Separation of arachidonic acid metabolites by on-line extraction and reversed-phase high-performance liquid chromatography optimized by computer simulation

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Abstract

A complex mixture of arachidonic acid metabolites was separated by reversed-phase HPLC using a multi-step gradient, which was modelled by computer-assisted HPLC method development. The metabolites were extracted on-line on a precolumn connected to the analytical column in the same HPLC system. The predictions of the resolution and also the retention times calculated by computer simulation were very accurate when compared with the corresponding experimental run (maximum deviation 0.86%). An appropriate HPLC method and additionally an on-line extraction procedure could be developed with just three experimental HPLC runs. This method could be useful for evaluating the concentrations of arachidonic acid metabolites involved in inflammatory diseases.

1. Introduction

Arachidonic acid (20:4) is one of the major lipid constituents of cell membranes, which can be converted into a variety of extremely potent mediators exhibiting important physiological roles [1,2]. For example, 20:4 can be metabolized to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) by 5-lipoxygenase, which is primarily found in cells of the immune system. 5-HPETE is further converted either into leukotriene A 4 (LTA 4 ) via 5-lipoxygenase [3] or into 5-hydroxyeicosatetraenoic acid (5-HETE) by unspecified peroxidases [4]. Leukotriene A 4 is further metabolized in two ways: (i) in human polymorphonuclear granulocytes (PMN) to leukotriene B 4 (LTB 4 ), which possesses chemotactic activity to PMN [5] and (ii) in mast cells and basophilic granulocytes to vasoconstrictive leukotrienes C 4 , D 4 , E 4 (LTC 4 , D 4 , E 4 ) [6,7]. In addition to these pathways, 20:4 is converted into 12-hydroperoxyeicosatetraenoic acid [12-H(PE)TE] by 12-lipoxygenase in human platelets [8,9] or porcine leukocytes [10]. Further, in PMN from various species 20:4 can be converted into 15-hydroperoxyeicosatetraenoic acid [15-H(PE)TE] [11,12], which can be further metabolized to biological active lipoxins [13].

Reversed-phase high performance liquid chromatography (RP-HPLC) has become a powerful...
tool for the separation of 20:4 metabolites from biological samples [14-17]. When investigating 20:4 metabolites it is highly desirable to examine a complex mixture of leukotrienes (LTs), hydroxyeicosatetraenoic acids (HETEs) and lipoxins. In order to resolve 20:4 metabolites from biological samples they have to be extracted prior to separation by HPLC. Various extraction techniques have been investigated such as solid-phase extraction and procedures with cartridges containing octadecylsilica (ODS) as the stationary phase [18-20] or liquid-liquid extraction with ethyl acetate [21] or diethyl ether [22]. The main disadvantage of these time-consuming techniques is the low recovery. Therefore, in recent studies on-line extraction methods have been investigated [23,24]. This approach is very effective in terms of the time reduction required for the analysis of samples. It also yields more reliable results as sample loss and deterioration could be excluded. Nevertheless, all of these procedures need additional equipment that is not available in most laboratories and additional time in HPLC method development.

For the development of a reversed-phase gradient method, technical experience with HPLC is necessary in order to establish appropriate separation conditions with a minimum of effort and time. Many experimental chromatograms have to be obtained in order to optimize the stationary phase, the mobile phase and the appropriate temperatures.

Taken together, every individual step during optimization needs a single chromatogram to be obtained without the prospect of success. To minimize the time for method development, without experimentally changing columns and mobile phases, the computer-assisted HPLC optimization program Drylab/Windows has been developed [25]. The software simulates experimental HPLC traces after entering the results of only two experimental runs. Completely new experimental conditions can be modelled. It is able to calculate the influence of gradient compositions for different column conditions with respect to column diameter, column length, particle size, temperature and flow-rate. Poor resolution of bands as a result of unsuitable elution conditions can be monitored early without wasting time on optimization. Finally, the optimum HPLC separation conditions simulated can usually be confirmed by one final experimental run.

The aim of this work was to develop an on-line extraction HPLC method for the detection of a complex mixture of arachidonic acid metabolites, without additional equipment, using Drylab for optimization.

