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PAPER IN FOREFRONT

Fast and quantitative analysis of branched-chain amino acids in biological samples using a pillar array column

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Abstract In this study, a fast and quantitative determination method for branched-chain amino acids (BCAAs), namely leucine, isoleucine, and valine, was developed using a pillar array column. A pillar array column with low-dispersion turns was fabricated on a 20×20-mm^2 microchip using multistep ultraviolet photolithography and deep reactive ion etching. The BCAAs were fluorescently labeled with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F), followed by reversed-phase separation on the pillar array column. The NBD derivatives of the three BCAAs and an internal standard (6-aminocaproic acid) were separated in 100 s. The calibration curves for the NBD-BCAAs had good linearity in the range of 0.4–20 μM, using an internal standard. The intra- and interday precisions were found to be in the ranges of 1.42–3.80 and 2.74–6.97 %, respectively. The accuracies for the NBD-BCAA were from 90.2 to 99.1 %. The method was used for the analysis of sports drink and human plasma samples. The concentrations of BCAAs determined by the developed method showed good agreements with those determined using a conventional high-performance liquid chromatography system. As BCAAs are important biomarkers of some diseases, these results showed that the developed method could be a potential diagnostic tool in clinical research.

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Introduction

Branched-chain amino acids (BCAAs) are a group of essential amino acids that have aliphatic side-chains with a branch (a carbon atom bound to more than two other carbon atoms). Among proteinogenic amino acids, there are three BCAAs: leucine (Leu), isoleucine (Ile), and valine (Val) (Fig. 1). BCAAs modulate protein synthesis and reduce protein catabolism [1] and also affect the mammalian target of rapamycin (mTOR) pathway, which could increase the basic metabolic rate [2]. Recently, BCAAs have been found to be closely related to diseases, such as chronic liver disease [3, 4], diabetes [5–7], obesity [8], and heart disease [9]. There is therefore a growing interest in the analysis of BCAAs to develop a better understanding of the mechanism, prediction, and diagnosis of diseases. Until now, many analytical methods have been developed for the analysis of only BCAAs, such as high-performance liquid chromatography (HPLC) with diode array [10], fluorescence [11], and chemiluminescence detection [12, 13] and liquid chromatography-tandem mass spectrometry [14]. Although analysis of amino acid mixtures including BCAAs has been performed within 10 min [15–18], the analysis times are longer for pure BCAAs when using these determination methods. In clinical diagnosis, faster analysis might be significant.

Some new types of columns, such as monolithic columns, sub-2-μm-particle columns, and core–shell columns, have recently been developed to shorten the analysis time [19–21]. For the analysis of BCAAs, using the sub-2-μm-particle column shortened the analysis time to 5 min [22]. Recently, with the development of precise fabrication technologies, pillar array

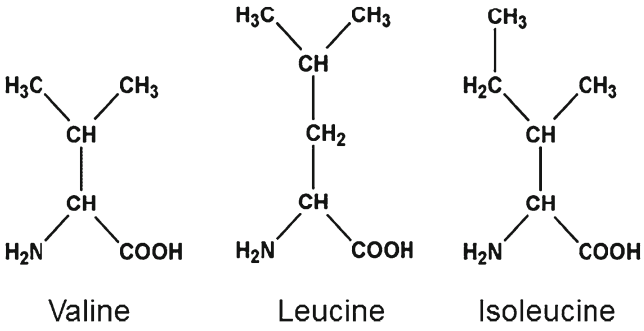

Fig. 1 Chemical structures of valine, leucine, and isoleucine

columns in which each pillar has exactly the same size have been developed [23–28]. Such structures could effectively decrease eddy diffusion, which results from non-ideal particle packing in the column and contributes to band broadening. We developed pillar array columns at a length of 110 mm, which were folded with low-dispersion turns [29]. The separation efficiencies were greatly improved, and the analysis of six fluorescently labeled amino acids could be achieved in about 140 s. However, pillar array columns have not been used for the quantitative analysis of food and biological samples, and there is little evidence as to whether pillar array columns can be applied to real samples. Eghbali et al. successfully used nonporous pillar array columns for the analysis of two different bacterial strains of fluorescent pseudomonads [30]; however, no quantitative data were reported. In this study, we used pillar array column for the fast analysis and quantification of BCAAs in sports drink and plasma samples.

