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Development of miniaturized multi-channel high-performance liquid chromatography for high-throughput analysis

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Abstract

We have developed miniaturized multi-channel high-performance liquid chromatography (HPLC) system. With this system, we can simultaneously separate multiple samples, using a single high-pressure gradient pump, a chip-based sample injection unit, a monolithic silica capillary column array, and a multi-channel UV detection unit based on fiber optics. The injection unit has a simplified structure composed of brass housing and a quartz microchip having microchannels and access ports, which enable a direct injection of sample to multi-channel by commercial multichannel micropipette. Moreover, that possesses a function of microvalve, and on-chip definition of sample injection plugs achieved with a cross channel injection method, providing each column of monolithic silica capillary array. The substances in channels were simultaneously detected with UV having multiple cells. Standard samples were analyzed for characterizing newly developed system, and sharp peaks were obtained with reproducibility data of < 0.9% (R.S.D.). Analysis of tryptic digestion of casein was also employed. These results show that the novel multi-channel HPLC system has the benefits for the high-throughput analysis in the post-genomic analysis/combinatorial chemistry.

Keywords: Miniaturized HPLC; Multi-channel HPLC; Monolithic silica column; High-throughput analysis

1. Introduction

As leading researchers begin to recognize the vast potential of biomarker discovery in the post-genomic research, the needs for the high-throughput analysis are on the rise. In order to understand the myriad of interactions, which occur between the proteins that participate in cellular control networks, the large numbers of protein complexes have to be analyzed [1–3]. This significant undertaking is only possible if the multi-samples were simultaneously determined in a one-time analysis. Also, the acceleration of drug discovery in recent years has proposed the significant analytical challenges. The introduction of the combinatorial chemistry with automated parallel synthesis increased dramatically the number of compounds that must be analyzed [4–6]. Therefore, the high-throughput analytical techniques have become critical for determining the identity and purity of synthesized substances.

To achieve high-throughput analysis, the researchers are focused on the chromatographic techniques of (1) increased efficiencies in shorter analytical time and (2) multi-sample detection method using a multi-channel high-performance liquid chromatography (HPLC). One possibility is to use the smaller particles as separation media for high-speed analysis [7,8]. However, difficulties are pointed out by the fact that pressure rises as the liner velocity is increased in high-throughput analysis. In order to overcome the problem indicated in the particle packing, a double-pore structure (macro-pore and meso-pore) silica gel monolith was developed by Nakanishi [9], which is manufactured by combining the sol–gel reaction with phase separation and a subsequent solvent exchange treatment. By controlling the composition of the starting mixture or changing the concentrations of

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alkoxysilane and polyethyleneglycol (PEG), it can control the size of silica skeletons and through-pores to produce monolithic silica having (through-pore-size)/(skeleton-size) ratios greater than those found in a particle-packed column [10]. Cabrera et al. [11] and Ishizuka et al. [12] applied the monolithic silica to the packings for conventional/capillary-sized HPLC, and proved its unique features, including low pressure-drop, total porosity higher than particle column. Shintani et al. [13] indicated that the monolithic silica had the application possibility as a solid-phase micro-extraction media. The peculiarity of monolithic silica column clarified by those reports has suitability for the separation media of micro-HPLC.

Another approach is to use multi-channel chromatography. de Biasi et al. [14] reported a four-channel multiplexed electrospray interface, using four HPLC columns and one multiple probe injector. This interface rapidly switches between multiple liquid streams. Unfortunately, the interface shortens the dwell times, and reduces the sensitivity. Additionally, since this approach requires the modifications to the MS ion source, the user may be limited. Korfmacher et al. [15] reported another system which was consisted of two separate LC pumping systems, autosamplers and columns connected via a divert valve to a single mass spectrometer. A major disadvantage of this system is that two conventional HPLC systems are required. In addition, the capillary-scale or micro-scale multi-channel HPLC has not been reported.

Recently, the micro total-analysis systems (μ-TAS) have attracted a great deal of attentions as possible means to increase the throughput and mass sensitivity of the analytical procedures via miniaturization [16]. A variety of microdevices has been constructed. Compared to the standard instrumentation, the microdevices offer the means for handling small sample amounts with no interferences from excessive dead volumes. However, most microfluidic devices are applied under the atmosphere pressure. No microfluidic device that could withstand the high-pressure exits.

