Evaluation of High Sensitive LC/MS Analysis of Basic Compounds using a Direct Injection Column and an Ion-Suppressor Device

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Introduction

- Reversed phase chromatography with an ion-pair reagent in the mobile phase is generally used to obtaining sufficient retention of positively charged hydrophilic compounds by HPLC. However, when using detection by mass spectrometry, ion-pair reagents often lead to (1) a reduction in sensitivity by suppressing solute ionization, and (2) contamination of the detector.
- Hydrophilic interaction chromatography (HILIC) is frequently used for the analysis of hydrophilic compounds without using ion-pairing reagent in the mobile phase. However, this method exhibits some limitations. The chemical structure of the analyte functional groups influence such chromatographic properties as retention time, peak shape and recovery, making HILIC not as widely applicable as RPC. It has also been reported that the lifetime of HILIC columns is limited when analyzing biological samples.
- To overcome these difficulties we investigated a new, high sensitivity LC/MS method for the determination of positively charged hydrophilic compounds in biological samples and compared it with the performance of hydrophilic interaction chromatography (HILIC).

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Experimental

- In this method, a suppressor device which contains strong ion exchange resin, is positioned between an analytical column and the MS detector. By this device, the ion-pair reagent is removed from the mobile phase before it enters the MS detector, thus minimizing the ion suppression effect.
- As a result, contamination of the detector is avoided and background baseline noise level and sensitivity of analytes are improved.
- A newly developed reversed phase column (TSKgel BSA-ODS-100V), in which the surface of the packing material was coated with denatured bovine serum albumin, was employed for analysis of analytes in serum samples.
- Prior to analysis, serum samples were diluted and filtered, which substantially reduce sample preparation time compared to liquid-liquid extraction or solid phase extraction (SPE) methods.

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Experimental, Continued

- Four kinds of basic hydrophilic compounds (nicotine, cotinine, nornicotine and anabasine) in human serum were measured.
- The separation of these compounds was performed on a TSKgel BSA-ODS-100V (2.0mm ID x 10cm, 5µm) analytical column.
- Eluent A and eluent B were 10mM HFBA (heptafluoro-n-butyric acid) solution and acetonitrile, respectively.
- After injecting the serum sample, proteins present in the sample were excluded from the pores of the packing material and were switched to waste. After rotating the switching valve, the solution was directed via the suppressor device to the MS detector.
- Analytes were eluted from the column by using gradient elution and then were detected on ESI ionization.

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Figure 1: Schematic Diagram of the LC/MS System