Experimental

Chemicals and reagents

4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) was obtained from Dojindo Laboratories (Kumamoto, Japan). 6-aminocaproic acid, used as an internal standard, was obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile (HPLC grade) was purchased from Honeywell Burdick & Jackson (Muskegon, MI). Trifluoroacetic acid (TFA) for HPLC analysis was provided by Kanto Chemical Co. (Tokyo, Japan). A Milli-Q system (Millipore, Bedford, MA) was used for water purification. A sports drink (Amino-Value 4000, Otsuka Pharmaceutical, Tokyo, Japan) was bought from a local store in Tokyo, Japan. Human plasma was purchased from Sigma-Aldrich.

Microchip fabrication

The same chip design was used for all the BCAA separations in the study. The microchip was fabricated using multistep ultraviolet photolithography and deep reactive-ion etching. Low-dispersion turns were used for folding the pillar array column in a microchip with a size of 20× 20 mm^2 (Fig. 2). The column length was 110 mm. The channel widths in the straight part and the turns were 400 and 110 μm, respectively. The depth of the pillar array column was 30 μm, and the injection channel depth was 60 μm. The pillars were square shape with a size of 3 μm, and the inter-pillar distance was 2 μm. After fabrication of the microchip, dimethyloctadecylchlorosilane was used to modify the surface of the separation channel for reversed-phase separation. Details of the fabrication and modification procedures were described in our previous papers [29, 31].

Fluorescence derivatization of branched-chain amino acids

Twenty microliters of a solution of BCAAs and 20 μL of an internal standard solution (0.1 mM 6-aminocaproic acid) were added to 180 μL of 0.2 M borate buffer solution (pH 9.0). Then, 40 μL of 10 mM NBD-F were added and the mixture was heated in a water bath at a temperature of

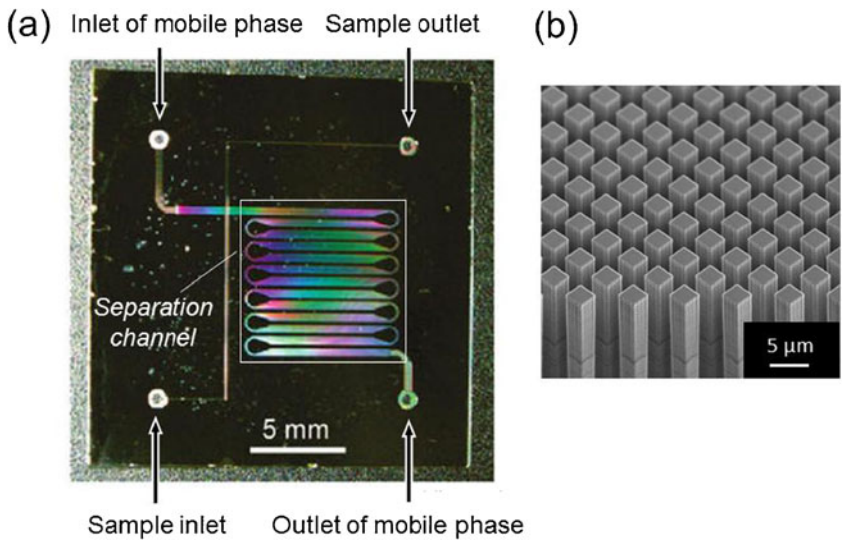

Fig. 2 (**a**) Photograph of the fabricated microchip and (**b**) scanning electron microscopy image of the pillar array structure