2. Experimental

2.1. Standards

Prostaglandin B\textsubscript{2}, LTB\textsubscript{4}, 20-OH-LTB\textsubscript{4}, 20-COOH-LTB\textsubscript{4}, 6-trans, 12-epi-LTB\textsubscript{4}, all-trans-LTB\textsubscript{4}, 12-HETE, 15-HETE, 5-HETE, and 5-HPETE were obtained from Cayman Chemicals (Ann Arbor, MI, USA). A mixture of the above 20:4 metabolites was diluted to a final concentration of 1 \(\mu\)g/ml in ethanol [cleaned by passing through SepPak C\textsubscript{18} solid-phase extraction columns (Waters/Millipore, Eschborn, Germany)]. The diluted mixture was thoroughly degassed with nitrogen in order to diminish the autooxidation of the 20:4 metabolites. Mobile phase components were of HPLC grade and purchased from Riedel-de Haën (Seelze, Germany). All other reagents were of analytical-reagent grade.

2.2. HPLC equipment

The mobile-phase delivery system consisted of a Sykam (Gilching, Germany) S1000 pump, an S2110 lower gradient mixer and an S4110 column oven. The S3300 UV detector was connected to an Axxiom integration system. For on-line extraction an Axxiom-controlled pneumatic switching valve (Rheodyne Model 7010) integrated into the column oven was used (Fig. 1). Sample injection was performed with an autosampler (Promis Spark-Holland, Friedrichsdorf, Germany). Drylab/Windows (LC Resources) was purchased from Molnar-Institut (Berlin, Germany).
2.3. Procedures for on-line extraction

The precolumn of 3-μm Sperhisorb ODS-2 (1 x 0.46 cm I.D.), (Grom, Herrenberg, Germany) was equilibrated with eluent C [acetonitrile–water–trifluoracetic acid (TFA) (10:90:0.02 (v/v/v))] for 10 min. at a flow-rate of 1.5 ml/min at 40°C before injection. After injection of the sample (50 μl) the precolumn was eluted with eluent C at a flow-rate of 1.5 ml/min at 40°C for 10 min before the precolumn was automatically switched in front of the 3-μm Ultrasphere ODS highspeed analytical column (7.5 x 0.46 cm I.D.) (Beckman Instruments, Palo Alto, CA, USA). A mixture of 20:4 metabolites (30 ng each of 20-COOH-LTB₄, 20-OH-LTB₄, PGB₂, LTB₄, 15-HETE, 12-HETE, 5-HETE, 25 ng each of 6-trans, 12-epi-LTB₄ and 10 μg of all-trans-LTB₄ diluted in 100% ethanol) was eluted with different compositions of eluent A [acetonitrile–water–TFA (20:80:0.02 (v/v/v))] vs. eluent B [acetonitrile–methanol–water–TFA (75:22:3:0.02 (v/v/v)]. Leukotrienes were monitored at 270 nm and HETEs at 235 nm. Finally, the columns were re-equilibrated.

3. Results

3.1. Mobile phase optimization

In order to optimize the mobile phase for RP-HPLC methods using the computer, two initial experimental runs had to be performed. These two runs had to differ at least three-fold with respect to their gradient run times \( t_G \). Therefore, the first run was performed by injecting a mixture of 20:4 metabolites (for concentrations see Section 2.3) on to the precolumn, which was flushed with eluent C (on-line extraction) for 10 min prior to elution of the metabolites from the analytical column with a linear gradient from 10% to 95% eluent B in 30 min. The resulting chromatogram is shown in Fig. 2. The second experimental run was performed by eluting the same mixture with a 90-min linear gradient from 10% to 95% B after on-line extraction (Fig. 3). The operating variables of the gradient ranges, such as dwell volume, flow-rate, column width, length and number of bands, were entered into the corresponding menus of the mobile-phase optimization option (Drylab). Finally, the retention times and band areas were entered (Table 1).

