	1.0000	-112.33	43342.8	-13.54±0.04	5027.0±11.5	0.9999	-113.29	41794.6	0.9	3.6
C_8												
I	-9.43±0.03	3208.2±9.3	0.9999	-78.40	26673.0	-9.79±0.06	3317.3±18.3	0.9999	-74.93	27580.0	4.4	3.4
II	-8.58±0.03	2993.3±9.7	0.9999	-71.33	24886.3	-8.91±0.05	3093.5±14.4	0.9999	-67.62	25719.4	5.2	3.3
III	-8.99±0.13	3148.6±40.4	0.9995	-74.74	26177.5	-9.32±0.10	3247.3±32.4	0.9997	-71.03	26998.1	5.0	3.1
IV	-9.56±0.05	3333.4±16.4	0.9999	-79.48	27713.9	-9.89±0.03	3429.7±8.1	0.9999	-75.77	28514.5	4.7	3.0
V	-9.76±0.17	3450.2±53.2	0.9992	-81.14	28685.0	-10.07±0.15	3542.4±46.3	0.9995	-77.26	29451.5	4.8	2.7
VI	-8.70±0.39	3131.1±121.3	0.9955	-72.33	26032.0	-9.00±0.37	3220.6±114.9	0.9962	-68.37	26776.1	5.5	2.9

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ODP											
Ⅰ	-10.65±0.32	3609.1±101.1	0.9977	-88.54	30006.1	-14.07±0.12	4632.3±36.3	0.9998	-103.98	38512.9	17.4	28.3
Ⅱ	-9.84±0.29	3389.5±89.9	0.9979	-81.81	28180.3	-13.04±0.10	4348.8±30.5	0.9999	-95.42	36155.9	16.6	28.3
Ⅲ	-10.18±0.32	3521.8±100.1	0.9976	-84.64	29280.2	-13.29±0.05	4454.1±14.7	0.9999	-97.50	37031.4	15.2	26.5
Ⅳ	-10.81±0.36	3735.1±113.3	0.9973	-89.87	31053.6	-13.90±0.02	4660.0±7.7	0.9999	-102.57	38743.2	14.1	24.8
Ⅴ	-9.88±0.42	3485.1±132.5	0.9957	-82.14	28975.1	-12.77±0.13	4350.6±39.1	0.9998	-93.17	36170.9	13.4	24.8
Ⅵ	-11.50±0.16	4024.1±51.0	0.9995	-95.61	33456.4	-14.39±0.19	4887.6±60.5	0.9995	-106.64	40635.5	11.5	21.5

$$RE_{(\Delta S)}\% = \frac{\Delta S_k - \Delta S_K}{\Delta S_K} \times 100\%\ ;\quad RE_{(\Delta H)}\% = \frac{\Delta H_k - \Delta H_K}{\Delta H_K} \times 100\%$$

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500 **Table 2.** Best-fit parameters of intercept and slope, as well as correlation coefficients

501 (R^2) of Eq. 7 for C_{18}, cholesterol, C_8 and ODP columns.

	Intercept ($\frac{\Delta(\Delta S)}{R}$)	Slope ($-\frac{\Delta(\Delta H)}{R}$)	R^2
C_{18}			
$\ln\alpha_1$-1/T	1.208±0.054	-314.7±16.9	0.9914
$\ln\alpha_2$-1/T	-1.362±0.006	470.2±1.7	1.0000
$\ln\alpha_3$-1/T	0.250±0.148	-76.1±46.4	0.3617
$\ln\alpha_4$-1/T	-0.884±0.095	349.9±29.6	0.9788
$\ln\alpha_5$-1/T	1.253±0.012	-353.0±3.8	0.9997
Cholesterol			
$\ln\alpha_1$-1/T	1.090±0.021	-312.0±6.6	0.9987
$\ln\alpha_2$-1/T	-1.169±0.022	408.2±6.9	0.9992
$\ln\alpha_3$-1/T	0.486±0.035	-127.8±11.1	0.9778
$\ln\alpha_4$-1/T	0.341±0.020	-49.7±6.1	0.9560
$\ln\alpha_5$-1/T	-1.359±0.024	469.8±7.3	0.9993
C_8			
$\ln\alpha_1$-1/T	0.854±0.026	-214.9±8.2	0.9956
$\ln\alpha_2$-1/T	-0.420±0.148	156.2±46.3	0.9400
$\ln\alpha_3$-1/T	-0.561±0.087	181.9±27.1	0.9714
$\ln\alpha_4$-1/T	-0.192±0.125	115.8±39.1	0.9573
$\ln\alpha_5$-1/T	1.058±0.258	-319.1±80.7	0.8299
ODP			
$\ln\alpha_1$-1/T	0.814±0.039	-219.6±12.2	0.9907
$\ln\alpha_2$-1/T	-0.338±0.037	132.2±11.5	0.9777