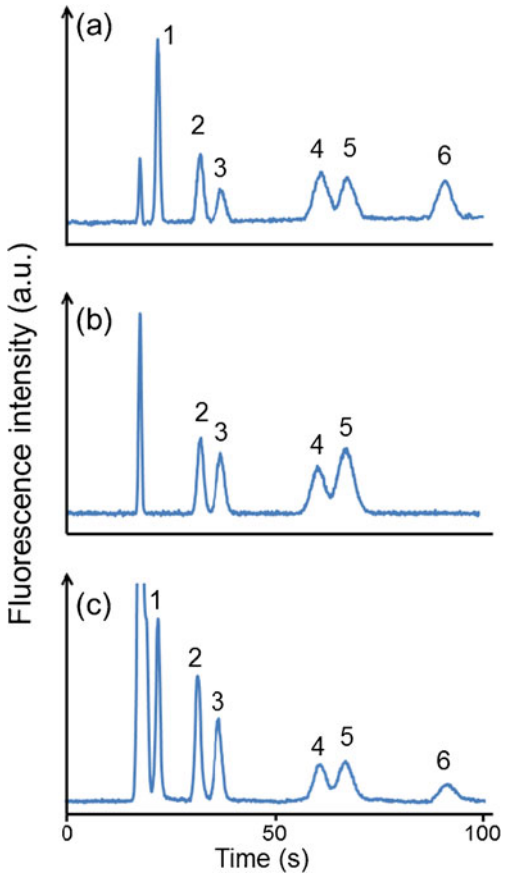

Fig. 3 Chromatograms of (**a**) NBD-amino acids solution, (**b**) sports drink, and (**c**) human plasma sample. Chromatogram peaks: *1* NBD-Pro, *2* NBD-Val, *3* NBD-6-aminocaproic acid, *4* NBD-Ile, *5* NBD-Leu, and *6* NBD-Phe

60 °C for 5 min. After cooling the reaction mixture in ice water, 740 μL of 50 mM HCl solution were added to the reaction mixture. The resultant solution was injected into the microchip LC system.

Pretreatment of sports drink sample

Twenty microliters of drink sample were diluted with 1,980 μL of 50 mM HCl. The diluted sample was fluorescently derivatized as described above.

Pretreatment of human plasma sample

In order to remove proteins in the plasma sample, 600 μL of methanol and 600 μL of acetonitrile were added to 200 μL of plasma sample. Then the sample was centrifuged at 10,000×*g* for 40 min. The supernatant was evaporated to dryness under reduced pressure at room temperature. The residue was derivatized with NBD-F as described above.

Chromatographic conditions

A water–acetonitrile–TFA (92:8:0.02, *v*/*v*/*v*) mixture was used as the mobile phase. The mobile phase was pumped into the microchip using a micro-HPLC pump (MP711, GL Sciences, Tokyo, Japan). The flow rate was set at 2 μL/min. An IX70 inverted microscope system (Olympus, Tokyo, Japan) was used for the determination of the fluorescence intensity. In this system, a UPlanApo ×20 (N.A. 0.70, Olympus) objective and an electron-multiplying charge-coupled device camera (iXon3, Andor Technologies, South Windsor, CT) were mounted for observation of the separation channel and fluorescence band. Fluorescence excitation was performed using a metal halide lamp. The filter cube had a BP460-490 excitation filter (Olympus), a 505DRLP dichroic mirror (Omega Optical, Brattleboro, VT), and an HQ 535-m emission filter (Chroma Technology, Rockingham, VT). A PSL-3F slit (Sigma Koki, Tokyo, Japan) was placed between the emission filter and the photocounter tube for confinement of the detection area. The detection point was set near the outlet of the pillar array column. The fluorescence intensity was analyzed using an H7421-40 photomultiplier tube equipped with a PHC-2500 photocounter. The sample was injected into the pillar array column using a four-port valve (Valco Instruments, Houston, TX) and a commercially available syringe (Hamilton, Reno, NV).