3.2. Determination of system variables

For the prediction of gradient runs it was essential to determine the system dwell volume, \( V_D \), which is the volume between the place of mixing A and B and the column inlet. The volume is responsible for the gradient delay, leading to different retentions of bands in different HPLC systems. The dwell volume was calculated by comparing an experimental run of 60 min with the prediction obtained by simulation. The dwell volume was changed manually in the Drylab options until the prediction of the retention times was close to those in the experimental run. The calculated value for the dwell volume of this HPLC system was \( V_D = 3.4 \) ml. Table 2 shows that following adaption of the dwell volume the maximum difference in retention time was less than 0.14 min for the bands of the 20:4 metabolite mixture.
3.3. Gradient optimization

In order to see how well the 20:4 metabolite mixture could be resolved by changing the values of %B/min during linear gradients without changing the mobile phase and the stationary phase, the $k^*$ value calculated by Drylab had to be taken into account. The $k^*$ value (see Eq. 1) affects the overall sample resolution and defines the retention behaviour of bands with respect to their chemical properties ($S$), the change in %B ($\Delta \phi$), the flow-rate ($F$), the column dead volume ($V_m$) and the gradient time ($t_0$). The $k^*$ value depends on the above-mentioned parameters and therefore directly influences the resolution ($R_s$) of bands (Eqs. 2–4).

$$k^* = \frac{t_0 F}{(1.15V_m \Delta \phi S)} \quad (1)$$

$$k' = \frac{(t_R - t_0)}{t_0} \quad (2)$$

$$\alpha = k'(\text{band 2}) / k'(\text{band 1}) \quad (3)$$

$$R_s = \frac{1}{4}(\alpha - 1)n^{0.5}[k^*/(k^* + 1)] \quad (4)$$

In order to obtain accurate separation of bands, $k^*$ (Eq. 1) should be in the range of 5–20 kbar; $k^* < 5$ would lead to poor resolution as a result of the sample bands bunching together, whereas bands with $k' > 20$ are more widely separated and would lead to a decrease in sensitivity.

To monitor the quality of equal band spacing of the 30 and 90-min runs the software simulated a relative resolution map (RRM). The RRM provides information on the resolution of the poorest separated band pair vs. gradient time, based on a selected column plate number.

The resolution of bands 5 and 6 is, experimentally, $R_s = 1.1$ for a 90-min linear gradient. To obtain the same resolution in the software chro-
Fig. 3. Experimental chromatogram for the separation of a mixture of 20:4 metabolites (for concentrations and RP-HPLC method, see Experimental) with a linear gradient over 90 min.

The RRM provides evidence that the most critical band pair 4 and 5 (all-trans-LTB⁴ and 6-trans, 12-epi-LTB⁴) could be resolved maxi-

Table 1
Retention data for the separation of 20:4 metabolite mixture (for concentrations and HPLC method, see Experimental) using experimental linear gradients over 30 and 90 min

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>20:4 metabolite</th>
<th>Retention time (min)</th>
<th>Area (V min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>30-min run</td>
<td>90-min run</td>
</tr>
<tr>
<td>1</td>
<td>20-COOH-LTB⁴</td>
<td>4.59</td>
<td>5.34</td>
</tr>
<tr>
<td>2</td>
<td>20-OH-LTB⁴</td>
<td>4.83</td>
<td>5.68</td>
</tr>
<tr>
<td>3</td>
<td>PGB2</td>
<td>10.13</td>
<td>16.41</td>
</tr>
<tr>
<td>4</td>
<td>All-trans-LTB⁴</td>
<td>11.91</td>
<td>21.22</td>
</tr>
<tr>
<td>5</td>
<td>6-trans-12-epi-LTB⁴</td>
<td>12.03</td>
<td>21.62</td>
</tr>
<tr>
<td>6</td>
<td>LTB⁴</td>
<td>12.26</td>
<td>22.14</td>
</tr>
<tr>
<td>7</td>
<td>15-HETE</td>
<td>17.50</td>
<td>36.29</td>
</tr>
<tr>
<td>8</td>
<td>12-HETE</td>
<td>18.32</td>
<td>38.53</td>
</tr>
<tr>
<td>9</td>
<td>5-HETE</td>
<td>19.02</td>
<td>40.46</td>
</tr>
<tr>
<td>10</td>
<td>5-HPETE</td>
<td>19.59</td>
<td>42.38</td>
</tr>
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</table>
Table 2
Differences in retention data based on an experimental 60-min linear gradient and Drylab-calculated retentions after adjustment of the system's dwell volume (for HPLC methods, see Experimental)