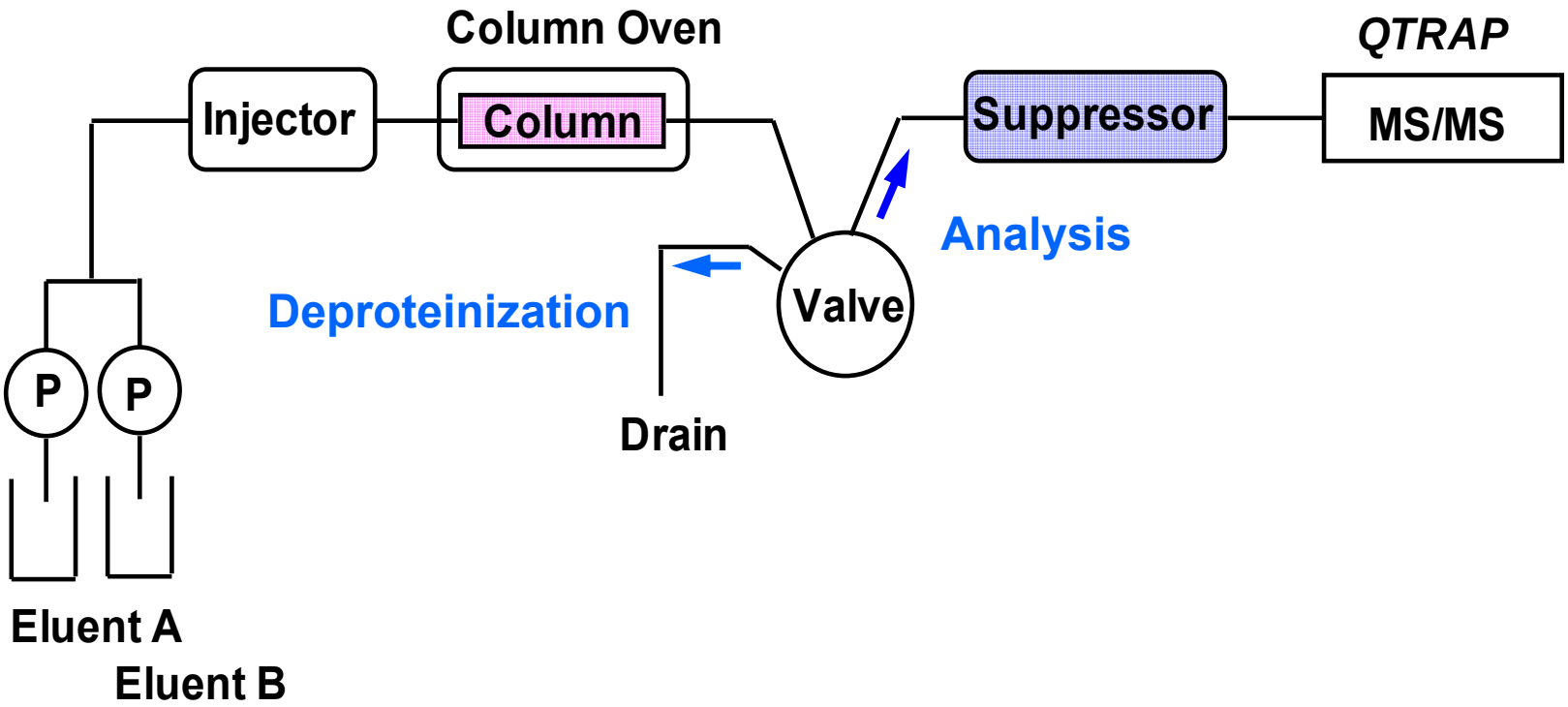

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Figure 2: Protocol to Renew the Ion Exchange Resin at the Suppressor Device after each Analysis

