

# Evaluation of Column Retentivity with large injection volume for high sensitive and High-Throughput LC-MS/MS Quantitative Analysis

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## INTRODUCTION

In the pharmaceutical industry, the necessity of high sensitivity and high-throughput is increasing. Easy sample preparation, for example, only 'Protein precipitation with organic solvent', is preferable for high-throughput and large injection volume is preferable for high sensitive LC-MS/MS analysis. Large volume injection with sample in which organic solvent concentration is high often makes column retentivity weaken, on the other hand, making sample in which organic solvent concentration is low needs additional step for sample preparation, which not only loses High Throughputness but may occur sample adsorption. We report here evaluation of various columns' retentivity with large injection volume and its improvement with online dilution method using UHPLC-MS/MS.



Figure 1. HTC-PAL AMR New Wash Station & Allegro-AMR UHPLC Pump & API 5000™ LC/MS/MS System

## Device & Method

Mixtures of three compounds, Reserpine, Verapamil, Propranolol were prepared in acetonitrile and several injection volumes, up to 50  $\mu$ L, of the sample were loaded on various columns, which were, ODS (solid phase), 1.7, 2.0, 2.2, 3.0, 5.0 $\mu$ m (particle size), 50, 75, 100x2.0mm (length). UHPLC-MS/MS system; AB/MDS Sciex API 5000™ triple-quadrupole mass spectrometer configuring with HTC PAL with AMR New Wash System autosampler and Allegro-AMR UHPLC pump. MS/MS Analyses were performed with positive electrospray ionization in multiple reaction monitoring (MRM) mode.

### Allegro-AMR UHPLC Pump

Only 70 $\mu$ L Delay volume  
Up to 100MPa  
Flow range 0.1 $\mu$ L --- 1000 $\mu$ L/min  
Binary or Quaternary  
Integration into Analyst® 1.4.2 (API 5000™ operation software)



Figure 2. Allegro-AMR UHPLC Pump

## EXPERIMENTAL

### 1. Evaluation of retentivity by difference of particle size of columns

First of all, analyses were performed by using columns of the same lengths with a variety of particle sizes.

The measurement conditions and the columns used are as follows.

#### HPLC System : HTC PAL AMR New Wash Station Allegro-AMR UHPLC Pump

Mobile phase : (A) H<sub>2</sub>O containing 0.1% Formic acid  
(B) Acetonitrile containing 0.1% formic acid

Gradient:  
Time (min) 0 1 2 2.01 3  
A (%) 90 10 10 90 90  
B (%) 10 90 90 10 10

Flow rate : 400  $\mu$ L/min Column Temperature : Ambient  
Injection volume : 2  $\mu$ L --- 50  $\mu$ L

#### Mass Spectrometry : AB/MDS-Sciex API 5000™ LC/MS/MS

Ion Source : TurbolonSpray  
Ionization Mode : MRM Positive  
Q1/Q3 : 455/165 (Verapamil)  
609/195 (Reserpine)  
260/116 (Propranolol)

Table 1. Columns used for analyses

Column	Length (mmID x mm)	Particle size ( $\mu$ m)	Max. pressure observed during analysis (Mpa)
Column A	2.1 x 50	1.7	34
Column B1	2.0 x 50	2	23
Column B2	2.0 x 50	2	24
Column C	2.0 x 50	2.2	22
Column D1	2.0 x 50	3	13
Column D2	2.0 x 50	3	10.5
Column E	2.1 x 50	3	10
Column F1	2.0 x 50	3	11
Column F2	2.0 x 50	3	11
Column F3	2.0 x 50	5	11

