Possibility of Large Volume Injection and Band Focusing in UHPLC

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New shell-type stationary phases are widely used in fast chromatographic measurements. These columns provide more efficient separation, when applied in a conventional high-performance liquid chromatography instrument, than columns with fully porous particles, and the volume overload of core-shell particles is 60% of the value obtained for fully porous particles. Additionally, to achieve adequate sensitivity, the injection volume cannot be significantly decreased.

This study presents a systematic evaluation of the possibilities of large volume injection onto columns packed with 2.6 μm Kinetex C18 shell particles. The effect of volume overload on performance of columns with different lengths (50, 100 and 150 mm) is studied. Column efficiency is compared under isocratic, pulse gradient and gradient conditions. The application of large volume injection in practice is also reported. The most suitable among the tested large volume injection techniques was the gradient elution, which was applied to determine amino acid enantiomers from fruit juice.

Introduction

The history of core-shell or superficially porous stationary phases started in the late sixties, when Horváth and co-workers applied glass bead particles covered with ion exchange resin (1). Today, vendors offer several types of core-shell stationary phases (e.g., Halo-C18, Kinetex C18, Poroshell and YMC Triart). Kinetex particles consist of a 1.9 μm solid silica core and a 0.35 μm thick layer of porous silica. Recently published studies deal with the kinetic performance (2, 3) and physical properties (4) of shell-type stationary phases. These columns can provide high efficiency, but the performance of core shell particles is much more sensitive to sample overload than fully porous particles (5). On the other hand, the sensitivity of the analytical method can be increased by injecting large volumes onto the chromatographic column.

The question arises whether it is possible to achieve high efficiency and to inject large volumes onto the chromatographic column at the same time. One solution is on-column focusing, when the injection solvent in which the sample is dissolved is of lower elution strength than the mobile phase (6). In extreme cases, the sample can be dissolved in a non-eluting solvent (typically in water, in the case of reversed-phase high-performance liquid chromatography (RP-HPLC) methods) (7). In some cases, on-column focusing causes problems. Layne and co-workers found that the injection of caffeine, dissolved in a solvent of lower eluting strength than that of the mobile phase, caused peak distortion (8). Lough et al. experienced the adsorption of indomethacin in an injection system in which the sample was injected in water (9).

Sample injection followed by a water plug is another possibility for sample band compression. In this case, a water plug, which is a non-eluting medium, focuses the sample at the front of the column. At the same time, this water plug decreases the efficient column length. Gritti et al. injected water immediately after injection of the sample to provide mixing of the water and the sample at the front of the column. Improvements in resolution and efficiency were reported (10). Li and co-workers applied a pulse gradient to achieve increased injection volume and lower limit of detection (11).

Gradient elution also has band focusing effects. On one hand, gradient elution is commonly used for shortening the analysis time of complex samples in which the retention factors of the compounds are very different. On the other hand, gradient elution is applied to decrease peak width. Schellinger et al. highlighted that gradient elution is also suitable for the separation of compounds with similar retention factors, because gradient elution provides shorter analysis time and narrower peak width than isocratic elution (12). The band compression effect of gradient elution has been studied from the early eighties (13) to today (14, 15).

In this work, serine enantiomer derivatives were used to study column performance under different conditions: isocratic, pulse gradient and gradient elution. L- and D-serines were derivatized with Marfey’s reagent [1-fluoro-2,4-dinitrophenyl-5-L-alanine-amide (FDAA)], which was synthesized and applied for the first time by Peter Marfey for the determination of D-amino acids (16). Later, his method was applied for the determination of the absolute configuration of amino acids from samples obtained by hydrolysis of different peptides, proteins and amino acid derivatives (17). Recently, Harada and co-workers further developed Marfey’s method, and instead of using ultraviolet-visible (UV-VIS) detection, they applied liquid chromatography–mass spectrometry (LC–MS) with fast atom bombardment or electrospray for the determination of derivatives. This method is referred to as advanced Marfey’s method (18).