In this paper, we describe the novel miniaturized multi-channel HPLC system, which is possible to simultaneously separate multiple samples. The key features are: (1) the single high-pressure gradient pump for delivering eluent to several channels; (2) the chip-based multi-sample injection unit; (3) the monolithic silica capillary column array; and (4) the high sensitive UV detection unit having multi-channel with based on fiber optics. First, each component was characterized and optimized. Then they were integrated to realize the multi-channel HPLC.

2. Experimental

2.1. Chemical and reagent

Ethylbenzene was supplied by Kishida Chemicals (Osaka, Japan). Butylbenzene, amilbenzene, *p*-hydroxybenzoic acid ethyl ester, *p*-hydroxybenzoic acid propyl ester and *p*-hydroxybenzoic acid butyl ester were purchased from Tokyo Kaseikogyo (Tokyo, Japan). 1-Phenylethanol and 4-methylbenzylacetate were purchased from Merck (Frankfurter, Germany) and Research Chemicals (USA), respectively. Sequencing Grade modified trypsin (bovine) was purchased by Promega (Tokyo, Japan). ß-Casen was provided from Sigma-Aldrich (USA). Polydimethylsiloxane elastomer (PDMS; Sylgard 184) was obtained from Dow Corning Asia (Tokyo, Japan). Acetonitrile used for the eluent was of HPLC grade and purchased from Kishida Chemicals. Deionized water was prepared with a Milli-Q system (Nihon Millipore Kogyo, Tokyo, Japan). All other reagents were of analytical grade, and purchased from Kishida Chemicals.

2.2. Equipment

Single-channel micro-HPLC analyses were performed using a MP-711 Micro-Flow Pump (GL Sciences, Tokyo, Japan) and a UV-702 UV–vis detector (GL Sciences) equipped with on-column cell or long-optical-path flow cell. The sample injector was model C4-1004-.1 injection valve (sample volume 0.1 μL; Valco, Schenkon, Switzerland). The chromatographic separation was typically performed on a MonoCap for fast flow monolithic silica capillary column (150 mm × 0.1 mm i.d., through-pore size 2 μm, meso-pore size 15 nm, skeleton size 1 μm; GL Sciences). Model P779-01 nanotight uinion (0.05 in. of thru-hole and 8 nL of inner volume; Upchurch Scientific, USA) was used for connecting capillaries. Data acquisition was performed using Ezchrom Elite (GL Sciences).

2.3. Fabrication of injection unit

The device configuration of injection unit is depicted in Fig. 1. The unit has a simplified structure composed of brass housing, and a quartz microchip having microchannels and four access ports. Techno Quartz Inc. (Yamagata, Japan) kindly produced and provided all quartz microchips used in this study. The microchannels (typically 100 μm width, 100 μm depth) were diced into a quartz wafer using a dicing saw, and taper connection ports were created by using a drilling machine. Two quartz wafers were welded in the following procedure: each grinding side of chips was matched and then was heated with high pressure in an electric furnace. Inlet port 1 enables a direct injection of sample to channel by using a commercial micropipette. Inlet port 2 and outlet port 2 adapt a direct connection of 1/16 in. fittings. The brass housing and the Teflon adaptor were piled up to the quartz chip. As for the brass housing and the microchip, the precise positioning was individually possible, and the function of microvalve was carried out by sliding the brass blocks.

2.4. Preparation of PDMS for the connection of microchips

PDMS for the connection of microchips was prepared according to the following procedure: a 10:1 combination of