Validation study

Standard stock solutions were prepared by dissolving amino acids in 100-mM HCl solution. Solutions with different concentrations of amino acids (20, 40, 100, 200, and 1,000 μM) were prepared from the stock solutions. Linear

Table 1 Limits of detection and limits of quantification for NBD-BCAAs

Amino acid	Pillar array column				Conventional LC			
	LOD (nM)	LOD (fmol)	LOQ (nM)	LOQ (fmol)	LOD (nM)	LOD (fmol)	LOQ (nM)	LOQ (fmol)
Pro	89.3	0.11	298	0.36	0.47	4.67	1.56	15.6
Val	107	0.13	355	0.43	0.29	2.94	0.98	9.8
Ile	130	0.16	434	0.52	0.43	4.27	1.42	14.2
Leu	123	0.15	412	0.49	0.44	4.40	1.47	14.7
Phe	161	0.19	538	0.65	0.50	4.97	1.66	16.6

Table 2 Precision and accuracy of proposed method for determination of BCAAs in sports drink

Amino acid	Intraday precision (%) (n=5)	Interday precision (%) (n=5)	Accuracy (%)
Val	2.69	6.97	99.1±1.9
Ile	1.42	4.64	96.9±1.2
Leu	3.03	3.80	90.2±2.4

calibration curves were produced by plotting the ratios of the peak heights of the NBD-amino acids to the internal standard versus the injection amounts of the NBD-amino acids. Intra- and interday precision experiments were performed by analyzing the plasma samples five times in the same day and once per day for five consecutive days, respectively. To evaluate the accuracy of the analytical method, standard amino acids of different concentrations (200, 400, and 600 μM) were added to a human plasma sample. The accuracy was calculated by dividing the value of the slope obtained from the standard-spiked plasma sample by that of the slope for the standard amino acids.

Analysis of branched-chain amino acids using conventional LC system

The analysis of BCAAs using conventional LC was based on our previous research [16].

Results and discussion

Optimization of chromatographic conditions

In our previous study [29], water–acetonitrile–TFA (90:10:0.02, *v*/*v*/*v*) was used as the mobile phase for the separation of six NBD-amino acids (proline (Pro), Val, 6-aminocaproic acid, Ile, Leu, and phenylalanine (Phe)). The analysis time was about 140 s. In order to achieve a faster analysis of only BCAAs, we optimized the chromatographic conditions, including composition of the mobile phase, flow rate, and the detection point on the chip. The optimized mobile phase was water–acetonitrile–TFA (92:8:0.02, *v*/*v*/*v*) at a flow rate of 2 μL/min. A longer pillar array column would ensure better-resolved separation; therefore, the detection point was set near the outlet of the separation channel. A representative chromatogram of NBD-BCAAs is shown in Fig. 3a. The separation was achieved within about 100 s, which was about two thirds of the time taken in our previous study [29]. The reproducibility of the retention time of each NBD-BCAA was below 2.82 %.

Linearity, limits of detection, and limits of quantification

A pillar array column is a powerful tool for improving separation efficiency. However, to obtain this advantage, extra-column broadening should be kept to a minimum, as such broadening lowers the detection sensitivity. In this study, therefore, we used on-chip detection with a fluorescence microscope. First, we investigated the reproducibility of the peak intensity detected on the photomultiplier tube because the injection amount was very small. The relative standard deviation of the peak intensity was 28.4 % when NBD-Val was injected at a concentration of 2 μM. 6-aminocaproic acid was added as an internal standard, and the ratio of the peak intensity of NBD-Val to that of the internal standard was calculated. We found that addition of an internal standard improved the reproducibility to 3.23 %. This result shows that quantitative analysis can be performed using a pillar array column with the help of an internal standard.

The calibration curve for each NBD-BCAA showed good linearity in the range 0.4 to 20 μM. The limits of detection (LODs) and limits of quantification (LOQs) for the NBD-BCAAs were each calculated as the concentration and the injection amount. For comparison, the values obtained with conventional LC in our previous research were used [32]. As shown in Table 1, when the LODs and LOQs for individual NBD-amino acids were calculated as the concentrations at signal-to-noise ratios of 3 and 10, respectively, the values obtained using the pillar array column were significantly higher than those obtained using conventional LC. However, when the LODs and LOQs were calculated as injection

Table 3 Concentrations of BCAAs in sports drink

Amino acid	Concentration reported in product brochure (mg/100 mL)	Concentration determined using pillar array column (mg/100 mL)	Concentration determined using conventional LC (mg/100 mL)
Val	200	188±3.5	200±3.4
Ile	200	214±2.6	200±6.6
Leu	400	392±10	409±13

(mean±SD, n=3)

