Prediction of retention time by combining multiple datasets with chromatographic parameter vectorization and transfer learning

Xiaohui Lin*[a], Yansong Li[a], Di Yu [b]
[a] *School of Computer Science & Technology, Dalian University of Technology, 116024 Dalian, China.*
[b] *Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China.*

*Email: datas@dlut.edu.cn

Liquid chromatography-mass spectrometry (LC-MS) is one of the most useful platforms for metabolomics study, but metabolite identification is still a bottleneck in nontargeted metabolomics. The identification of metabolites is usually based on mass spectrometry information, and has a high false positive rate. Retention time (RT) in chromatography can provide information orthogonal to mass spectra, helping improve the accuracy. But, under given chromatographic conditions, the number of compounds with known RTs is small. This data sparsity often leads to insufficient training of machine learning models, seriously affecting the performance of RT prediction.

We propose an effective method (MDL-TL) to address the data sparsity in RT prediction and enhance the RT prediction performance. MDL-TL vectorize chromatographic parameter by autoencoders and Word2Vec, and marks the datasets from different chromatographic experiments by adding the chromatographic parameter vectors into the compound representation. Then MDL-TL combines multiple datasets to pretrain the base model and learn the prior knowledge. For a certain target task, the RT prediction model is obtained by fine tuning the base model.

Experiments on the 14 RPLC datasets and 14 HILIC datasets have shown that MDL-TL outperforms the compared machine learning and deep learning methods (including transfer learning methods) in most cases. Experiments also show that the base model pre-trained with the multiple datasets from chromatographic modes that are the same as the target task usually has a better performance than the model pretrained by combining small datasets from different chromatographic modes. Since MDL-TL incorporates the chromatographic parameter into the RT prediction model, the experiment on the 14 mixed datasets shows that MDL-TL still outperforms the baseline methods in most cases, even using the datasets from different chromatographic modes for pre-training. Further, even seven datasets are combined (total 2240 compounds) for pretraining, MDL-TL can also get a better performance than the compared transfer learning method based on SMRT dataset (containing 80038 compounds) in most cases.

Keywords: retention time, chromatographic parameter vectorization, transfer learning, metabolomics

Automatic solid phase extraction with coated microfibrous sorbent as a front-end to UHPLC – case of determination of xenobiotic residues in surface water

Lucie Chocholoušová Havlíková*[a], Petr Chocholouš[a], Jakub Erben[b], Pavel Holec[b], František Švec[a], Dalibor Šatínský[a]

[a] *Charles University, Faculty of Pharmacy, Department of Analytical Chemistry, Akademika Heyrovského 1203, 50003, Hradec Králové, Czechia*
[b] *Technical University of Liberec, Faculty of Textile Engineering, Department of Nonwovens and Nanofibrous Materials, Studentská 1402/2, 46001, Liberec 1, Czechia*

*Email: chocholousova@faf.cuni.cz

Automation of multiple steps was used for the sensitive determination of xenobiotic residues in surface waters. The online hyphenated sequential injection analysis instrumentation used surface-modified microfibrous sorbent for the effective solid phase extraction of 1.0 mL of sample into a 100 µL zone as a front-end of reversed-phase ultra-high performance liquid chromatography (SIA-SPE-UHPLC). The SPE column was packed with polypropylene microfibers coated with polydopamine. The freshly prepared column was used for extraction of more than 200 samples in the SIA-SPE-UHPLC method with high reproducibility. The performance of the developed method was demonstrated with the analysis of fifteen river water samples. Our study highlights the advantages of online SPE systems and their contribution to the advancement of water quality monitoring through automation and improved analytical sensitivity.

The authors gratefully acknowledge the financial support of the Czech Science Foundation, project GAČR No 23-05586S.