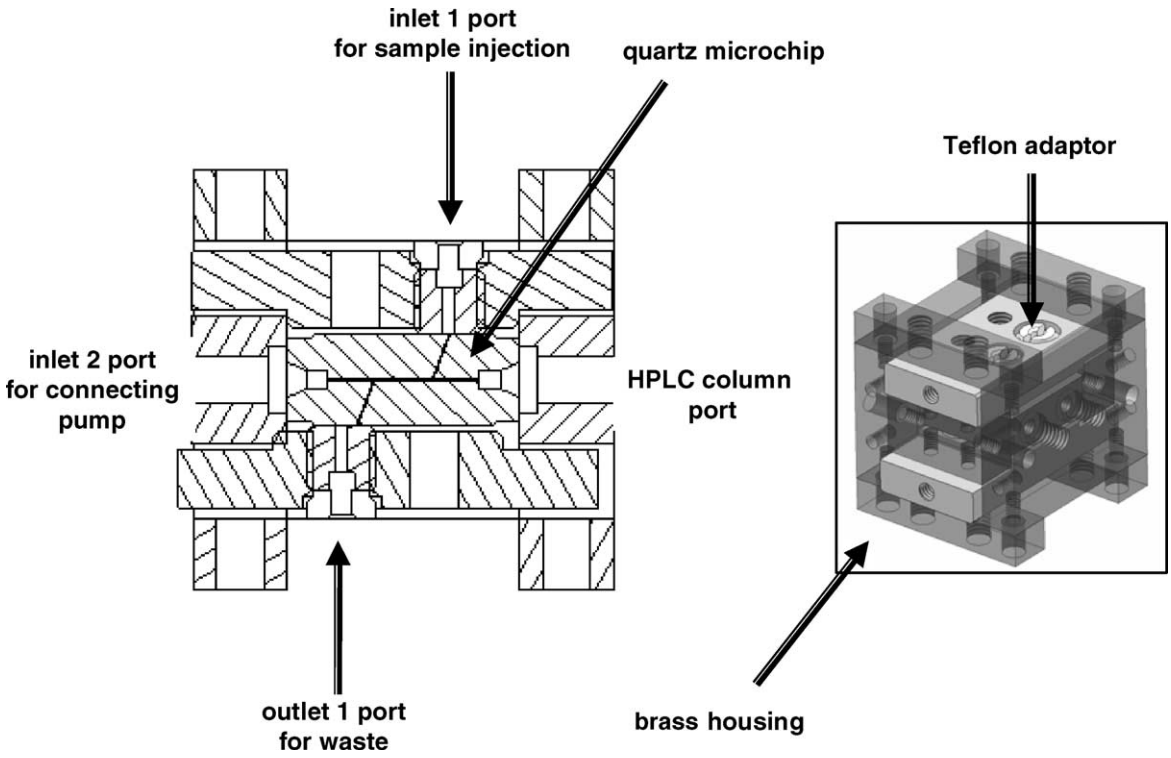

Fig. 1. Schematic diagram of chip-based multi-channel injection unit equipped with microvalve.

PDMS elastomer and curing agent were mixed and then degassed thoroughly. The polymer mixture was cast into the mould or was spread on the seal side of the chips, and then cured for 6 h at 120 °C in an oven.

2.5. Fabrication of UV detection unit

Two types of the UV detector were developed for the high sensitive detection and for the simultaneous multichannel detection. The light from a 30 W deuterium lamp (L6301; Hamamatsu Photonics, Shizuoka, Japan) was delivered to a long-path-length cell using 300 mm fiber optics (coreϕ/claddingϕ of 360/400 μm; 32 optical fibers were bundled). The transmission factor of fiber used in this study was ca. 99% at 254 nm and > 80% at 210 nm. The flow cell had 4-mm path length and 177 nL detection volume. The transmitted beam was received in a diode array (S4111-16Q; Hamamatsu Photonics). For the delivery of light to plural channels, a "1–2 channel" or a "1–4 channel" divergence fiber optics were also employed.

3. Result and discussion

3.1. Multi-channel injection unit

For the injection unit of a multichannel HPLC, the simultaneous introduction of several samples into each channel of multichannel is basically required. In addition, no sample loss and high reproducibility is essential. We newly developed the multichannel injection unit, which achieved sample introduction by a commercial multichannel micropipette. The characteristics of multi-sample injector were examined. The procedure of sample introduction was performed as follows: first, the channels were filled with eluent, and then sample solution was loaded from inlet 1 to outlet by using a micropipette (Fig. 2a). Next, the brass housing was slid to close the both of inlet 1 and outlet port (Fig. 2b). Last, the eluent was pumped from inlet 2, and then pushed the sample plug defined by the intersection of the two channels (typically 50 nL) to HPLC column for separation/detection (Fig. 2c). Fig. 3 shows the analytical result of a mixture of 1-phenylethanol and 4-methylbenzylacetate that was achieved by using the 2-channel injection unit. The sharp peaks were obtained with reproducibility data of < 0.9% (R.S.D., $n = 9$). No liquid leakage was observed under the pressure of 2 MPa.