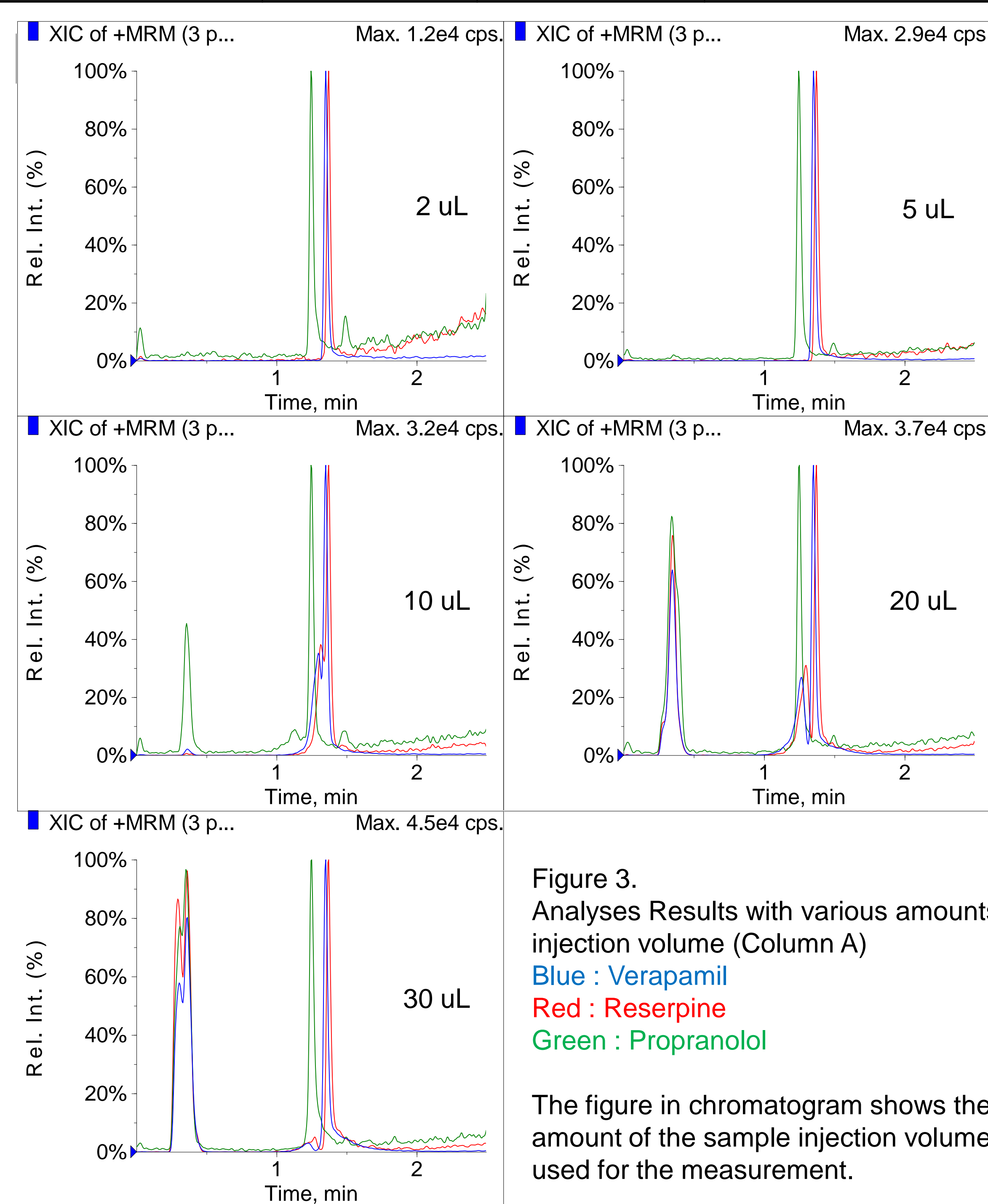


Figure 3. Analyses Results with various amounts of injection volume (Column A)  
Blue : Verapamil  
Red : Reserpine  
Green : Propranolol  
The figure in chromatogram shows the amount of the sample injection volume used for the measurement.

The results performed by column A were shown in Figure 3.

These compounds were eluted to certain retention time with good peak shape at the small amounts of injection volume (2 $\mu$ L, 5 $\mu$ L), eluted with leading-shape at 10 $\mu$ L (especially a part of Propranolol was eluted to void volume) and then, almost of them were eluted to void volume at the large amounts of injection volume (~30 $\mu$ L). As for these compounds, Reserpine, Verapamil, Propranolol, the up-limited amount of injection volume to keep certain retention time was found to be 5 $\mu$ L.