Keywords: online solid phase extraction; sequential injection analysis; ultra-high performance liquid chromatography; water analysis; microfibers

Efficient enrichment and rapid determination of pyrrolizidine alkaloids by novel microporous organic network extraction coupled with miniature mass spectrometry

Liying You [a], Xiyue Yang [a], Chengxiong Yang [b], Zhengtao Wang[a], Li Yang*[a], Linnan Li* [a]

[a] State Key Laboratory of Discovery and Utilization of Functional Components in Traditional Chinese Medicine, The MOE Key Laboratory of Standardization of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

[b] School of Pharmaceutical Sciences & Institute of Materia Medica, Medical Science and Technology Innovation Center, Shandong First Medical University & Shandong Academy of Medical Sciences, Jinan, Shandong 250117, China

Email: yl7@shutcm.edu.cn; linnanli@shutcm.edu.cn

Pyrrolizidine alkaloids (PAs) are hepatotoxic natural toxins found in numerous plants, and their ingestion can lead to severe liver injury. For patients with suspected PA-induced liver damage, the rapid detection of these toxins in biological samples is crucial for accurate diagnosis and timely clinical intervention. This study presents a novel method that combines a microporous organic network (MON) as a selective adsorbent for sample pretreatment with a miniature mass spectrometer for rapid analysis. We demonstrate that this approach enables the efficient enrichment and determination of PAs in complex matrices. Compared to conventional protein precipitation, the MON-based dispersive solid phase extraction (DSPE) significantly enhances detection sensitivity. Furthermore, the MON material exhibited superior performance for PA enrichment and purification over other commonly used adsorbents. The use of miniature mass spectrometry, in place of traditional liquid chromatography-mass spectrometry (LC-MS) systems, offers key advantages in analytical speed and operational simplicity. This integrated strategy holds considerable promise for the rapid, point-of-care screening of PA exposure and the clinical diagnosis of related toxicities.

Keywords: pyrrolizidine alkaloids; miniature mass spectrometry; microporous organic network; urine

References

[1] Xiyue Yang, et al., *Analyst*, **2025**, 150

[2] Shuowen Gao, et al., *JOURNAL OF CHROMATOGRAPHY A*, **2024**, 1721

Highly Sensitive Microchip Electrophoresis of Cationic Analytes by LDMS

Fumihiko Kitagawa[1]*, Kotaro Ishikawa[1], Takayuki Kawai[2]

[1]*Graduate School of Science and Technology, Hirosaki University, Japan*
[2]*Graduate School of Science, Kyushu University*

To achieve highly sensitive electrophoretic analysis, in our research group, on-line sample preconcentration by large-volume sample stacking with an electroosmotic flow (EOF) pump (LVSEP)[1], which allows the entire volume of sample introduced into the channel has been applied to microchip electrophoresis (MCE). However, the application of LVSEP has mainly been limited to anionic analytes. For LVSEP analyses of cations, it is necessary to reverse the EOF. To address this issue, polyallylamine-modified microchips, which generates a positive zeta potential on the channel surface, was employed to the analysis of cationic species. However, it was difficult to control the EOF in the polyallylamine-immobilized microchannel, resulting in insufficient enrichment efficiency. In this study, large-volume dual-sample stacking by micelle collapse and sweeping (LDMS)[2], which utilizes micelle collapse by organic solvents, was used to enrich cationic analytes in poly(vinyl alcohol) (PVA)-modified microchannels, generating a stable EOF.

PDMS straight-channel microchips with 150 μm width and 100 μm depth were fabricated, and the channel surface was modified with PVA using a vacuum-drying method[3]. NBD-histamine (HA) was used as a standard cationic analytes. 10 mM MES buffer (pH 5.5), 10 mM sodium dodecyl sulfate (SDS) and acetonitrile (ACN) were employed as a background electrolyte (BGE), a micellar solution and organic solvent for micelle collapse, respectively. In the MCE analysis, a voltage of +1.5 kV was applied, and fluorescence detection was performed with excitation at 488 nm and monitoring at 520 nm.