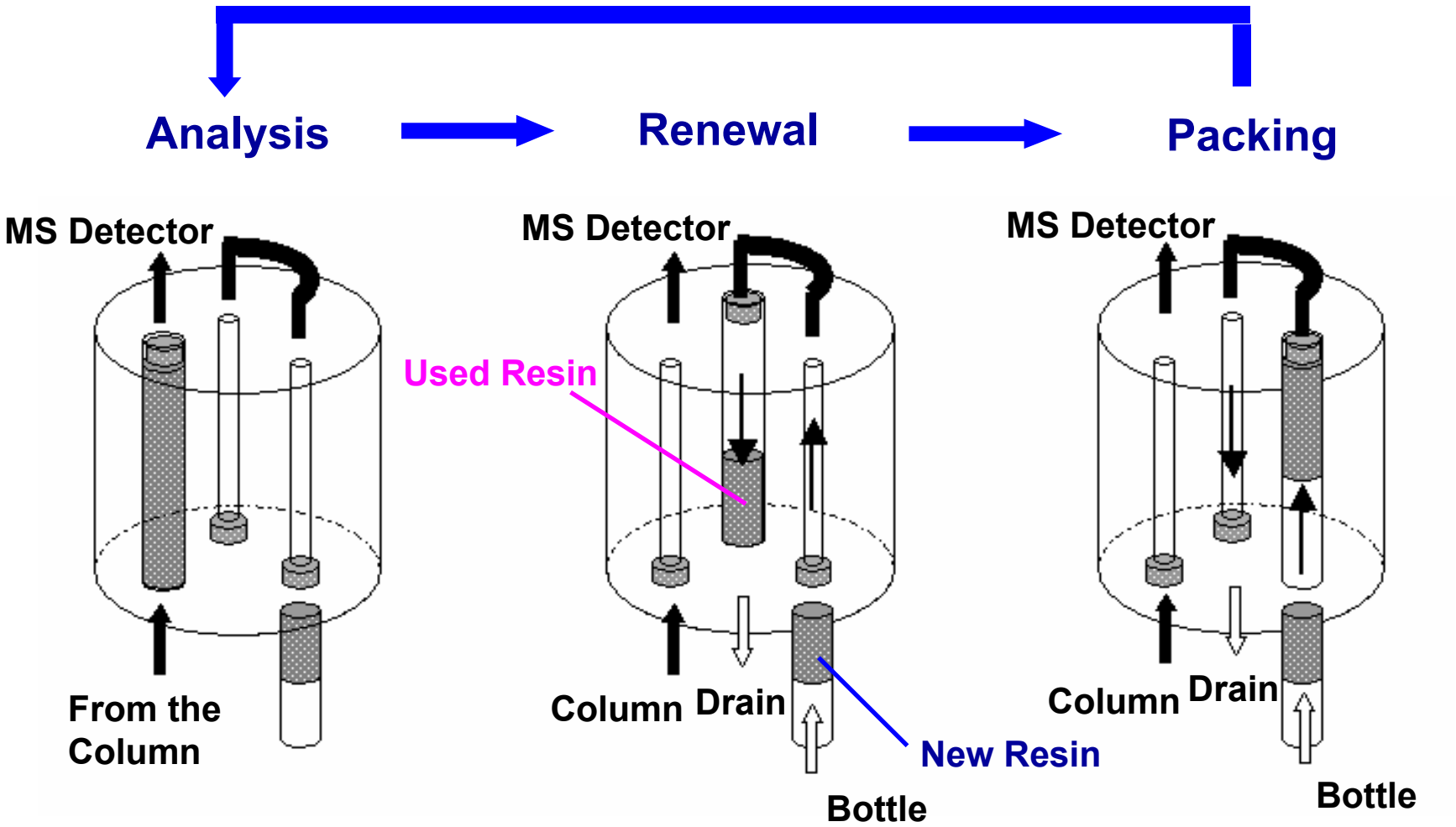

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