Other columns were also performed by various amounts of injection volume. The results by each column with amount 5 $\mu$ L and 10 $\mu$ L of injection volume was shown in Figure 4(a). This resulted that these compounds were eluted to certain retention time with good peak shape until 5 $\mu$ L of injection volume, but not over 10 $\mu$ L of injection volume, which didn't depend on the particle size at fixed column length (2 x 50mm). However, column D1 (Cadenza CD-C18; Imtakt Corporation, Kyoto, Japan) and column D2 (Unison UK-C18; Imtakt Corporation, Kyoto, Japan) could keep their retention time from small amount of injection volume to large (up to 50 $\mu$ L) shown in Figure 4(b).

These results indicated that column retentivity for sample in organic solvent was independent of its particle size and that considerably large injection volume was available depending on the selection of the column.

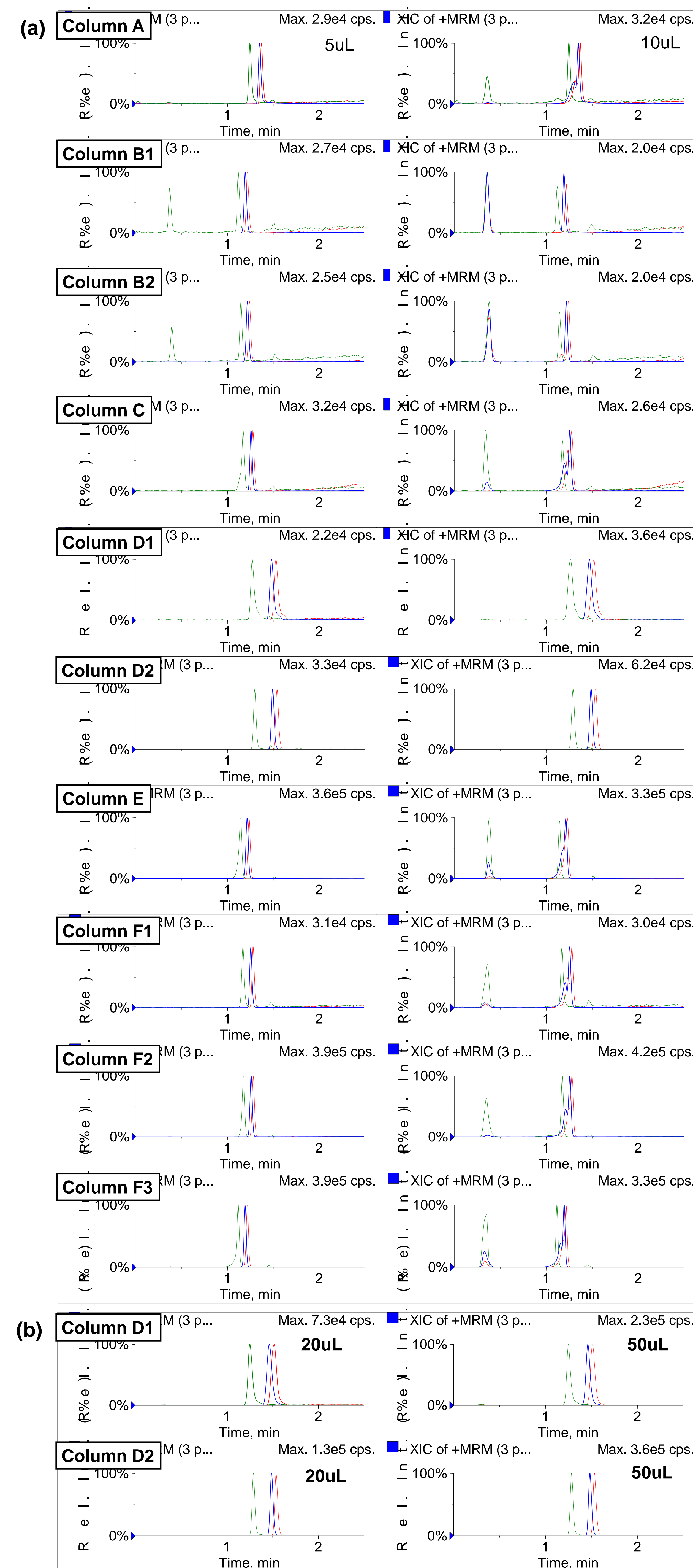


Figure 4. Results with various amounts of injection volume  
(a) left chromatograms: injection volume 5 $\mu$ L, right: 10 $\mu$ L.  
(b) Results of column D1, D2 at large amounts of injection volume (20 $\mu$ L, 50 $\mu$ L)

### 2. Evaluation of retentivity by difference of column length

Next, To evaluate the effect of the difference of column length, three columns, 2.0x 50, 75, 100mm (same manufacturer, same solid phase, only different length), were tested (Figure 5).