In the LDMS method, the entire microchannel is initially filled with a sample solution, followed by the injection of a short plug of SDS micelle from the outlet reservoir. By applying a positive electric field, the SDS micelles migrate electrophoretically toward the inlet while capturing and sweeping cationic analytes. By raising the liquid level of the inlet reservoir, an organic solvent is gradually introduced into the channel via pressure-driven flow, which causes the micelle collapse into SDS monomers. When the micelle collapses occur around the inlet reservoir, cationic analytes captured by the SDS micelle were released to create a narrower zone. When NBD-labeled histamine (HA) was used as the model analyte, and the LDMS analysis was performed using a BGE containing acetonitrile (ACN) in the inlet reservoir and 10 mM MES buffer (pH 5.5) in the outlet reservoir, a single sharp-peak was observed. Under conditions where the liquid levels of both reservoirs were equal, the sensitive enhancement factor (SEF) reached to 3000. When the inlet liquid level was raised by 0.25 mm, the SEF was increased to 3500 with significant improvements in the analytical reproducibilities.

Under the initial experimental conditions, it was required as long as 15 min to detect the target peaks. To overcome this limitation, the channel length was reduced from 6 cm to 3 cm. When the liquid level difference between the inlet and outlet reservoirs was varied from 0 to 0.50 mm, the highest peak was obtained at 0.25 mm difference, resulting in the SEF of 1080. The detection time was successfully reduced to *ca.* 6 min, and the peak width was as narrow as 0.4 s. In our enrichment studies for cationic analytes, this was the best SEF and peak width. In the present LDMS condition, the anionic SDS micelles effectively captured the positively charged HA through electrostatic interaction, contributing to sharper peak profiles.

1) F. Kitagawa, *et al. Anal. Sci.* 29 (2013) 1129-39: DOI 10.2116/analsci.29.1129.
2) S. Koganemaru, *et al. Br. J. Pharmacol.* 180 (2023) 762-774: DOI 10.1111/bph.15988.
3) F. Kitagawa, *et al. Anal. Sci.* 31 (2015) 1171-75: DOI 10.2116/analsci.31.1171.

Non-invasive diagnostics of Barrett's esophagus - analysis of bile acids in saliva and exhaled breath condensate

Jiri Volanek*[a, b], Petr Kuban[a], Julia Lacna[a], Marketa Lastovickova[c], Vera Dosedelova[a], Stefan Konecny[d], Jiri Dolina[d]

[a] *Department of Bioanalytical Instrumentation, Institute of Analytical Chemistry of the Czech Academy of Sciences, v.v.i., Veveří 97, Brno, 602 00, Czech Republic*

[b] *Department of Chemistry, Faculty of Science, Masaryk University, Kamenice 5, Brno, 625 00, Czech Republic*

[c] *Department of Environmental Analytical Chemistry, Institute of Analytical Chemistry of the Czech Academy of Sciences, v.v.i., Veveří 97, Brno, 602 00, Czech Republic*

[d] *Internal Gastroenterology Department, University Hospital Brno and Faculty of Medicine, Masaryk University, Jihlavská 20, Brno, 625 00, Czech Republic*