3.2. Connection between modules

Since HPLC is a composition thing of units such as injector, column and detector, the method of connecting them is the significant research topic. We developed the new interconnecting method "PDMS connection method", which could withstand the pressure of 4 MPa. Fig. 4 shows the typical connection between an injection unit and a capillary column array. The mixture of PDMS elastomer and curing agent was spread on the seal side of the chips at the proportion of 15 mg/cm^2, and then they were baked. The influence

Table 1
The influence of PDMS connection method to the theoretical plate

	Ethylbenzene		Butylbenzene		Amylbenzene	
	Plate (l5 cm)	Ratio to the direct connection	Plate (15 cm)	Ratio to the direct connection	Plate (15 cm)	Ratio to the direct connection
Direct connection	5073	1.000	6484	1.000	7415	1.000
Union connection	4593	0.905	6073	0.937	7151	0.964
PDMS connection	4451	0.877	5939	0.916	6918	0.933

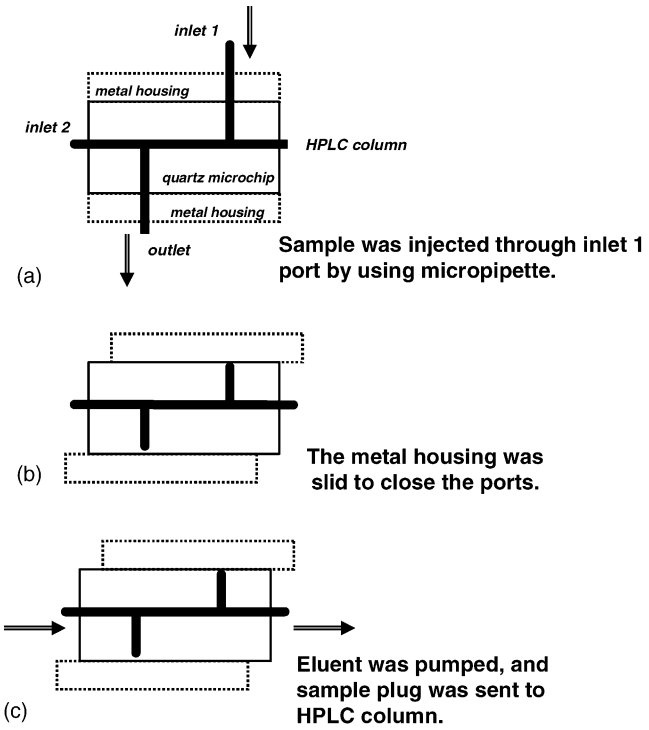

Fig. 2. Procedure of sample injection using multichannel injection unit.

of PDMS connection method concerning the extra column effect on chromatographic separation was evaluated. The influence of PDMS connection and typical union fitting for micro-HPLC was examined based on the case where an injector, a column and a UV detector were directly connected. The typical chromatogram is shown as Fig. 5. The ratio of the plate of the PDMS connection and the union to the direct connection is indicated in Table 1. The theoretical plate of PDMS connection was decreased compared with the union fitting. The value of plate decreased by ca. 5% at 1.4–2.6 of retention factor (k). However, considering the advantage of user-friendlily one-touch connection, PDMS connection method obviously had the potential from a practical viewpoint.