It resulted that column retentivity had increased as the column became long, but the improvement of the retentivity was a little (the up-limited amount: 10 $\mu$ L)

\* The maximum pressure during analysis with a 2.0x100mm column was 39MPa.

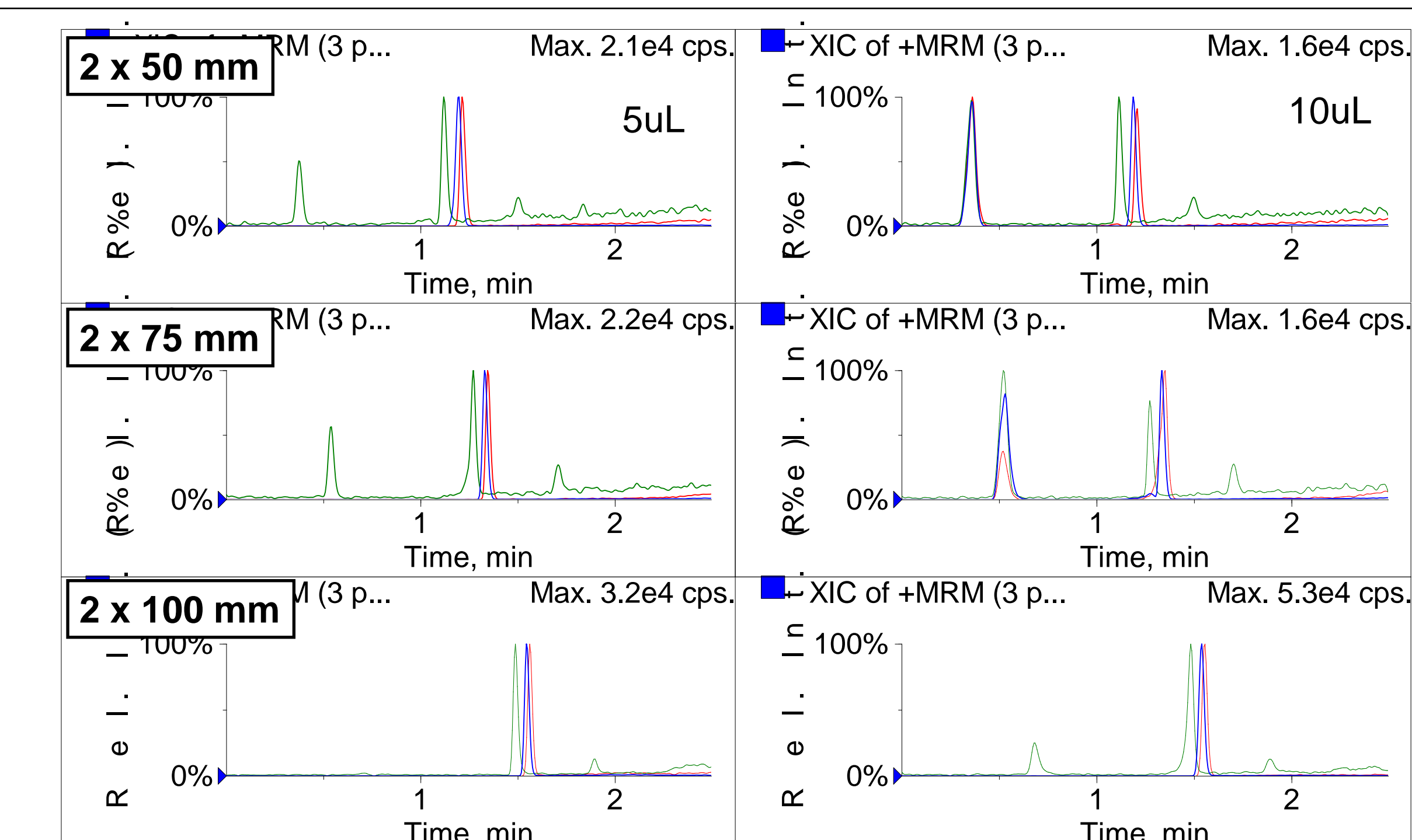


Figure 5. Results of three columns, 2.0x50, 2.0x75, 2.0x100mm  
left chromatograms: injection volume 5 $\mu$ L, right: 10 $\mu$ L.