*Email: volanek@iach.cz

Bile acids (BA) are a group of steroid compounds essential for lipid digestion. However, when the BAs are refluxed into the stomach and the esophagus during the duodenogastroesophageal reflux, they could have a detrimental effect on the esophageal epithelium. This could lead to the development of pathological changes in esophageal tissue, e.g. Barret's esophagus (BE) [1]. The levels of BA in non-invasive samples, such as saliva or exhaled breath condensate (EBC), might serve as a possible biomarker for the BE diagnostics and indicate potential for BE development. For non-invasive sampling, two methods were selected. Saliva sampling was performed by simple spitting. The EBC collection was performed using a lab-developed sampling device. The EBC sampler consists of three serially connected cooled glass tubes, enabling efficient sampling, e.g. a healthy male individual with an approximate lung capacity of 4-5 L can typically collect 100-150 µL of EBC per exhalation. Quantification of 15 bile acids (unconjugated, glycine-conjugated and taurine-conjugated) was performed. The samples were collected as a part of an ongoing clinical study, including patients with gastroesophageal reflux disease (GERD), patients with Barrett's esophagus and healthy subjects. For the purpose of non-invasive diagnosis of BE patients, as well as potential prediction of BE development, we have optimized a preconcentration method utilizing SPE sample treatment and UHPLC-MS analysis of bile acids in the above non-invasive samples [2]. Results show that the ratio for tauro-conjugated bile acids, glyco-conjugated bile acids and total bile acids shows significant statistical difference between patients with BE and healthy subjects ($p < 0.018$, $p < 0.023$, $p < 0.021$) and between patients with BE and GERD ($p < 0.027$, $p < 0.041$, $p < 0.034$). Of all the BA analytes, most promising biomarkers appear to be glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA). There were no statistical differences between patients with GERD and healthy individuals, suggesting that the ratio pattern is altered specifically in Barret's esophagus.

The authors acknowledge the financial support from the Ministry of Health of the Czech Republic (Grant no. NU23-08-00303) and the institutional support RVO: 68081715.

Keywords: bile acids, saliva, exhaled breath condensate, Barret's esophagus, GERD

References

[1] D.S. Oh, J.A. Hagen, M. Fein, C.G. Bremner, C.M. Dunst, S.R. DeMeester, J. Lipham, T.R. DeMeester, ***J. Gastrointest. Surg.*** 10, 787–796, 2006. [2] V. Dosedelova, M. Lastovickova, J.F. Ayala-Cabrera, J. Dolina, S. Konecny, O.J. Schmitz, P. Kuban, ***J. Chromatogr. A***, 1676, 2022.

On-line electrochemical synthesis of fluorescently labeled glycans utilizing a microfluidic chip with electrodes

Sachio Yamamoto*[a], Ryuki Kosaka[a], Asaka Tamari[a], Sakura Ida[a], Mitsuhiro Kinoshita[a, b]
[a] *Faculty of Pharmaceutical Sciences, Kindai University, 3-4-1, Kowakae, Higashi-osaka, Osaka, 577-8502, Japan.*
[b] *Antiaging Center, Kindai University, 3-4-1, Kowakae, Higashi-osaka, Osaka, 577-8502, Japan.*

*Email: yamamoto@phar.kindai.ac.jp

Post-translational modifications (PTMs), which involve the covalent attachment of chemical entities to the side chains of modifiable residues, act as molecular switches that allow cells to respond to diverse conditions. PTMs play a vital role in the control of protein activity, stability, and subcellular localization, thereby contributing to intracellular regulation [1]. Microfluidic devices have contributed to several remarkable innovations in both chemistry and biology. The miniaturization of these devices results in reduced reagent consumption, rapid analysis times, and ease of automation. We previously reported a method for PTMs analysis such as glycosylation and phosphorylation that did not require preconcentration by fabricating functional acrylamide gels within the channels of a microfluidic chip [2,3]. However, these methods cannot achieve online fluorescent labeling and require glycans that have been fluorescently labeled in advance. In addition, conventional fluorescent labeling of glycans requires the reaction of glycans with a high concentration of fluorescent reagents for several hours while heating, followed by purification of the excess fluorescent reagent.

Here, we present an online electrochemical synthesis of fluorescently labeled glycans utilizing a microfluidic chip with electrodes (Fig. 1). The microfluidic chip had a microchannel sandwiched between the top and bottom electrodes. A sample solution containing a mixture of fluorescent reagents and glycans of the same concentration was introduced into this microchannel under pressure, and when a voltage was applied to the electrodes, a reductive amination reaction occurred at the cathode, resulting in the production of a fluorescently labeled glycan sample. We report the results of optimizing various conditions using 7-amino-4-methylcoumalin as a fluorescent reagent and maltotriose as a model sample.