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$\ln\alpha_3$-1/T	-0.634±0.043	213.3±13.5	0.9880
$\ln\alpha_4$-1/T	0.933±0.072	-250.0±22.5	0.9760
$\ln\alpha_5$-1/T	-1.628±0.278	539.0±86.8	0.9260

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503 **Table 3.** The comparison between fitted and calculated $\Delta(\Delta S)$ and $-\Delta(\Delta H)$ values

504 obtained on on C_{18}, cholesterol, C_8 and ODP columns.

	$\Delta(\Delta S)^a$	$\Delta(\Delta S)^b$	$RE_{\Delta(\Delta S)}$ (%)	$-\Delta(\Delta H)^a$	$-\Delta(\Delta H)^b$	$RE_{\Delta(\Delta H)}$ (%)
C_{18}						
$\ln\alpha_1$-1/T	10.047	10.039	0.1	-2616.7	-2613.8	0.1
$\ln\alpha_2$-1/T	-11.328	-11.304	0.2	3909.0	3901.4	0.2
$\ln\alpha_3$-1/T	2.081	2.038	2.1	-633.2	-620.1	2.1
$\ln\alpha_4$-1/T	-7.349	-7.282	0.9	2909.1	2888.3	0.7
$\ln\alpha_5$-1/T	10.416	10.390	2.5	-2935.1	-2926.8	0.3
Cholesterol						
$\ln\alpha_1$-1/T	9.064	9.063	0.01	-2593.9	-2593.0	0.03
$\ln\alpha_2$-1/T	-9.719	-9.712	0.07	3393.8	3393.8	0.00
$\ln\alpha_3$-1/T	4.045	4.044	0.02	-1062.4	-1062.2	0.02
$\ln\alpha_4$-1/T	2.832	2.832	0.00	-413.1	-413.1	0.00
$\ln\alpha_5$-1/T	-11.295	-11.295	0.00	3906.0	3906.0	0.00
C_8						
$\ln\alpha_1$-1/T	7.100	7.067	0.46	-1786.7	1786.7	0.00
$\ln\alpha_2$-1/T	-3.489	-3.409	2.29	1298.6	1291.2	0.57
$\ln\alpha_3$-1/T	-4.660	-4.739	1.70	1512.1	1536.4	1.61
$\ln\alpha_4$-1/T	-1.599	-1.663	4.00	963.1	971.1	0.83
$\ln\alpha_5$-1/T	8.794	8.813	0.22	-2653.4	2653.0	0.02
ODP						
$\ln\alpha_1$-1/T	6.765	6.73	0.52	-1825.6	1826.2	0.03
$\ln\alpha_2$-1/T	-2.814	2.83	0.57	1099.5	1099.9	0.04
$\ln\alpha_3$-1/T	-5.268	5.23	0.72	1773.7	1773.4	0.02

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$\ln\alpha_4$-1/T	7.755	7.73	0.32	-2078.6	2078.5	0.00
$\ln\alpha_5$-1/T	-13.533	13.47	0.47	4481.6	4481.3	0.00

505 [a] Best-fit parameters via Eq. 7; [b] calculated values obtained by relating data from

506 ln$K$-1/T plots in Table 1.

507 $$RE_{\Delta(\Delta S)}(\%) = \frac{\Delta(\Delta S)^b - \Delta(\Delta S)^a}{\Delta(\Delta S)^a} \times 100\% \; ; \; RE_{\Delta(\Delta H)}(\%) = \frac{-\Delta(\Delta H)^b - (-\Delta(\Delta H)^a)}{\Delta(\Delta H)^a} \times 100\%$$

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508 **Table 4.** Predicted and experimental values of selectivity factor on on C_{18}, cholesterol, C_8 and ODP columns at different temperatures.