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>20:4 metabolite</th>
<th>Retention time (min)</th>
<th>DrylabG-predicted, 60-min run</th>
<th>Experimental, 60-min run</th>
<th>Predicted – experimental (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20-COOH-LTB4</td>
<td>5.12</td>
<td>4.98</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>20-OH-LTB4</td>
<td>5.41</td>
<td>5.32</td>
<td>0.09</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PGB2</td>
<td>13.83</td>
<td>13.81</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>All-trans-LTB4</td>
<td>17.21</td>
<td>17.30</td>
<td>-0.09</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>6-trans-12-epi-LTB4</td>
<td>17.47</td>
<td>17.50</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>LTB4</td>
<td>17.85</td>
<td>17.88</td>
<td>-0.03</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>15-HETE</td>
<td>27.71</td>
<td>27.71</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>12-HETE</td>
<td>29.26</td>
<td>29.24</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>5-HETE</td>
<td>30.59</td>
<td>30.56</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5-HPFETE</td>
<td>31.82</td>
<td>31.80</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Optimum system dwell volume calculated by DrylabG = 3.4 ml.

Changes in %B/min had only a marginal influence of the resolution of bands 5 and 6, (6-trans, 12-epi-LTB4 and LTB4) (Fig. 3). Hence the separation of bands 4–6 is not much affected by the gradient steepness. The remaining bands were less influenced by changes in %B/min.

3.4. Simulation of multi-segmented gradient runs

The gradient was theoretically performed in four steps starting with the isocratic elution of the metabolites after on-line extraction with 10% B for 1 min. Bands 1 and 2 were eluted shortly after the system dwell volume (dwell volume 3.425 ml = 2.28 min) and they were separated with a resolution $R_s = 1.87$ shortly after initiating the next step (see modelled chromatogram, Fig. 4).

![Relative resolution map for bands 4 and 5 in the separation of the 20:4 metabolite mixture. The calculated resolutions ($R_s$) are based on the input data for 30- and 90-min linear gradients ($t_o$) from 10% to 90% B. For input data, see Table 1.](image)
Fig. 5. Simulated chromatogram for the separation of 20:4 metabolite mixture based on a four-step gradient (for gradient, see Table 3).

5). This was achieved by changing from 10 to 37% B in 11 min. The slope of the %B/min change was flatter than for the 30-min linear gradient in order to obtain a better resolution of bands 4 and 5. During this gradient time band 3 eluted without overlapping bands (Fig. 5).

Bands 4 and 5 could only be resolved with $R_s = 0.85$. The system gave a non-baseline separation of these three peaks. However, the resolution was slightly better compared with the 30-min linear gradient run ($R_s = 0.62$). Bands 5 and 6 were only resolved with $R_s = 1.11$.

After the elution of leukotrienes and prostaglandins, the wavelength of the detector was changed to 235 nm in order to monitor eicosatetraenoic acids. This next gradient step was performed by an increase from 23% to 58% B in 13 min until all eicosatetraenoic acids were eluted. Finally, the column was cleaned with 95% B. Bands 7–10 were all eluted with a resolution of at least 2.89.

The last band was eluted with a retention time of 24.66 min. Instead of a rapid increase of %B/min a more shallow gradient was selected for the elution of bands 7–10 in order to decrease the baseline drift after changing the detector wavelength to 235 nm. However, on comparing the resulting resolutions of bands 7–10 examined in the 90-min linear gradient with those in the four-step gradient, the conclusion was drawn that essentially the same resolution was obtained but the retention time was 20 min less. Hence about 30 ml less of mobile phase were needed to achieve the same resolution compared with the 90-min linear gradient run.