Table 4 Precision and accuracy of developed method for determination of BCAAs in human plasma sample

Amino acid	Intra-day precision (%) (n=5)	Inter-day precision (%) (n=5)	Accuracy (%)
Val	1.93	2.74	91.6±1.0
Ile	3.80	3.63	92.6±1.1
Leu	3.19	4.86	92.1±0.8

amounts, the values obtained using the pillar array column were significantly lower than those obtained using conventional LC. This was clearly because of the difference between the injection amounts in the two methods. When conventional LC was used, the injection amount was 10 μL, whereas the injection volume was only about 1 nL in the microchip LC system. In order to improve the sensitivity, more studies are necessary. A blue laser, which matches the excitation wavelength of the NBD-BCAAs, might be useful for fluorescence irradiation, to obtain a stronger signal [33]. As there are pillar arrays at the detection point in this study, the design of the detection area on the microchip needs to be optimized. On-chip sample preconcentration before separation with the pillar array column could also greatly increase the detection sensitivity and simplify sample pretreatment [34].

Determination of branched-chain amino acids in sports drink sample

As an intake of BCAAs before exercise can delay the production of lactic acid and lead to maintenance of endurance exercise capacity, sports drinks containing BCAAs are useful [35]. We used the developed method to determine BCAAs in a sports drink; the BCAA concentration had to be sufficiently high so that this case serves as a good example for the first application of the method to real samples. After dilution and derivatization with NBD-F, the sample was analyzed using pillar array columns. The chromatogram obtained from the sports drink sample is shown in Fig. 3b. The peaks of the NBD-BCAAs and NBD-6-aminocaproic acid were well separated. There were no other peaks interfering with the analysis of the NBD-BCAAs. The sports drink also contained arginine, but because arginine is hydrophilic, NBD-arginine was easily eluted and overlapped with the peak of NBD-OH. Precision and accuracy data are listed in Table 2. These results showed that the method was valid for the determination of BCAAs in a sports drink. The concentrations of amino acids determined using pillar array column and conventional LC are presented in Table 3. These values showed good agreement with the concentrations reported in the product brochure.

Table 5 Concentrations of BCAAs in human plasma sample

Amino acid	Concentration determined using pillar array column (μM)	Concentration determined using conventional LC (μM)
Val	191±4.0	195±5.5
Ile	72.7±1.6	77.0±3.5
Leu	111±1.5	121±7.8

(mean±SD, n=3)

Determination of branched-chain amino acids in human plasma sample

Next, we applied the developed method to a human plasma sample. This is more difficult because the plasma sample has many other endogenous compounds and the concentrations of BCAAs are much lower than in a sports drink. The chromatogram obtained from the human plasma sample is shown in Fig. 3c. Because most of the NBD-amino acids were very hydrophilic, they were easily eluted and overlapped with the NBD-OH peak when the optimized mobile phase was used, as mentioned above. Precision and accuracy experiments were carried out to evaluate whether the developed method could be useful for routine analysis of BCAAs in human plasma samples. As shown in Table 4, both the intra- and interday precisions (n=5) were below 5 %. The accuracies for the three BCAAs were about 90 %. The results indicate that the developed method using the pillar array column should be applicable to biological samples. The concentrations of amino acids determined using the developed method and conventional LC are presented in Table 5. From the table, we can conclude that the concentrations obtained using the pillar array column were similar to the data obtained using conventional LC.

Conclusions

A 110-mm pillar array column, which was folded with low-dispersion turns, was used for fast analysis of BCAAs. The analysis time of fluorescently labeled BCAAs was about 100 s, which was much faster than in previous studies. For quantitative determinations of BCAAs in sports drink and human plasma samples, fluorescence microscopy was used for sensitive detection. With the help of an internal standard, the concentrations of BCAAs in these samples were quantitatively determined. The concentrations determined using the developed method showed good agreement with data obtained using conventional LC. The developed method should be

useful for clinical diagnosis. Although the sensitivity still needs to be improved, we believe that the method using pillar array columns could be used for fast analysis of biological compounds in biosamples, such as plasma, urine, and tissue.