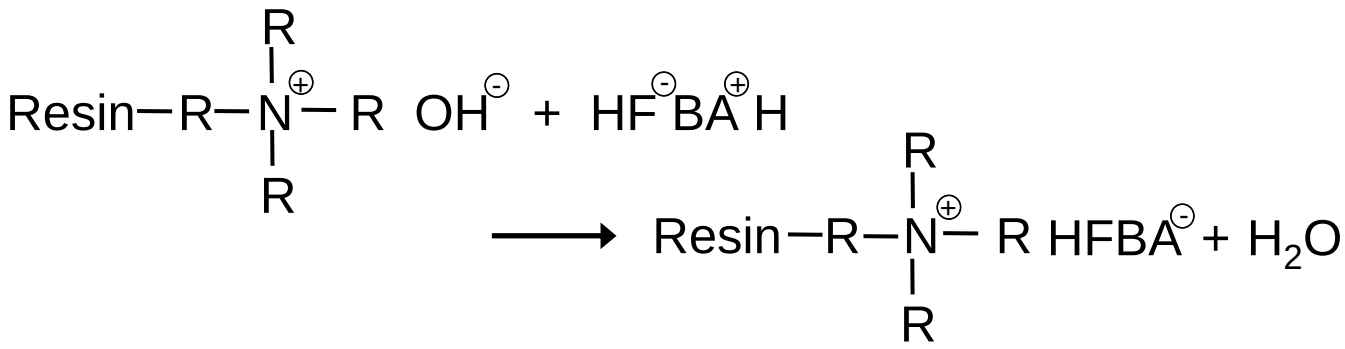
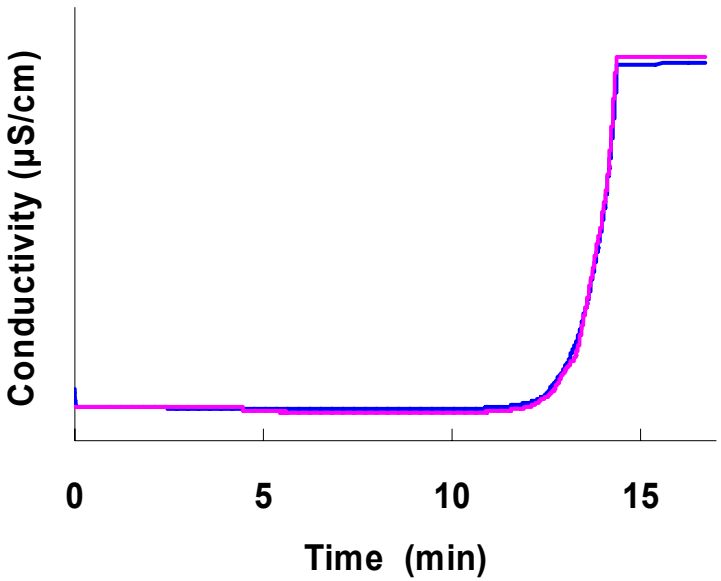
Figure 3: Function of Ion Exchange Resin