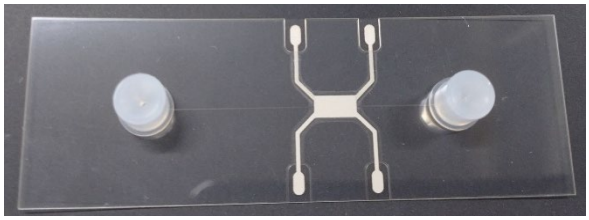

Fig. 1 A photograph of the microfluidic chip with electrodes used in this study.

Keywords: electrochemical synthesis, microfluidic chip, glycans, 7-amino-4-methylcoumalin

References

[1] S. Yamamoto, M. Kinoshita, S. Suzuki, ***J. Pharm. Biomed. Anal.,*** 130, 273-300, 2016. [2] S. Yamamoto, S. Suzuki, S. Suzuki, ***Analyst****, 137*, 2211-2217, 2012. [3] S. Yamamoto, M. Himeno, M. Kobayashi, M. Akamatsu, R. Satoh, M. Kinoshita, R. Sugiura, S. Suzuki, ***Analyst***, 142, 3416-3423, 2017.

Bioactive Glycan Motif Library Built on Structure-Based Separation for Rapid Profiling of Therapeutic Glycoproteins

Myung Jin Oh [a,b] and Hyun Joo An* [a, b]
[a] *Graduate School of Analytical Science and Technology, Chungnam National University, 99 Daehak-ro, Daejeon,34134, Korea*
[b] *Asia-Pacific Glycomic Reference Site, 99 Daehak-ro, Daejeon,34134, Korea*

*Email: hjan@cnu.ac.kr

Abstract body (up to 350 words)

The importance of glycosylation towards optimal drug bioactivity in biotherapeutics design is increasingly underscored. In biotherapeutic products, structure, specific modifications, and abundance of glycans can significantly affect the quality and safety of drugs. Therefore, detailed glycomic analysis is a key step in assessing quality control and tuning the glycosylation pathway of therapeutic glycoproteins. However, the inherent structural diversity and complexity of glycosylation significantly makes glyco-analysis extremely challenging. Here, we presented a strategy to facilitate the rapid identification and structural annotation of glycans in biotherapeutics using a catalog of glycans bearing bioactive motifs. Catalogue entries include not only typical glycans found in biotherapeutics, but also atypical glycans such as sialic acid O-acetylation, mannose phosphorylation, polylactosaminylation, NeuGc sialylation, HexNAc sulfation, and galactose-alpha-1,3-galactose. To build this resource, LC-MS/MS-based isomer specific separation and structural elucidation were performed on glycan representative of diverse glycoprotein classes such as monoclonal antibodies, cytokines, and enzymes. Diagnostic fragments were identified to enable rapid rapid and user-independent annotation of MS/MS spectra. Our strategy was successfully applied to structurally characterize glycans from biotherapeutics produced by various cell-based expression systems including CHO, HEK, and SP2/0. This approach enables rapid, yet highly accurate identification of both desired and undesired glycans containing bioactive motifs in various therapeutic glycoproteins, offering a valuable tool for biotherapeutic protein development, quality assurance, and glycoengineering.

Keywords: Therapeutic glycoprotein, glycan, structure, LC/MS/MS (up to 5)

References

[1] MJ Oh, S Hua, BJ Kim, HN Jeong, SH Jeong, R Grimm, JS Yoo, HJ An, ***Bioanalysis***, 5, 545-559, 2013, [2] MJ Oh, Y Seo, N Seo, HJ An, ***Mass Spectrom. Rev.,*** https://doi.org/10.1002/mas.21925, 2025. [3] AS Rathore, A Guttman, A Shrivastava, S Joshi, ***TrAC,*** 169, 117397, 2023.