In this work, Kinetex C18 columns with different column lengths (50–4.6, 100–4.6 and 150–4.6 mm) were studied in a Perkin Elmer Flexar UHPLC system. The possibilities of large-volume injection under isocratic, gradient and pulse gradient conditions were studied by the injection of derivatives of serine enantiomers. The most efficient method was applied for the determination of alanine, asparagine, glutamine and serine enantiomers from fruit juice.
Experimental

Instrumentation and reagents

UHPLC analyses were performed on a Perkin Elmer Flexar UHPLC system equipped with binary pump, autosampler, column oven and UV detector controlled by Chromera-Flexar-15 software.

Kinetex C18 columns packed with 2.6 μm shell particles (50–4.6, 100–4.6 and 150–4.6 mm) were obtained from Gen-Lab (Budapest, Hungary).

Amino acid standards (L- and D-alanine, L- and D-asparagine, L- and D-glutamine and L- and D-serine) and FDAA (Marfrey’s reagent) were purchased from Sigma-Aldrich (Budapest, Hungary). Acetonitrile, hydrochloric acid (to stop the derivatization reaction), acetic acid and sodium hydrogen carbonate (for sample buffering) were obtained from Merck (Darmstadt, Germany). Triethylamine was purchased from Riedel-de-Haën (Seelze, Germany). For the measurements, water was freshly prepared every day by using a MilliQ Synergy UV apparatus Millipore (Billerica, MA). Membrane filters with average pore sizes of 0.22 μm were obtained from La-Pha-Pack (Budapest, Hungary).

Methods

The buffered mobile phase for isocratic measurements was prepared by mixing acetonitrile and triethylammonium acetate buffer (pH 4; 3 g/L) in proportions of 20:80 (v/v), then filtering through a 0.22 μm membrane filter before use. This mobile phase composition was also applied in the pulse gradient study as mobile phase A, with purified water as mobile phase B.

In the gradient elution study, mobile phase A was a premixed 10% acetonitrile–90% triethylammonium acetate buffer (pH 4; 3 g/L) and mobile phase B was pure acetonitrile.

Volume overload of the column was tested by injecting regularly varied volumes from 10 to 50 μL in steps of 10 μL, and the smallest injection volume was 5 μL. Each measurement was triplicate.

In the case of pulse gradient (10), the effect of the pulse time on column efficiency was also studied. Five different pulse times (0.1, 0.2, 0.3, 0.5 and 1 min) were applied to acquire efficiency data, injection volume was set to 5 μL.

In the gradient elution study, the ratio of aqueous buffer and organic modifier was adjusted to provide the same apparent retention factor, approximately 2.1 and 2.3, for serine derivatives.

For the experiments of fruit juice, the same mobile phase was applied as for gradient elution. Gradient started from 0 to 25% B in 30 min. The temperature of the Kinetex C18 column (150–4.6 mm) was maintained at 30°C. The injection volume was 50 μL and detection was performed at 340 nm.

Results and Discussion

Column performance under isocratic conditions

The increase in the apparent theoretical plate number was 1.3–1.5 times higher for the 5 cm column than those obtained under isocratic conditions, theoretical plate numbers decreased with increasing injection volumes. Under the experimental conditions described here, the lowest efficiency was observed for the 5 cm long column with theoretical plate number ~165,000 per meter. The 10 and 15 cm columns provided theoretical plate number of ~200,000 per meter. The negligible difference in N of 10 and 15 cm long columns is expected solely from the packing efficiency of the columns.

Figure 1 shows a typical series of chromatograms as the injection volume is increased from 5 to 50 μL.

In comparison with 10 and 15 cm columns, in which an injection of 10 instead of 5 μL caused a slight decrease in efficiency, more than 10% efficiency loss was obtained with a 5 cm column.

<table>
<thead>
<tr>
<th>Column length</th>
<th>5 cm</th>
<th>10 cm</th>
<th>15 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume injected (μL)</td>
<td>L-ser</td>
<td>D-ser</td>
<td>L-ser</td>
</tr>
<tr>
<td>5</td>
<td>165,327</td>
<td>170,793</td>
<td>203,350</td>
</tr>
<tr>
<td>10</td>
<td>156,987</td>
<td>164,120</td>
<td>203,060</td>
</tr>
<tr>
<td>20</td>
<td>125,653</td>
<td>137,573</td>
<td>184,210</td>
</tr>
<tr>
<td>30</td>
<td>100,940</td>
<td>119,927</td>
<td>196,873</td>
</tr>
<tr>
<td>40</td>
<td>98,907</td>
<td>115,380</td>
<td>194,714</td>
</tr>
<tr>
<td>50</td>
<td>69,080</td>
<td>91,900</td>
<td>191,940</td>
</tr>
</tbody>
</table>

*Note: Theoretical plate numbers were normalized to column length and measured with three replications.