### 3. Examination of retentivity improvement by online dilution

Moreover, it was tested in the system that sample was diluted online with installing the by-pass line road before and behind autosampler (injection valve). The scheme was shown in Figure 6. The bypass line was installed to set dilute factor 5 times.

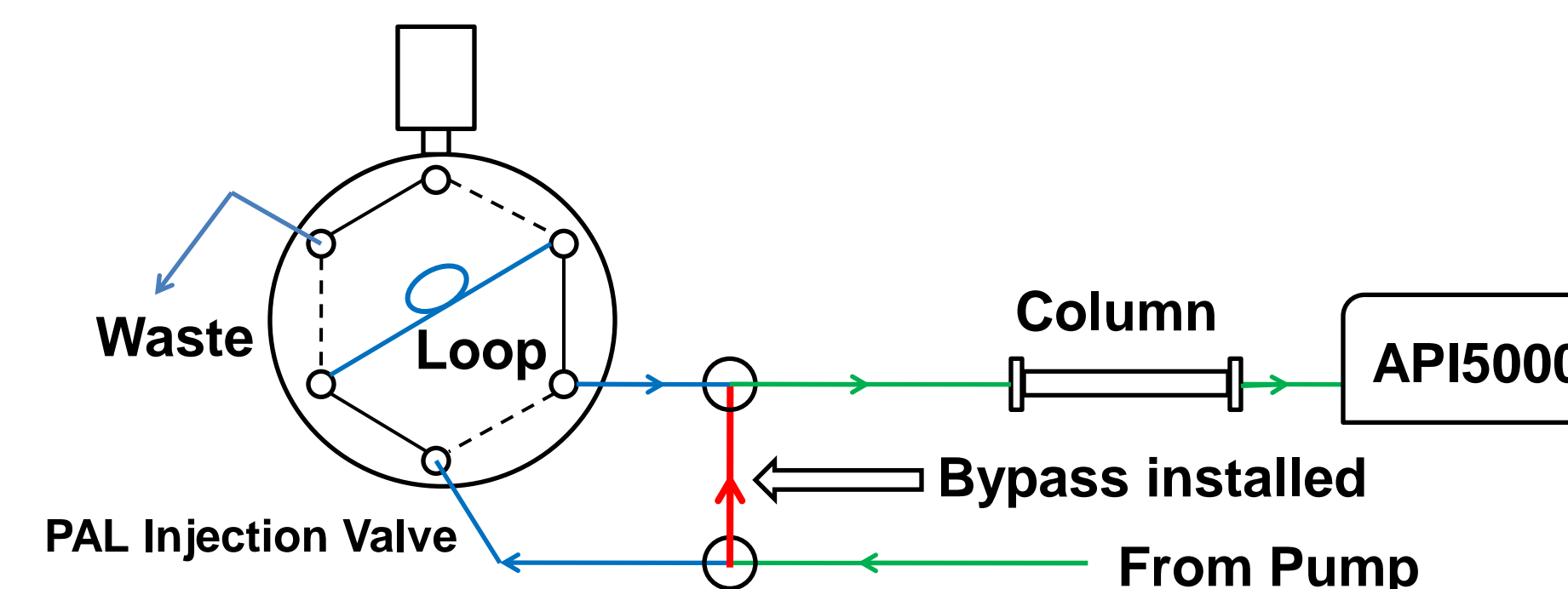


Figure 6. Scheme of online dilution system  
Red line was additionally installed. The flow rate to red line and to blue line was set 4 : 1

The tests were performed using this system. While the up-limited amount of injection volume was 5 $\mu$ L without the online dilution system, an excellent peak shape was able to be maintained in 10 $\mu$ L with the system (2.0x50 mm column). Furthermore, at the results of 2.0x100 mm column, compounds were eluted to certain retention time with good peak shape up to 20 $\mu$ L of injection volume (Figure 7).