Metabolomic analysis of tear fluid from patients with diabetic retinopathy using chemical isotope labeling liquid chromatography-mass spectrometry (CIL LC-MS)

Lei Zhou*[a, b], Xinyue Wang[c], Xuelei Liu[b], Thomas Chuen Lam[b, c], Allen MY Cheong[b, c], W. Scott Hopkins[d], Gavin Tan[e]

[a] *School of Optometry; Department of Applied Biology and Chemical Technology; Research Centre for SHARP Vision (RCSV), The Hong Kong Polytechnic University, Hong Kong.*
[b] *Centre for Eye and Vision Research (CEVR), The Hong Kong Polytechnic University, 17W, Hong Kong*
[c] *School of Optometry, The Hong Kong Polytechnic University, Hong Kong.*
[d] *Department of Chemistry, University of Waterloo, Canada.*
[e] *Singapore Eye Research Institute, Singapore.*

*Email: lei.henry.zhou@polyu.edu.hk

Abstract

Diabetic retinopathy (DR) is a progressive and globally prevalent complication of diabetes leading to vision impairment. Early detection of DR is crucial for successful disease management, aiming to prevent vision loss and related complications in individuals with diabetes mellitus (DM). However, the early diagnosis of DR and monitoring its progression pose significant challenges. This highlights the urgent necessity for discovering reliable biomarkers for intervention strategies and monitoring the prognosis of the disease. Herein, we developed a robust method of combining metabolomics and machine learning (ML) to discover dependable biomarkers for efficient risk stratification in DR. We utilized tear film as a non-invasive sample and employed the chemical isotope labelling (CIL) liquid chromatography-mass spectrometry (CIL LC-MS) technique [1] with a 13C-labelled reference sample as an internal standard for improving separation, ionization efficiency, and quantification accuracy. A total of 822 peak pairs were detected across four channels of CIL LC-MS after blank treatment and data cleansing. This included 212 peak pairs from channel A (amines/phenols), 171 from channel C (carboxylic acids), 308 from channel H (hydroxyl groups), and 131 from channel K (carbonyl metabolites). The detection of these 822 peak pairs suggests a diverse range of metabolites is present in the tear samples. Predictive models based on three ML algorithms, using tear metabolomics data, were extensively tested and compared. These models demonstrated a high degree of accuracy, with the best model - eXtreme Gradient Boosting - achieving a score 89% accuracy in distinguishing patients with DR from healthy control. Additionally, SHAP (SHapley Additive exPlanations) analysis was used to interpret the ML model and identify novel metabolites that have the potential to serve as biomarkers for DR. Our work presents the potential of using the tear metabolomics data to construct an AI-enabled disease diagnostic system that can be applied in real-world clinical settings.

Keywords: Metabolomics, Chemical isotope labeling liquid chromatography-mass spectrometry, Diabetic retinopathy, Machine Learning

References

[1] S. Zhao, H. Li, W. Han, W. Chan, L. Li, ***Anal. Chem.,*** 2019, 91 (18), 12108-12115.

Specific Separation of Halogenated Aromatic Compounds via Molecularly Imprinted Polymers Based on Halogen Bonding

Ryo Yamaguchi[a], Takuya Kubo[a,b]

[a] *Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan*

[b] *Graduate School of Life and Environmental Science, Kyoto Prefectural University, Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan.*

*Email: yamaguchi.ryo.36n@st.kyoto-u.ac.jp

Endocrine-disrupting chemicals have a strong influence on abnormal thyroid function, and there is an urgent need to identify and legally regulate these chemicals. However, no efficient *in vitro* screening method has been established for TR active compounds, and we strongly require to develop a simple screening method based on an engineering approach.

In our previous study, we succeeded in separating TR active substances from the mixture with TR inactive substances using a column packed with a molecularly imprinted polymer (MIP) via hydrogen bonding. [1] On the other hand, we selectively separated only the TR compounds having thyronine structure, such as triiodothyronine (T3) and thyroxine (T4), and the other active compounds, which have a number of halogen atoms, were not retained on the MIP. Therefore, we newly prepare the MIPs using halogen bonding as main interaction and we used them as packed columns for liquid chromatography (LC) to evaluate the retention selectivity.