3.3. UV detection unit

For the UV detector of miniaturized micro-channel HPLC, the high sensitivity, which led the detection of a small amount of sample and simultaneous detection of each channel, become a key technology. We developed two types of UV detector, introducing long optical path flow cell to achieve the high sensitive detection, and divergence fiber to divide light of a light source into plural flow cell. The on-column detection method, which is commonly applied in the field of micro-HPLC, frequently leads the sensitivity shortage

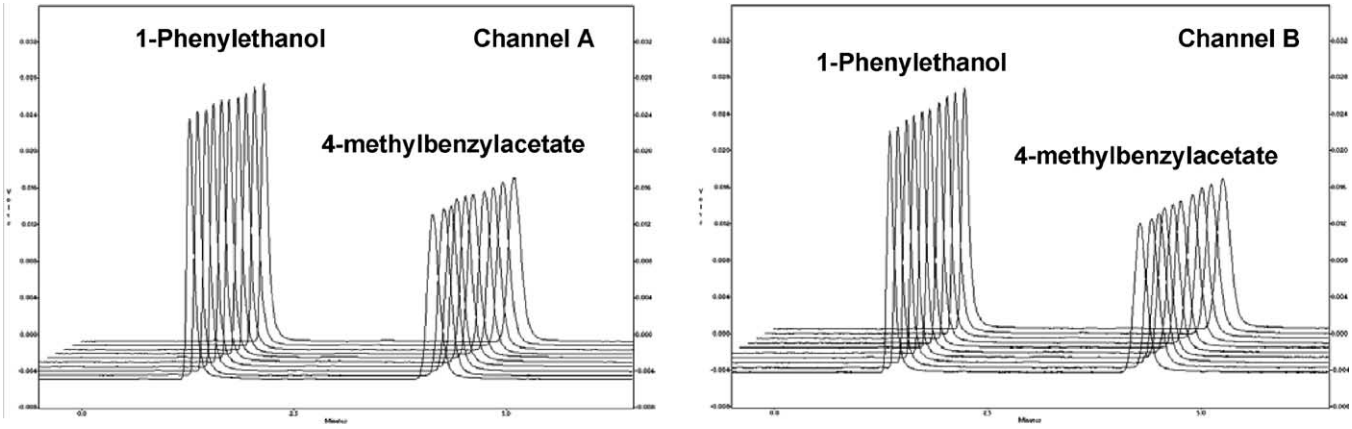

Fig. 3. Evaluation of the difference between channels. The chromatographic condition is as follows: separation column, Monocap® for fast flow (150 mm × 0.1 mm i.d.); eluent, 40% acetonitlile; flow rate, 2 μL/min; column temperature, ambient; detection, UV 210 nm; sample injection, 0.05 μL; sample, each 0.11 μL/mL of 1-phenylethanol and 4-methylbenzylacetate.

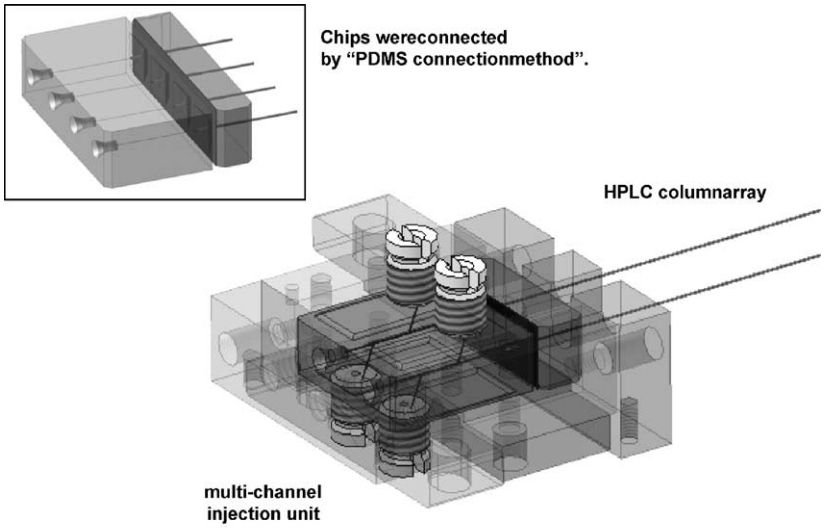

Fig. 4. PDMS connection between injection unit and capillary column array.

because of its insufficient optical path length. The long optical path leads to improve S/N ratio. Fig. 6 shows the comparison between the conventional on-column detection (0.075 mm of optical path) and the novel long-optical-path flow cell (4 mm of optical path). More than 10 times sensitivity improvement was confirmed despite the same range of noise (ca. 1×10^5 AU at UV 254 nm). Moreover, a prototype of multi-channel UV detector was also developed utilizing a "1–4 channel" or a "1–2 channel" fiber optics. Fig. 7 indicates the evaluation of sensitivity difference between channels for the case that a "1–4 channle" fiber optics was performed for delivering light to multi-channels. The difference of the peak height and the peak area between channels was less than 1% (R.S.D., $n = 5$), where noise level was 2×10^5 AU at UV 254 nm.