Resin:	**Anion exchange resin**
Resin Volume:	**102µL**
Flow rate:	**200µL/min**
Detector:	**Conductivity detector**
Solvent:	**10mM HFBA in (water/acetonitrile =1/99)**
	10mM HFBA in (water/acetonitrile =30/70)

HFBA: Heptafluoro-n-butyric acid

Removal of ion-pair reagent from mobile phase in 12 minutes

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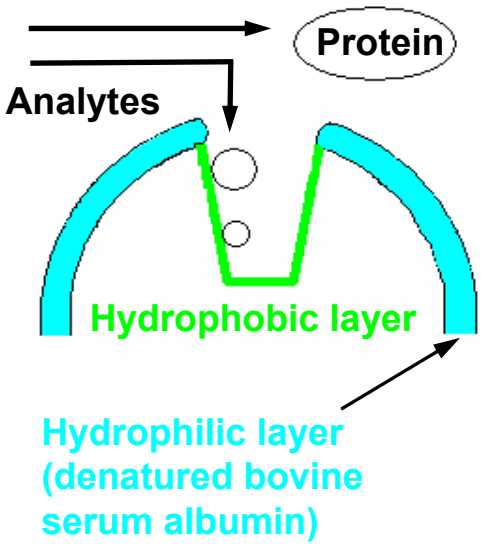
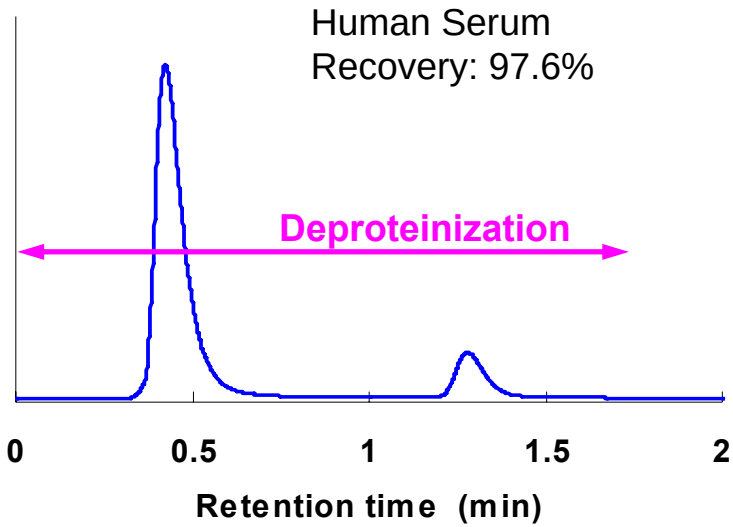
Figure 4: Function of Internal Surface of Reversed Phase Packing Material





Column:	**TSKgel BSA-ODS-100V (2.0mm ID x 5cm)**
Eluent:	**10mM HFBA in (water/acetonitrile=99/1)**
Flow rate:	**200µL/min**
Detector:	**UV@280nm**
Column temp.:	**40°C**
Sample vol.:	**2µL**

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Conditions for Figures 5 - 7

1. RPC-Suppressor Mode

Column: TSKgel BSA-ODS-100V (2.0mm ID x 5.0cm)