	α_1		α_2		α_3		α_4		α_5	
	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental	Predicted	Experimental
C_{18}										
40°C	3.812	3.798	1.145	1.145	1.137	1.134	1.085	1.093	1.770	1.750
RE%	0.4		0.0		0.3		0.7		1.1	
50°C	3.755	3.728	1.143	1.142	1.113	1.102	1.077	1.073	1.853	1.894
RE%	0.7		0.1		1.0		0.4		2.2	
Cholesterol										
40°C	6.713	6.702	1.142	1.141	1.194	1.194	1.083	1.084	2.399	2.389
RE%	0.2		0.1		0.0		0.1		0.4	
50°C	6.245	6.261	1.136	1.136	1.144	1.144	1.070	1.081	2.507	2.492
RE%	0.3		0.0		0.0		1.0		0.6	

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C_8										
40°C	8.509	8.489	1.186	1.187	1.169	1.167	1.112	1.111	3.002	3.196
RE%	0.2		0.01		0.2		0.1		6.1	
50°C	7.869	8.070	1.184	1.185	1.127	1.140	1.095	1.101	3.185	3.125
RE%	2.5		0.1		1.1		0.5		0.0	
ODP										
40°C	8.662	9.131	1.186	1.189	1.099	1.100	1.155	1.159	1.528	1.530
RE%	5.1		0.2		0.1		0.4		0.1	
50°C	7.646	7.389	1.178	1.178	1.073	1.073	1.152	1.153	1.581	1.581
RE%	3.5		0.0		0.0		0.1		0.0	

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Figures

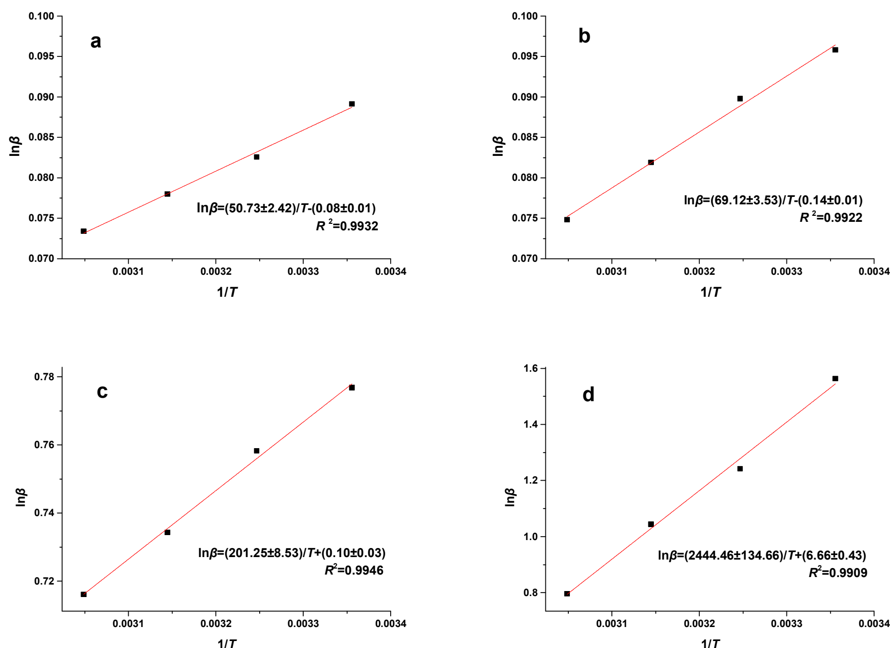

Figure 1. Effect of temperature on the phase ratio on C_{18} column (a), cholesterol column (b), C_8 column (c) and ODP column (d). Chromatographic conditions of C_{18} column: methanol-0.02 mol/L acetic acid (30:70, v/v), flow rate 1.0 mL/min, injection volume 10 μL; chromatographic conditions of cholesterol column: methanol-0.02 mol/L acetic acid (25:75, v/v), flow rate 0.4 mL/min, injection volume 10 μL; chromatographic conditions of C_8 column: methanol-0.02 mol/L acetic acid (30:70, v/v), flow rate 1.0 mL/min, injection volume 10 μL; chromatographic conditions of ODP column: methanol-0.02 mol/L acetic acid (30:70, v/v), flow rate 0.8 mL/min, injection volume 10 μL.