3.5. Predicted separation vs. experiment

Comparison of the chromatogram modelled by Drylab (Fig. 5) with the experimental run (Fig. 6) showed that the prediction was very close to the experimental data with a maximum deviation of 0.86% (Table 3).
4. Discussion

On-line extraction combined with RP-HPLC provides an efficient method for the analysis of complex mixtures of 20:4 metabolites in a single run. This method can readily be used for automation, resulting in additional time saving. An important advantage of the on-line extraction

Table 3
Comparison and differences in retention data obtained from experimental and Drylab-calculated separation of 20:4 metabolites using a four-step gradient (0–1 min, 10% B; 1–12 min, 10–37% B; 12–25 min, 37–58% B; 25–26 min, 58–95% B)

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>20:4 metabolite</th>
<th>Retention time (min)</th>
<th>Difference, predicted – experimental (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Experimental run</td>
<td>DrylabG-predicted</td>
</tr>
<tr>
<td>1</td>
<td>20-COOH-LTB4</td>
<td>5.32</td>
<td>5.36</td>
</tr>
<tr>
<td>2</td>
<td>20-OH-LTB4</td>
<td>5.59</td>
<td>5.61</td>
</tr>
<tr>
<td>3</td>
<td>PGB2</td>
<td>11.63</td>
<td>11.72</td>
</tr>
<tr>
<td>4</td>
<td>All-trans-LTB4</td>
<td>13.72</td>
<td>13.80</td>
</tr>
<tr>
<td>5</td>
<td>6-trans-12-epi-LTB4</td>
<td>13.83</td>
<td>13.95</td>
</tr>
<tr>
<td>6</td>
<td>LTB4</td>
<td>14.08</td>
<td>14.20</td>
</tr>
<tr>
<td>7</td>
<td>15-HETE</td>
<td>21.20</td>
<td>21.28</td>
</tr>
<tr>
<td>8</td>
<td>12-HETE</td>
<td>22.44</td>
<td>22.55</td>
</tr>
<tr>
<td>9</td>
<td>5-HETE</td>
<td>23.54</td>
<td>23.65</td>
</tr>
<tr>
<td>10</td>
<td>5-HPETE</td>
<td>24.52</td>
<td>24.63</td>
</tr>
</tbody>
</table>
RP-HPLC is that the sample is transferred directly from the precolumn to the analytical column. Therefore, sample extraction procedures followed by evaporation of the eluent prior to injection are no longer necessary. This technique provides accurate method in order to save time and decrease sample losses and also to decrease sample deterioration (unpublished data). Additionally, the same precolumn could be used several times because it is always cleaned in connection with the analytical column.

The computer modelled very accurate predictions and was a rapid and reliable tool for optimizing the separation of a complex mixture of 20:4 metabolites.

Most of the 20:4 metabolites were well resolved using linear gradients. Nevertheless, the separation of three bands had to be optimized. These critical band pairs correspond to all-trans-LTB₄ (band 4), 6-trans, 12-epi-LTB₄ (band 5), which are non-enzymatic hydrolysis products of LTA₄, and the main LTA₄-hydrolase product LTB₄ (band 6). The structures of these LTs are closely related. All three stereoisomeric compounds are hydroxylated at positions 5 and 12 of the arachidonic acid. The conformational structures of these leukotrienes, however, differ: 5(S),12(S)-DiHETE = 6-trans, 12-epi-LTB₄, 5(S),12(R)-DiHETE with a trans conformation = all-trans-LTB₄, and 5(S),12(R)-DiHETE with a cis conformation = LTB₄.

The retention behaviour and band width of biological samples are influenced by the chemical properties of the sample [26,27]. All injections were performed with acidic samples in order to protonate the 20:4 metabolites and to exclude silanol interactions, which affect the separation. Parameters such as the ionic strength of the mobile phase and the ionic strength of the samples cannot be entered for column optimization by Drylab. However, the predicted resolutions, after changing the column plate number in Drylab, were close to the calculated resolutions from the experimental run. As LTB₄ (band 6), the major LTA₄-hydrolase product of human PMN, which was the metabolite of main interest, was eluted 1.2 min after the two stereoisomeric LTs, the reduced column plate number of the column used was of minor importance.