3.4. Application to peptides

To realize miniaturized multi-channel HPLC, the newly developed injection unit, monolithic capillary column array

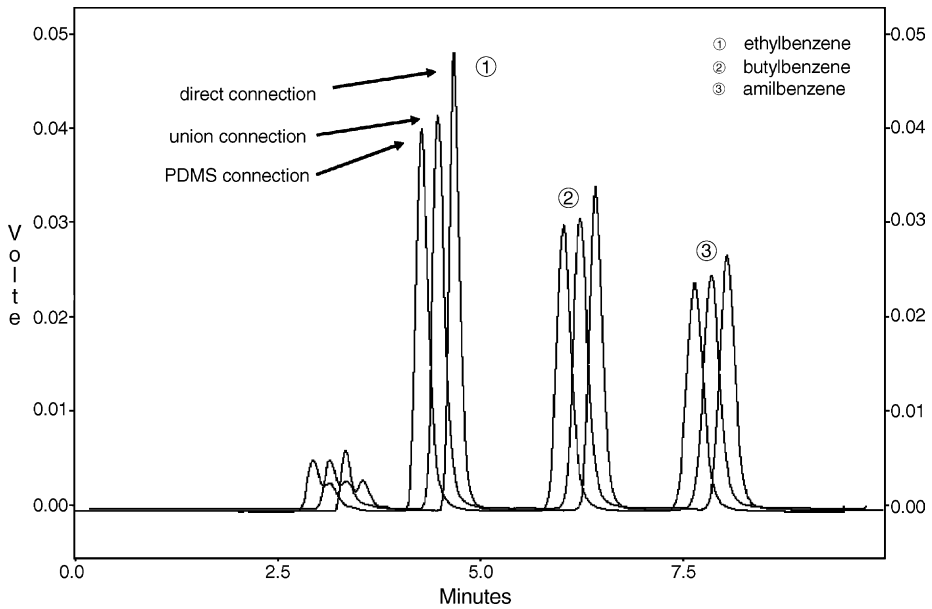

Fig. 5. Comparison of peak shapes between PDMS connection method and union. The chromatographic condition is as follows: separation column, Monocap® for fast flow (150 mm × 0.1 mm i.d.); eluent, 80% MeOH; flow rate, 2 μL/min; column temperature, ambient; detection, UV 210 nm; sample injection, 0.05 μL; sample, each 0.11 μL/mL of 1-phenylethanol and 4-methylbenzylacetate.