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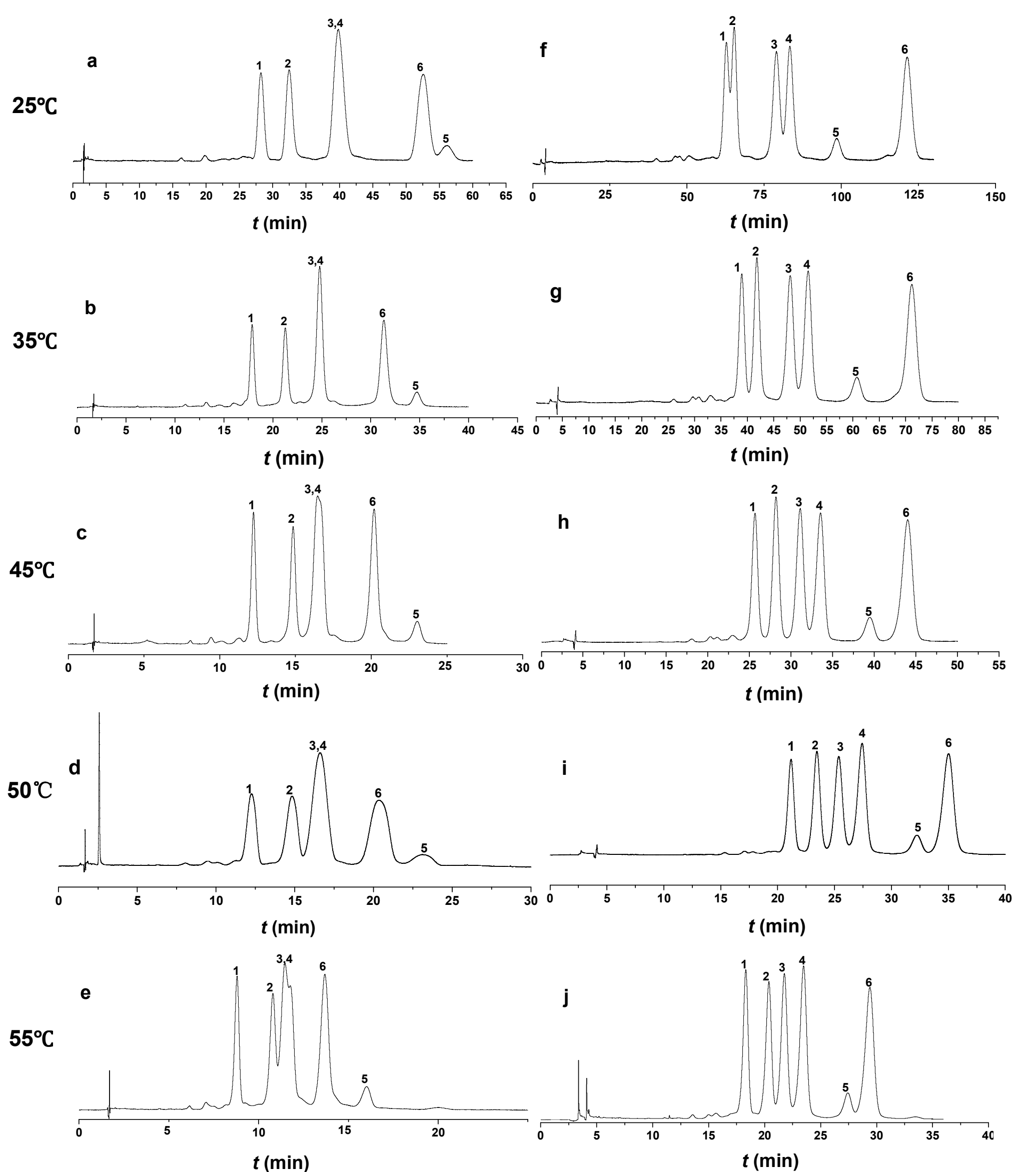

Figure 2. Chromatograms of the six flavonoid glycosides on C_{18} column (a, b, c, d and e) and cholesterol column (f, g, h, i and j) under different temperatures. See Fig. 1 for chromatographic conditions. Peaks: 1. flavonoid glycoside I (Schaftoside); 2. flavonoid glycoside II; 3. flavonoid glycoside III (Isoschaftoside); 4. flavonoid glycoside IV; 5. flavonoid glycoside V; 6. flavonoid glycoside VI.