References

1. De Bandt JP, Cynober L (2006) Therapeutic use of branched-chain amino acids in burn, trauma, and sepsis. J Nutr 136(1):308S–313S
2. Cota D, Proulx K, Woods S, Seeley R (2005) Mammalian target of rapamycin (mTOR) and branched-chain amino acids: role in the hypothalamic circuits regulating energy balance. Obes Res 13: A21–A21
3. Suzuki K, Suzuki K, Koizumi K, Ichimura H, Oka S, Takada H, Kuwayama H (2008) Measurement of serum branched-chain amino acids to tyrosine ratio level is useful in a prediction of a change of serum albumin level in chronic liver disease. Hepatol Res 38 (3):267–272
4. Kawaguchi T, Izumi N, Charlton MR, Sata M (2011) Branched-chain amino acids as pharmacological nutrients in chronic liver disease. Hepatology 54(3):1063–1070
5. Kuzuya T, Katano Y, Nakano I, Hirooka Y, Itoh A, Ishigami M, Hayashi K, Honda T, Goto H, Fujita Y, Shikano R, Muramatsu Y, Bajotto G, Tamura T, Tamura N, Shimomura Y (2008) Regulation of branched-chain amino acid catabolism in rat models for spontaneous type 2 diabetes mellitus. Biochem Biophys Res Commun 373(1):94–98
6. Belalcazar LM, Ballantyne CM (2011) Nutrition and metabolism—sphingolipids and branched chain amino acids: indicators and effectors of adipose tissue function and diabetes risk. Curr Opin Lipidol 22 (6):503–504
7. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, Lewis GD, Fox CS, Jacques PF, Fernandez C, O'Donnell CJ, Carr SA, Mootha VK, Florez JC, Souza A, Melander O, Clish CB, Gerszten RE (2011) Metabolite profiles and the risk of developing diabetes. Nat Med 17(4):448–453
8. Felig P, Marliss E, Cahill GF (1969) Plasma amino acid levels and insulin secretion in obesity. N Engl J Med 281(15):811–816
9. Huang Y, Zhou M, Sun H, Wang Y (2011) Branched-chain amino acid metabolism in heart disease: an epiphenomenon or a real culprit? Cardiovasc Res 90(2):220–223
10. Gioia MG, Andreatta P, Boschetti S, Gatti R (2007) Development and validation of a liquid chromatographic method for the determination of branched-chain amino acids in new dosage forms. J Pharm Biomed Anal 45(3):456–464
11. Kand'ar R, Zakova P, Jirosova J, Sladka M (2009) Determination of branched chain amino acids, methionine, phenylalanine, tyrosine and alpha-keto acids in plasma and dried blood samples using HPLC with fluorescence detection. Clin Chem Lab Med 47(5):565–572
12. Kiba N, Oyama Y, Kato A, Furusawa M (1996) Postcolumn co-immobilized leucine dehydrogenase-NADH oxidase reactor for the determination of branched-chain amino acids by high-performance liquid chromatography with chemiluminescence detection. J Chromatogr A 724(1–2):354–357
13. Uchikura K (2003) Determination of aromatic and branched-chain amino acids in plasma by HPLC with electrogenerated Ru (bpy)(3)(3+) chemiluminescence detection. Chem Pharm Bull 51 (9):1092–1094
14. Sowell J, Pollard L, Wood T (2011) Quantification of branched-chain amino acids in blood spots and plasma by liquid chromatography tandem mass spectrometry for the diagnosis of maple syrup urine disease. J Sep Sci 34(6):631–639
15. Shimbo K, Kubo S, Harada Y, Oonuki T, Yokokura T, Yoshida H, Amao M, Nakamura M, Kageyama N, Yamazaki J, Ozawa S, Hirayama K, Ando T, Miura J, Miyano H (2009) Automated precolumn derivatization system for analyzing physiological amino acids by liquid chromatography/mass spectrometry. Biomed Chromatogr 24(7):683–691
16. Song Y, Funatsu T, Tsunoda M (2012) Rapid determination of amino acids in biological samples using a monolithic silica column. Amino Acids 42(5):1897–1902