5. Conclusions

The prediction of the retention times of the 20:4 metabolites, using the four-step gradient, was very accurate. The maximum difference in retention time between the prediction and the corresponding experimental run was 1.21%.

The on-line extraction RP-HPLC method described here is suitable for the detection of less than 1 ng of LTs and 1 ng of HETEs in biological samples. The 5-lipoxygenase product 5-HPETE is one of the most critical metabolites in the 20:4 metabolite mixture. It is unstable and will be reduced to 5-HETE. Additionally, 5-HETE could not be resolved from 5-HPETE by RP-HPLC using gradients with only acetonitrile as the organic eluent [16]. Therefore, until now 5-HPETE has been reduced by NaBH₄ [28] in order to examine the 5-lipoxygenase activity or to separate 5-HPETE by normal-phase HPLC (NP-HPLC). The reported detection limit of 5-HPETE was above 10 ng owing to quenching effects of the organic eluents used [16]. In contrast, the on-line extraction RP-HPLC method reported here demonstrated that 5-HPETE, in addition to 5-HETE, could be resolved and determined. The detection limit (5 ng) of 5-HPETE was lower than in NP-HPLC.

In addition to the above-mentioned separation of the 20:4 metabolite mixture, this four-step gradient optimized by Drylab is also capable of separating peptido-leukotrienes (LTC₄, LTD₄, LTE₄), LTA₄ hydrolase products (5,6-DiHETEs) and the corresponding lipoxygenase products 15-HPETE and 12-HPETE together with the 20:4 metabolites in a single run. These data will be published in subsequent paper.

In summary, Drylab was useful and accurate in this application, but not much improvement in resolution was achieved by computer simulation. The use of the software avoided the need for additional experimental runs and showed that with these separation conditions no improvement in performance could be obtained.
Abbreviations and symbols

20-COOH-LTB₄

\[ [5(S),6Z,8E,10E,12(R),14Z]-5,12\text{-dihydroxy}-20\text{-carboxy} \text{eicosatetraen-1-oic} \text{acid} \]

20-OH-LTB₄

\[ [5(S),6Z,8E,10E,12(R),14Z]-5,12\text{-20-trihydroxy} \text{eicosatetraen-1-oic} \text{acid} \]

LTB₄

\[ [5(S),6Z,8E,10E,12(R),14Z]-5,12\text{-dihydroxyeicosatetraen-1-oic} \text{acid} \]

6-trans,12-epi-LTB₄

\[ [5(S),6E,8E,10E,12(S),14Z]-5,12\text{-dihydroxyeicosatetraen-1-oic} \text{acid} \]

All-trans-LTB₄

\[ [5(S),6E,8E,10E,12(R),14Z]-5,12\text{-dihydroxyeicosatetraen-1-oic} \text{acid} \]

5-HETE

\[ [5(S),6E,8Z,11Z,14Z]-5\text{-hydroxyeicosatetraen-1-oic} \text{acid} \]

12-HETE

\[ [5Z,8Z,10E,12(R),14Z]-12\text{-hydroxyeicosatetraen-1-oic} \text{acid} \]

15-HETE

\[ [5Z,8Z,11Z,13E,15(S)]-15\text{-hydroxyeicosatetraen-1-oic} \text{acid} \]

5-HPETE

\[ [5(S),6E,8Z,11Z,14Z]-5\text{-hydroperoxyeicosatetraen-1-oic} \text{acid} \]

20:4

\[ (5Z,8Z,11Z,14Z)\text{-eicosatetraen-1-oic} \text{acid} \]

(archachidonic acid)

\( k' \)
capacity factor

\( k^* \)
average \( k' \) value during gradient runs

\( N \)
thoretical column plate number

\( R_s \)
resolution

\( t_R \)
retention time for samples (min)

\( t_0 \)
column dead time (min)

\( t_G \)
gradient time (min)

\( F \)
flow-rate (ml/min)

\( V_m \)
column dead volume (ml) = \( t_0F \)

\( S \)
100S is the negative slope of log \( k' \) vs. % organic component plots

\( \Delta \phi \)
change in organic volume fraction of the mobile phase (%B/100)

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References