Eluent A: 10mM HFBA in (water/acetonitrile = 99/1) (HFBA: Heptafluoro-n-butyric acid)

Eluent B: 10mM HFBA in acetonitrile

Flow Rate: 200µL/min

Column Oven: 40°C

Sample Volume: 50µL

Linear Gradient: 0min (B 0%), 2min (B 0%), 9min (B 30%), 11min (B 30%)

Valve Control: 0min (b), 2min (a), 11min (b)

2. HILIC Mode

Column: TSKgel Amide-80, 3µm (2.0mm ID x 5.0cm)

Eluent A: 10mM ammonium formate (pH 3.75)

Eluent B: acetonitrile

Linear Gradient: 0min (B 95%), 10min (B 50%), 12min (B 50%), 13min (B 95%)

Flow Rate: 200µL/min

Column Oven: 40°C

Sample Volume: 10µL

MS: QTRAP (Applied Biosystems)

Ionization: MRM mode (ESI+)

Samples: Nicotine (163.130)

Cotinine (177.80)

Nornicotine (149.130)

Anabasine (163.118)

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Figure 5: Effect of Injection Volume on Chromatogram

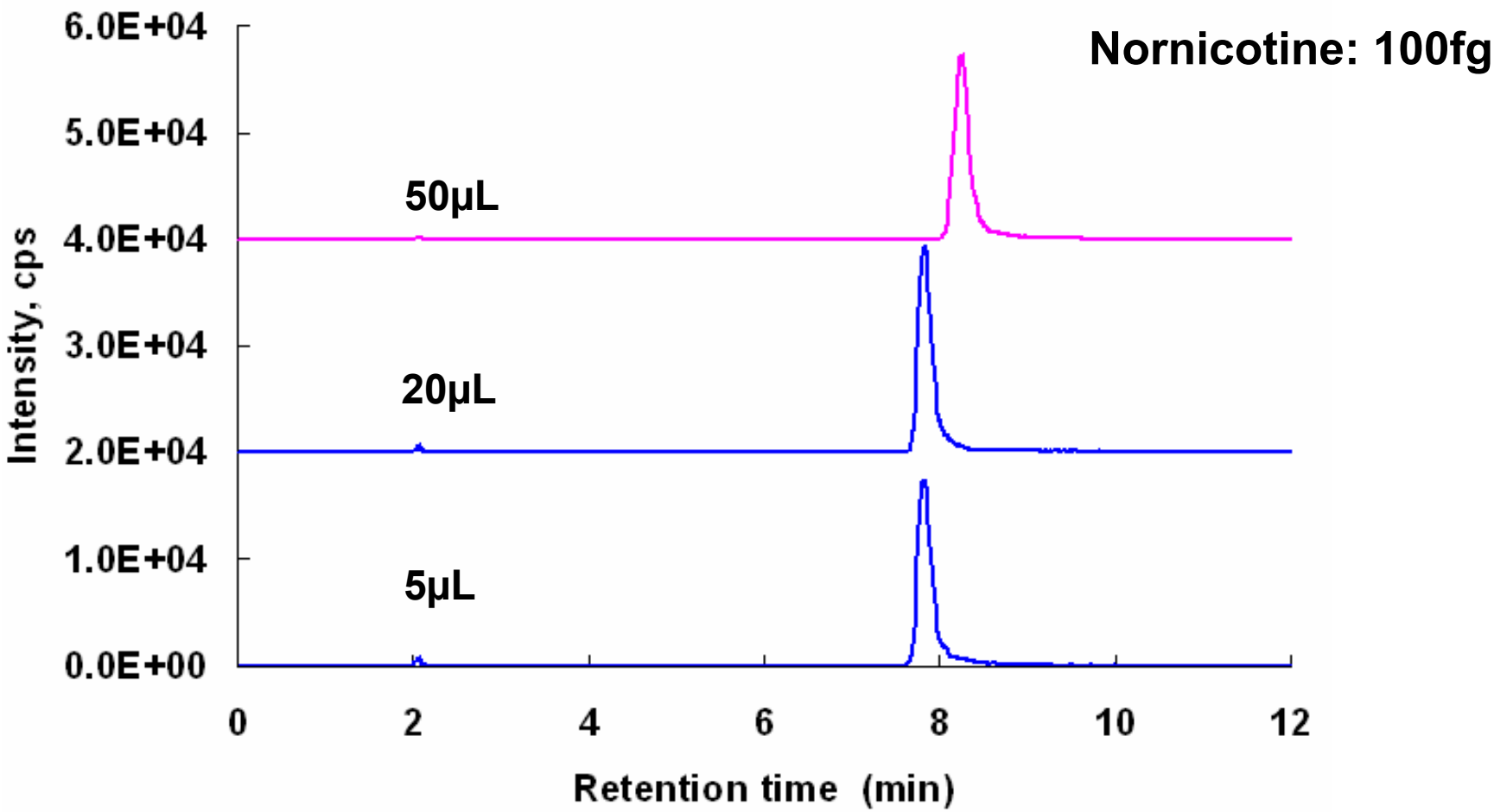

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Figure 6: Comparison of Chromatograms of Compounds Under Each Condition

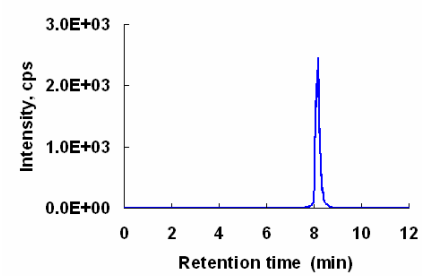
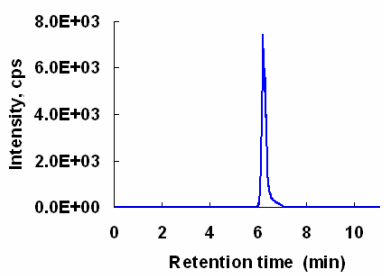
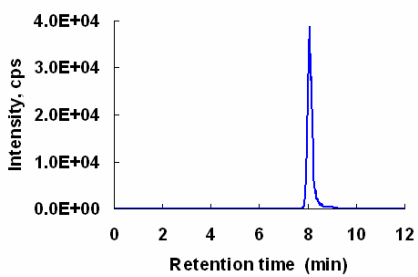
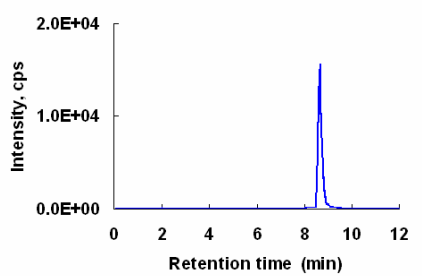
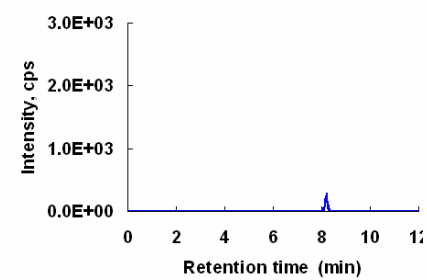
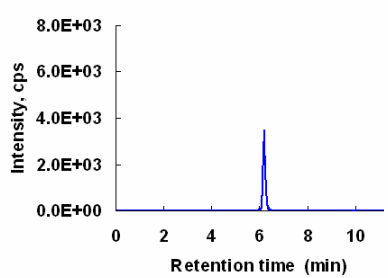
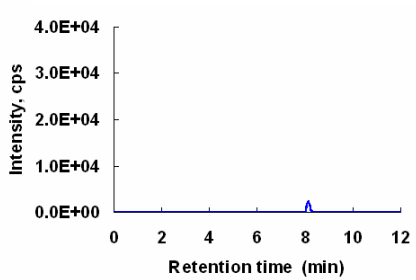
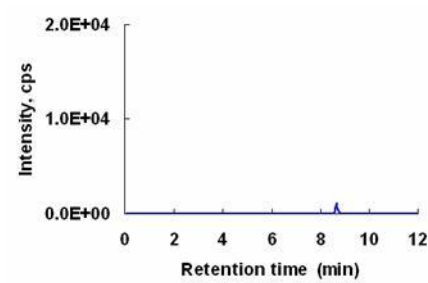
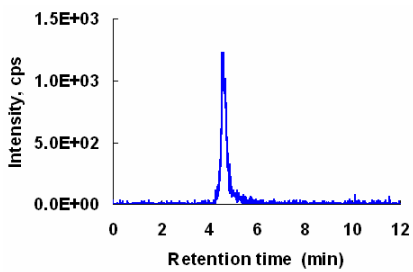
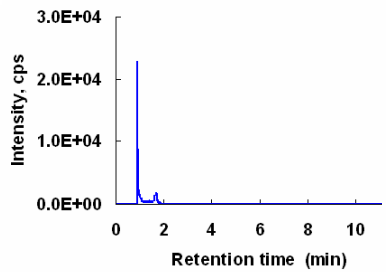
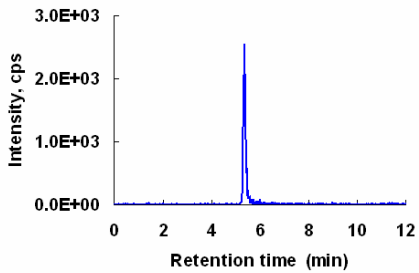
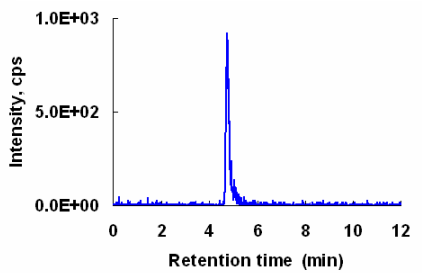
(Sample: 5µg/L)	Nicotine	Cotinine	Nornicotine	Anabasine
With Suppressor				
Without suppressor				
HILIC column				