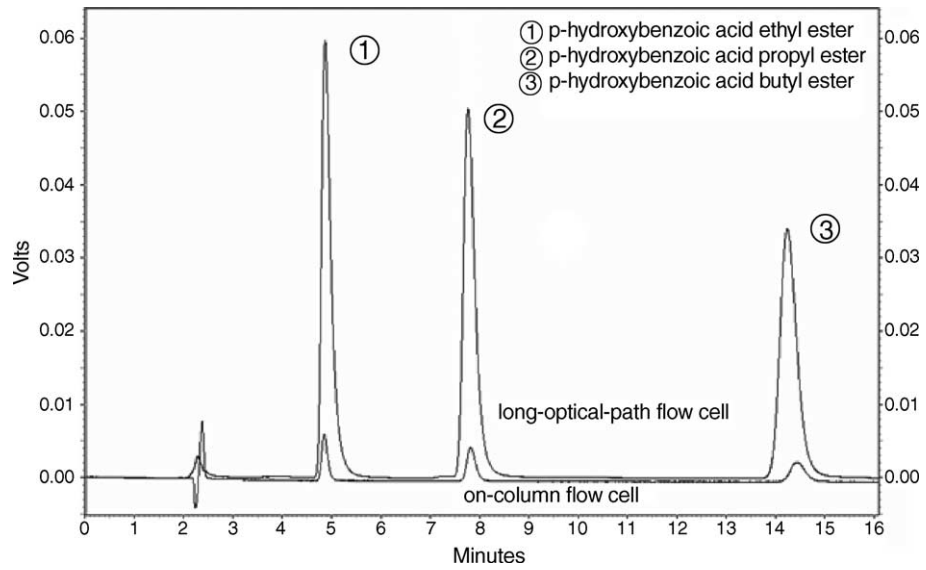

Fig. 6. Comparison of the sensitivity. The chromatographic condition is as follows: separation column, Monocap® for fast flow (150 mm × 0.2 mm i.d.); eluent, 30% acetonitlile; flow rate, 2 μL/min; column temperature, ambient; detection, UV 254 nm; sample injection, 0.1 μL; sample, each 0.1 mg/mL of *p*-hydroxybenzoic acid ethyl ester, *p*-hydroxybenzoic acid propyl ester and *p*-hydroxybenzoic acid butyl ester.

and multichannel detection unit were integrated by applying the PDMS connection method. The effectiveness was evaluated with ß-casein as a typical bio-sample. Obtained chromatogram is shown in Fig. 8. The multiple samples were simultaneously detected with excellent separation and sensitivity. This system was allowed to successfully analyze 100 fmol ß-casein. Thus, the 10-times sensibility and the two-times throughput compared with the conventional HPLC