Figure 1. Chromatograms showing the effects of injection volume on peak shape. Stationary phase: Kinetex C18 (50–4.6 mm, 2.6 μm), mobile phase, 20:80 acetonitrile–triethylammonium acetate buffer (pH 4, 3 g/L), temperature, 30°C, detection wavelength, 340 nm.
with the 10 and 15 cm columns. The pulse gradient time has a significant effect on column performance in each case. The best results were acquired with a 1 min gradient time: approximately 6–700,000 per meter apparent theoretical plates for 5 and 10 cm long columns and approximately 400,000 per meter for 15 cm long column (data are detailed in Table II).

Under pulse gradient conditions, band and peak width is affected by peak compression, and by extra-column and column contributions to band broadening. This peak focusing effect is significant in the case of the 10 cm long column, but in the case of 15 cm long column, the effect is not considerable.

Retention times of the compounds increased with pulse time (Figure 3).

After optimization of the pulse gradient time, the volume overload was studied. For these measurements, a 1 min pulse time was set, which provided the best column performance.

In the case of the 5 µL injection volume, the 5 cm Kinetex C18 column showed the best column performance (N approximately 6–700,000), which was approximately 1.5 times higher than observed with longer columns.

### Table II

**Effect of Pulse Gradient Time**

<table>
<thead>
<tr>
<th>Column length</th>
<th>5 cm</th>
<th>10 cm</th>
<th>15 cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N (1/m)</td>
<td>N (1/m)</td>
<td>N (1/m)</td>
</tr>
<tr>
<td>Water plug (min)</td>
<td>L-ser</td>
<td>D-ser</td>
<td>L-ser</td>
</tr>
<tr>
<td>0</td>
<td>182,360</td>
<td>196,467</td>
<td>253,300</td>
</tr>
<tr>
<td>0.1</td>
<td>170,900</td>
<td>258,560</td>
<td>286,993</td>
</tr>
<tr>
<td>0.2</td>
<td>231,140</td>
<td>357,080</td>
<td>361,753</td>
</tr>
<tr>
<td>0.3</td>
<td>263,553</td>
<td>395,253</td>
<td>391,883</td>
</tr>
<tr>
<td>0.5</td>
<td>360,327</td>
<td>479,627</td>
<td>465,823</td>
</tr>
<tr>
<td>1</td>
<td>575,447</td>
<td>703,580</td>
<td>606,000</td>
</tr>
</tbody>
</table>

*Note: Theoretical plate numbers were normalized to the column length and measured with three replications.

### Figure 2

Effect of pulse gradient time on the efficiency of columns with different lengths. L: 5, L: 10 and L: 15 are the lengths of the columns in cm; ser: 1 and ser: 2 stand for L-serine and D-serine, respectively. Water: 0, 1, 2, 3, 5 and 10 are equal to 0, 0.1, 0.2, 0.3, 0.5 and 1 min pulse time.

### Figure 3

Effect of pulse time on the retention times of the compounds: under isocratic conditions (A); 1 min pulse time (B). Stationary phase, Kinetex C18 (50–4.6 mm, 2.6 µm); mobile phase A, 20:80 acetonitrile–triethylammonium acetate buffer (pH 4, 3 g/L); mobile phase B, water; temperature, 30°C; detection wavelength, 340 nm.

Above a 30 µL injection volume, 5, 10 and 15 cm columns provided similar apparent theoretical plate numbers (Table III). The largest injection volume, for which the efficiency loss was no more than 10%, was found to be approximately 10 µL.

### Column performance under gradient condition

Gradient elution has a significant peak compression effect because elution strength ahead of the sample brand is weaker than the elution strength of the mobile phase behind the peak. A stronger mobile phase makes desorption faster and the apparent retention factor is decreased throughout the measurement.

In the gradient elution study, the ratios of aqueous buffer and organic modifier were adjusted to provide the same
apparent retention factors, approximately 2.1 and 2.3 for serine derivatives on the different columns. Results are reported in Table IV.