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Figure 7: Chromatograms of Human Serum Spiked with Standards

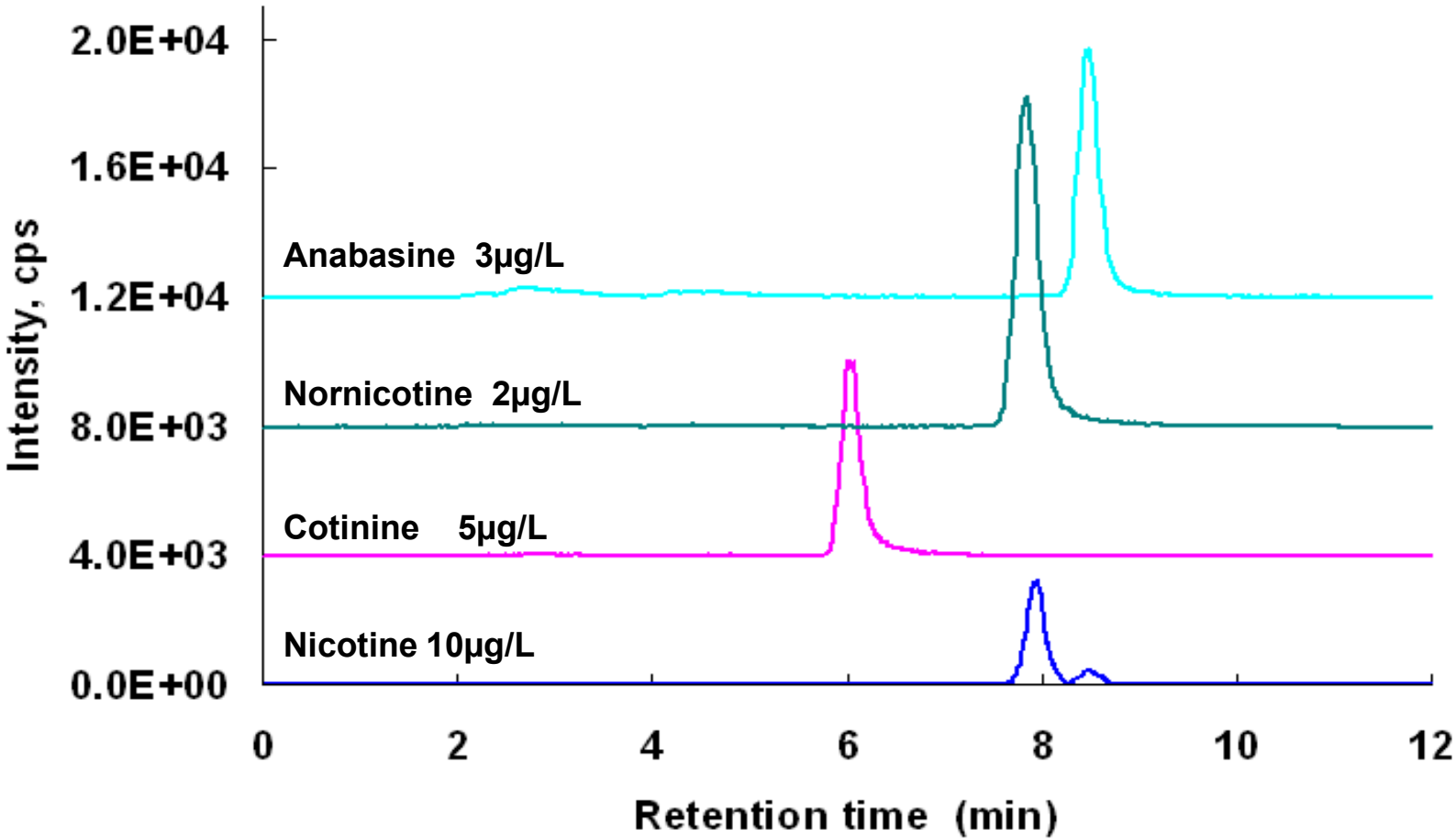

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Table 1: Linearity, Limits of Quantification and Limits of Detection

TSKgel BSA-ODS-100V				HILIC Mode	
Calibration range µg/L	**R^2**	**Precision, RSD 5µ/L, n=6**	**LOQ µg/L**	**Calibration range µg/L**	**LOQ µg/L**
0.2-50	0.996	0.65	0.2	1.0-50	1.5
0.1-50	0.998	0.52	0.1	No Retention	
0.1-50	0.999	0.68	0.05	1.0-50	0.6
0.1-50	0.999	0.53	0.1	1.0-50	1.5

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Table 2: Precision and Recovery in the Determination of Compounds Spiked in Human Serum

	Concentration, µg/L (Spiked Human Serum)	Precision, RSD n=6	Recovery %
Nicotine	10	1.5	92.1
	1	4.1	88.4
Cotinine	5	2.1	98.3
	0.5	2.6	96.1
Nornicotine	2	1.3	96.4
	0.2	3.1	91.5
Anabasine	5	1.6	94.7
	0.5	2.4	92.1

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Results

- Deproteinization using a direct injection reversed phase column
  - Protein content in human serum was measured after separation. Recovery was more than 97.6%. This means that it is possible to analyze biological samples by direct injection without performing a lengthy deproteinization step.
- Sensitivity of method
  - Removal of HFBA from the eluent improved the background baseline noise level and peak heights of analytes became higher. Sensitivity of analytes was improved 5-25 times when compared to analysis without a suppressor device.
  - Compared with HILIC mode, with the analysis using formic acid solution as the eluent, sensitivity was improved 3 times. Furthermore, when using a TSKgel BSA-ODS-100V column, a large amount of sample could be loaded. As a result, sensitivity was improved 10-fold compared with a HILIC analysis. Additionally, cotinine was poorly retained on the HILIC column.
- Measurement of human serum
  - The level of spiked standards in human serum could be measured after simple pretreatment (10-fold dilution with eluent A followed by filtration) on this system. There were no interference peaks and recovery of all compounds was in the range of 88 to 98%.

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Discussion

- By installing the ion-suppressor device before the MS detector, many compounds were analyzed using the MS detector under chromatographic conditions containing non-volatile salts or ion-pair reagents in the eluent.
- Furthermore, the ion-suppressor device improved the sensitivity of the MS detector because of a lack of contamination and ion suppression by these interferences.

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