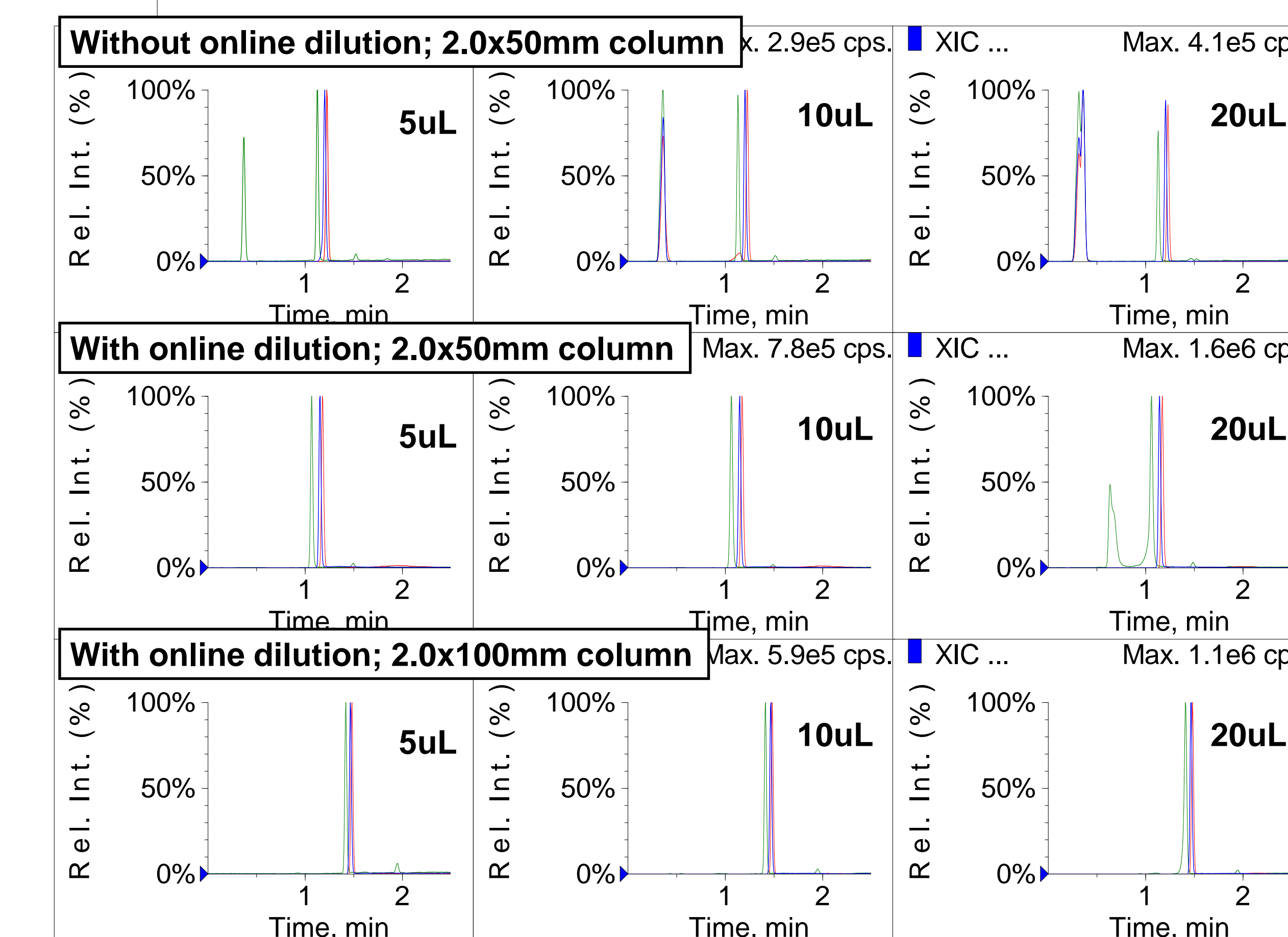


Figure 7. Results with and without online dilution system

## CONCLUSION

As the results of evaluation tests of column retentivity for organic solvent using ultra high pressure LC-MS/MS system, Allegro-AMR UHPLC Pump & HTC PAL AMR New Wash Station & API 5000™ LC/MS/MS System,

1. Column retentivity was independent of its particle size. Some columns were available with large injection volume, so it indicated that the column packing technology (uniformity of the solid phase surface, etc.) may be significant for the retentivity.
2. Column retentivity had increased as the column became long, but the improvement of the retentivity was a little.
3. Column retentivity had improved with online dilution system.