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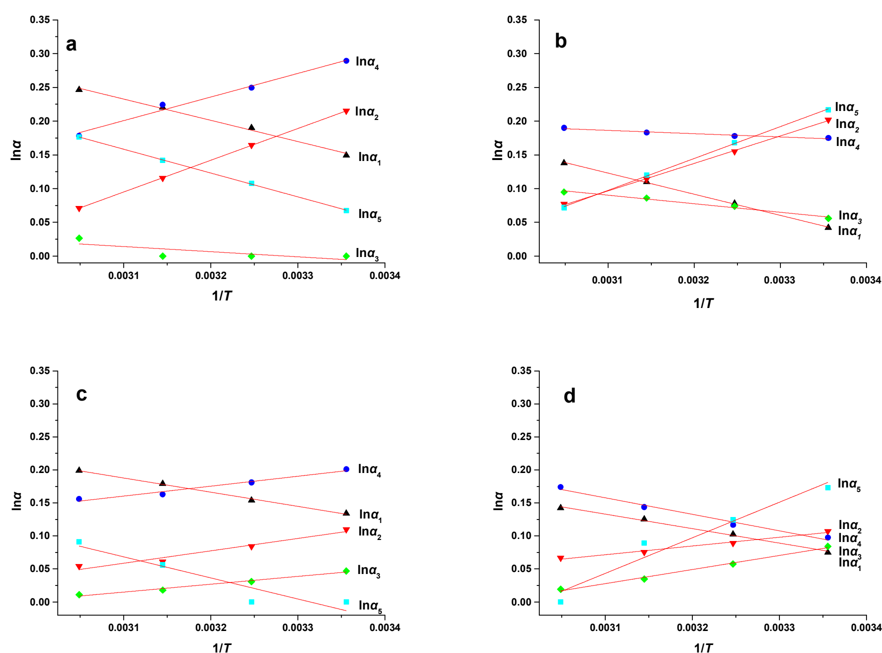

Figure 3. Effect of temperature on the selectivity factor on C_{18} column (a), cholesterol column (b), C_8 column (c) and ODP column (d). See Fig.1 for chromatographic conditions. ▲ α_1 (selectivity factor of I and II); ▼ α_2 (selectivity factor of II and III); ♦ α_3 (selectivity factor of III and IV); ● α_4 (selectivity factor of IV and V; ■ α_5 (selectivity factor of V and VI).

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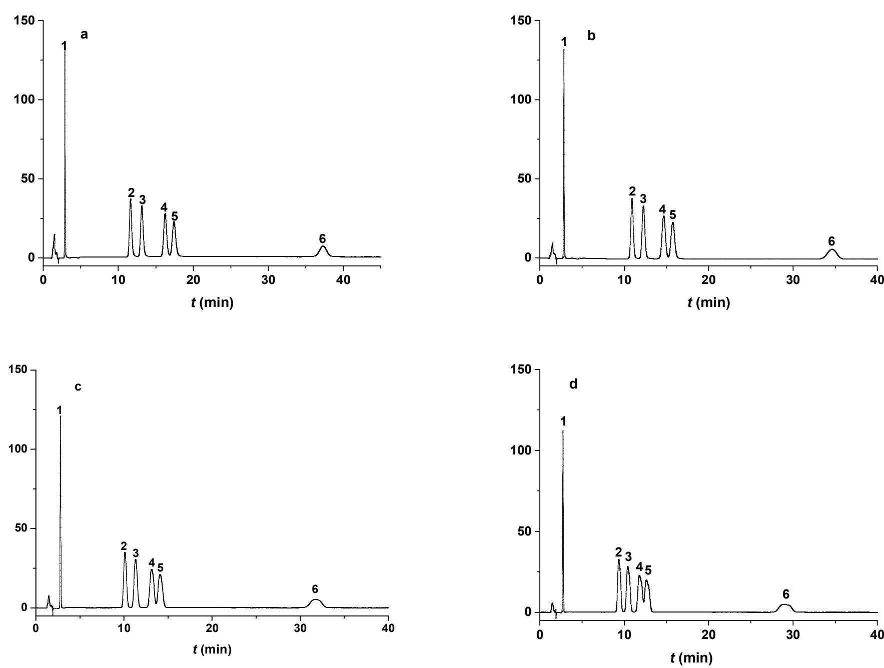

Figure 4. Chromatograms of the six steroid hormones on cholesterol column at different temperatures. (a) 20°C; (b) 30°C; (c) 40°C and (d) 50°C. Peaks: 1. Estriol (E3); 2. 17α-Estradiol (17α-E2); 3. 17β-Estradiol (17β-E2); 4. Estrone (E1); 5. Ethinyl estradiol (EE); 6. Progesterone (P4).

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