When apparent retention factor is kept constant, gradient slope is changed; therefore, the performance of the columns with different column lengths cannot be compared. The data show that increasing injection volume causes no significant efficiency loss.

**Measurement of amino acid content in fruit juice**

The performance of this method was studied in terms of precision, limit of detection (LOD), limit of quantitation (LOQ) and linearity.

The precision was determined using nine independent test solutions in three different concentrations.

The LOD and LOQ for amino acid enantiomer derivatives were determined by injecting diluted solutions with known concentrations. LOD is expressed as a concentration at 3:1 signal-to-noise ratio, LOQ is defined as a concentration at 10:1 signal-to-noise ratio.

The linearity test was measured in five different concentrations with three replications.

**Precision**

The precision was assessed at three concentrations in replications of three, on three separate occasions. Relative standard deviations (RSDs) were below 5% in each case. The results are summarized in Table V.

**LOD, LOQ and linearity**

LOD and LOQ were in the range of nmol/L for each amino acid derivative. The linear regression fit well for the data with a multiple \( R \) value above 0.995. The results are presented in Table VI.

Among the D-amino acids, D-asparagine, D-alanine were detected in pineapple juice, whereas D-serine and D-glutamine were found in pomegranate juice. The results are summarized in Table VII.

A typical chromatogram of pineapple and pomegranate juice is shown in Figure 4.

**Conclusions**

The aim of the study was to compare the volume overload of 4.6 mm i.d. Kinetex C18 columns with different lengths (50, 100 and 150 mm) under isocratic, pulse gradient and gradient conditions.
Under the experimental isocratic condition, the least efficient column was the 5 cm column. In this experiment, 10 and 15 cm columns provided slightly different theoretical plate numbers.

The objective of the pulse gradient experiments was two-fold: (i) to study the effect of the pulse gradient time, and (ii) to determine volume overload.

According to the experimental findings, increasing the pulse gradient time has an increasing band compression effect. This effect decreased with increasing column length. This is in accordance with the theory that the longer the column, the less the additional contribution (extra-column contribution) to the total peak broadening. In this part of the work, it was unexpected that the D-serine derivative, which was more retained on the 5 cm long column, provided greater apparent theoretical plate numbers than the less retained L-serine derivative; meanwhile, in the case of longer columns, the less retained derivatives gave greater apparent theoretical plate numbers.

In the volume overload study under pulse gradient conditions, column efficiency decreased with increasing injection volume, as was the case under isocratic conditions. For the injection volume above 30 μL, columns with 5, 10 and 15 cm length provided similar apparent theoretical plate numbers normalized to column length (1/m). The injection volume could be doubled (from 5 to 10 μL) without creating more than 10% efficiency loss under pulse gradient conditions.

In the case of gradient elution, the column performance was independent from the injection volume.

To achieve adequate sensitivity, the injection volume was set to 50 μL to determine amino acid enantiomers from fruit juice. Due to this large injection volume, the nmol/L range was

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Pineapple</th>
<th>Pomegranate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L-AA μmol/L</td>
<td>D-AA μmol/L</td>
</tr>
<tr>
<td>Serine</td>
<td>90</td>
<td>ND</td>
</tr>
<tr>
<td>Asparagine</td>
<td>72.3</td>
<td>1.7</td>
</tr>
<tr>
<td>Glutamine</td>
<td>87.6</td>
<td>ND</td>
</tr>
<tr>
<td>Alanine</td>
<td>116.9</td>
<td>4</td>
</tr>
</tbody>
</table>

Table VII
Quantities of Amino Acid Enantiomers in Pineapple and Pomegranate Juice

![Figure 4](image_url)

Figure 4. Typical chromatograms: pineapple juice (A); pomegranate juice (B). Stationary phase, Kinetex C18 (150–4.6 mm); mobile phase A, 10:90 acetonitrile–triethylammonium acetate (pH 4; 3 g/L); mobile phase B, acetonitrile. Gradient started from 0% B and ended at 25% B in 30 min. Injection volume was 50 μL, temperature of the column was 30°C and detection was performed at 340 nm.

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achieved for LOD and LOQ for L,D-asparagine, L,D-alanine, L,D-serine and L,D glutamine derivatives. These enantiomers were determined from pineapple and pomegranate juices.

Acknowledgments
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References
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