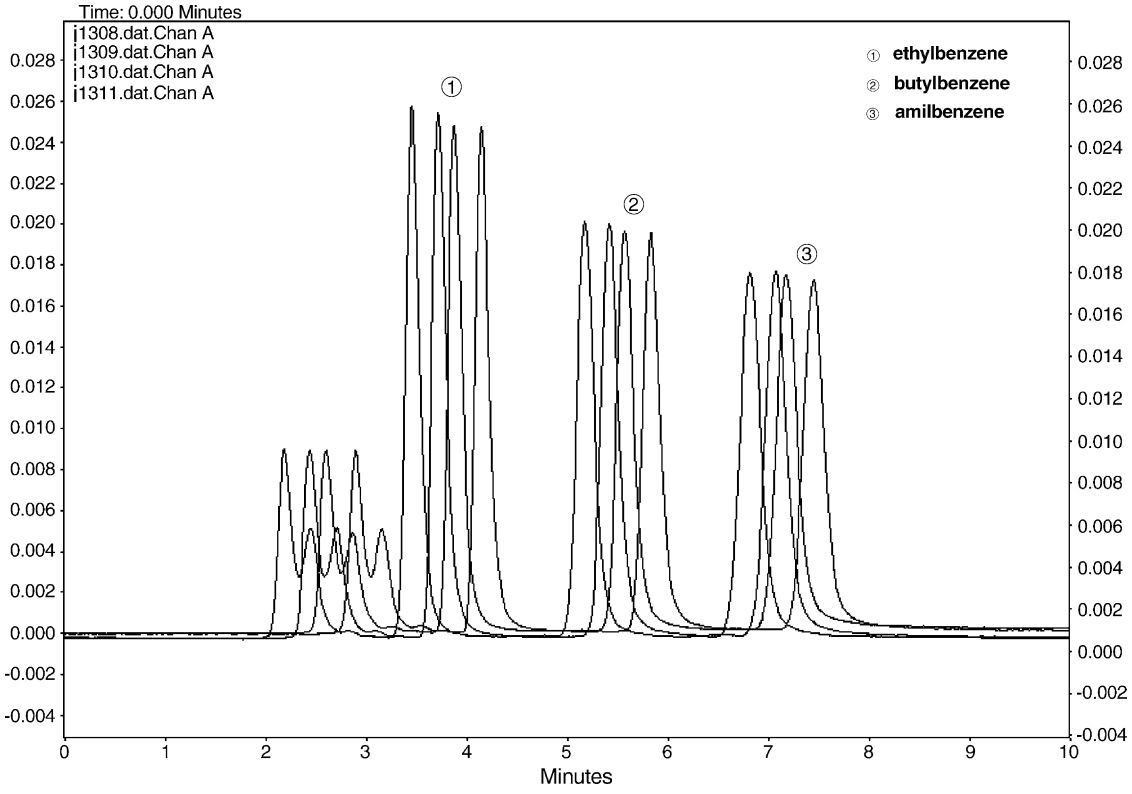

Fig. 7. Evaluation of the difference between channels using UV detector having multi-channel flow cell. The chromatographic condition is as follows: separation column, Monocap® for fast flow (150 mm × 0.1 mm i.d.); eluent, 80% methanol; flow rate, 2 μL/min; column temperature, ambient; detection, UV 210 nm; sample injection, 0.05 μL; sample, each 0.5 μL/mL of alkylbenzenes.

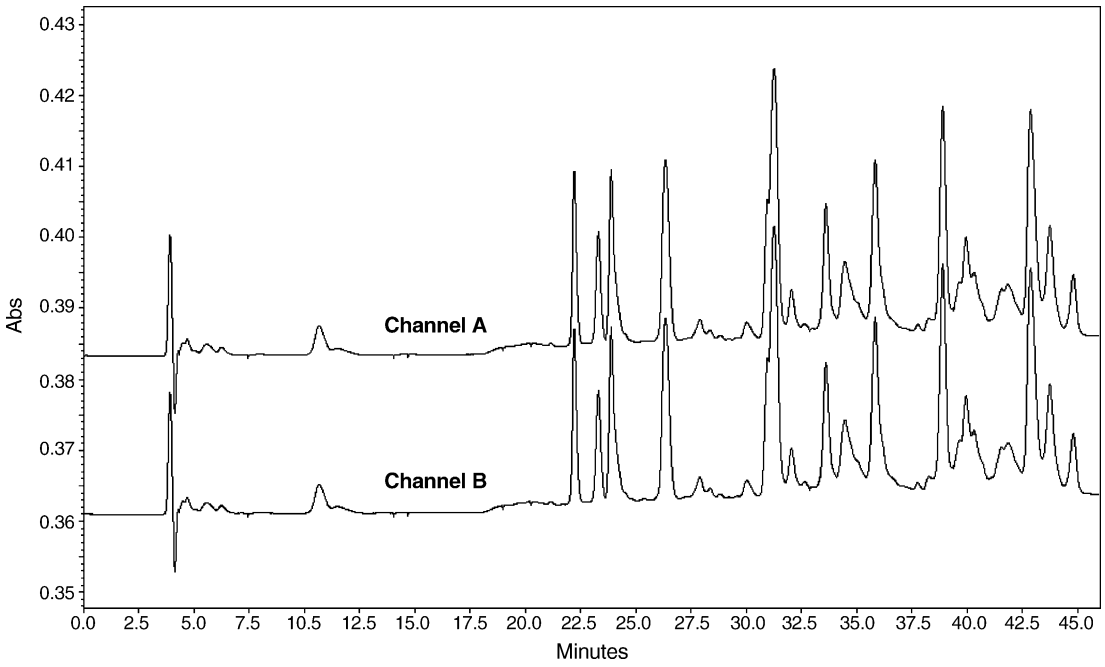

Fig. 8. Chromatogram of simultaneous multi-channel analysis of ß-casein The chromatographic condition is as follows: separation column, Monocap® for fast flow (150 m × 0.1 mm i.d.); eluent. (A) H_2O, (B) acetonitlile; gradient program, 5% (B) (initial), 3 min; 5% (B), 45 min; 45% (B); flow rate, 2 μL/min; column temperature, ambient; detection, UV 210 nm; sample injection, 0.05 μL; sample, tryptic digest of ß-casein.

analysis were achieved. It proves that the multichannel HPLC has a powerful ability for the high-throughput analysis of biosamples.

4. Conclusion

Novel miniaturized multi-channel HPLC was developed. Our findings suggest that the multi-channel approach allowing the parallel analysis of multiple samples can be employed to increase the bioanalytical throughput. It was also demonstrated that the multichannel system had enough capability of suitable detection limit and sample throughput. Further modifications of individual unit would be required for coping with the analysis of biological samples having wide variety of properties. It is also recommended that miniaturized multi-channel HPLC is applied for the routine MS analysis in biological media.

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