17. Pumera M (2007) Microfluidics in amino acid analysis. Electrophoresis 28(13):2113–2124
18. Poinsot V, Carpene M-A, Bouajila J, Gavard P, Feurer B, Couderc F (2012) Recent advances in amino acid analysis by capillary electrophoresis. Electrophoresis 33(1):14–35
19. Gritti F, Guiochon G (2012) The current revolution in column technology: how it began, where is it going? J Chromatogr A 1228:2–19
20. Gritti F, Tanaka N, Guiochon G (2012) Comparison of the fast gradient performance of new prototype silica monolithic columns and columns packed with fully porous and core–shell particles. J Chromatogr A 1236:28–41
21. Nunez O, Gallart-Ayala H, Martins CPB, Lucci P (2012) New trends in fast liquid chromatography for food and environmental analysis. J Chromatogr A 1228:298–323
22. Alodaib A, Carpenter K, Wiley V, Sim K, Christodoulou J, Wilcken B (2011) An improved ultra performance liquid chromatography-tandem mass spectrometry method for the determination of alloisoleucine and branched chain amino acids in dried blood samples. Ann Clin Biochem 48:468–470
23. He B, Tait N, Regnier F (1998) Fabrication of nanocolumns for liquid chromatography. Anal Chem 70(18):3790–3797
24. De Malsche W, Eghbali H, Clicq D, Vangelooven J, Gardeniers H, Desmet G (2007) Pressure-driven reverse-phase liquid chromatography separations in ordered nonporous pillar array columns. Anal Chem 79(15):5915–5926
25. Yan XH, Wang QW, Bau HH (2010) Dispersion in retentive pillar array columns. J Chromatogr A 1217(8):1332–1342
26. Vangelooven J, Schlautman S, Detobel F, Gardeniers H, Desmet G (2011) Experimental optimization of flow distributors for pressure-driven separations and reactions in flat-rectangular microchannels. Anal Chem 83(2):467–477
27. Lavrik NV, Taylor LT, Sepaniak MJ (2011) Nanotechnology and chip level systems for pressure driven liquid chromatography and emerging analytical separation techniques: a review. Anal Chim Acta 694(1–2):6–20
28. De Malsche W, De Beeck JO, De Bruyne S, Gardeniers H, Desmet G (2012) Realization of 1×10(6) theoretical plates in liquid chromatography using very long pillar array columns. Anal Chem 84 (3):1214–1219
29. Aoyama C, Saeki A, Noguchi M, Shirasaki Y, Shoji S, Funatsu T, Mizuno J, Tsunoda M (2010) Use of folded micromachined pillar array column with low-dispersion turns for pressure-driven liquid chromatography. Anal Chem 82(4):1420–1426
30. Eghbali H, Matthijs S, Verdoold V, Gardeniers H, Cornelis P, Desmet G (2009) Use of non-porous pillar array columns for the separation of *Pseudomonas pyoverdine* siderophores as an example of a real-world biological sample. J Chromatogr A 1216 (49):8603–8611
31. Song Y, Noguchi M, Takatsuki K, Sekiguchi T, Mizuno J, Funatsu T, Shoji S, Tsunoda M (2012) Integration of pillar array columns into a gradient elution system for pressure-driven liquid chromatography. Anal Chem 84(11):4739–4745
32. Song Y, Funatsu T, Tsunoda M (2011) Amino acids analysis using a monolithic silica column after derivatization with 4-fluoro-7-

nitro-2,1,3-benzoxadiazole (NBD-F). J Chromatogr B 879(5–6):335–340
33. Tsunoda M, Kato M, Fukushima T, Santa T, Homma H, Yanai H, Soga T, Imai K (1999) Determination of aspartic acid enantiomers in bio-samples by capillary electrophoresis. Biomed Chromatogr 13(5):335–339
34. Song S, Singh AK (2006) On-chip sample preconcentration for integrated microfluidic analysis. Anal Bioanal Chem 384(1):41–43
35. Calders P, Pannier JL, Matthys DM, Lacroix EM (1997) Pre-exercise branched chain amino acid administration increases endurance performance in rats. Med Sci Sports Exerc 29(9):1182–1186