In this study, we prepared MIPs and non-imprinted polymers (NIP) which were prepared without template molecules were prepared. 4-vinylpyridine was used as the functional monomer, divinylbenzene as the cross-linker, and a couple of halogenated biphenyls as the template molecules. Cyclohexane was used as the mobile phase, and the alterations in the retention behavior of halogenated aromatic compounds were evaluated by LC. The results demonstrated that the retention behavior differed depending on the numbers and the orientation of the template molecules. It was also found that precise separation using halogen bonding was possible for TR active compounds.

Literature:

[1] Enhanced molecular recognition with longer chain crosslinkers in molecularly imprinted polymers for an efficient separation of TR active substances, T. Kubo, M. Yagishita, T. Tanigawa, S. K-Yamada, D. Nakajima, *RSC Adv.*, 14, 12021-12029, 2024

Quality Control of Synthetic Cyclic Peptides using Two Different Chromatographic Modes

Kiyoshi Kakiya*[a], Ryosuke Kunitani[a], Yoshitaka Nemoto[a]
[a] *PeptiStar Inc., 2-5-1, Mishima, Settsu, Osaka, 566-0022, Japan.*

*Email: k-kakiya@peptistar.co.jp

Abstract body

Peptide pharmaceuticals are one of the new modalities currently attracting attention. PeptiStar, a very young company, was established in September 2017 and began operations in May 2019 as a CDMO (Contract Development Manufacturing Organization) that combines all-Japan technology to efficiently and high-quality manufacture peptide APIs with the latest equipment. In peptide API manufacturing, impurity analysis is important because various impurities are produced as byproducts. Additionally, impurity control is essential in drug development as it affects safety. Particularly for peptides containing non-natural amino acids, the structure becomes complex, and stricter control similar to small molecule drugs is required. Therefore, a lot of time is needed developing analytical methods for impurity analysis. The main separation method used is chromatography, with the reversed-phase high-performance liquid chromatography (RP-HPLC) being the first choice due to its high peak capacity. However, since impurities elute near the main peak, it may not be possible to control its purity with RP-HPLC alone. Therefore, HILIC was selected as another mode, and orthogonality was confirmed using four different cyclic peptides. As HILIC columns, DCpak® P4VP, DCpak® PTZ, and DCpak® PMPC from Daicel Corporation, a joint research partner, were used. This poster introduces the latest regulations important for developing peptide drugs and examples of cyclic peptide analysis using two separation modes.

Keywords: Cyclic Peptide, Regulation, Quality Control, RP-HPLC, HILIC

Separation of IgG fragments utilizing peptidomimetic polymer-modified resins

Koichi Deura*[a], Daniel Citterio[a], Yuki Hiruta[a]
[a] *Department of Applied Chemistry, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan.*

*Email: 0241koichi@keio.jp

Immunoglobulin G (IgG) consists of two antigen-binding regions (Fab) and a constant region (Fc). Their fragments without Fc have attracted attention as therapeutic agents smaller in size than full IgG and retain the antigen binding ability. They have several advantages over full IgG, such as rapid penetration and clearance. However, affinity ligands that can bind to both *κ* and *λ* light chains of Fab have not been developed, leaving challenges for low-cost production. IgG fragments can be genetically engineered to display peptide tags to enable purification by immobilized metal affinity chromatography, but this has the drawback of requiring the inclusion of peptide tag cleavage and removal steps in production [1]. We have previously developed a column for the chromatographic purification of IgG by modifying column packing materials with a polymer that mimics the amino acid composition of Z34C, the IgG-binding peptide derived from protein A, and the structure of histidine (Figure 1a) [2]. The prepared Z34C mimetic column showed pH-dependent retention of IgG like protein A and achieved IgG purification from cell culture supernatants with high purity and recovery by switching the mobile phase pH from neutral to acidic. In this study, we aimed to apply the Z34C mimetic column to the separation of IgG fragments. First, Fab and Fc fragments of IgG were generated by papain digestion of three types of monoclonal antibodies with different light chain classes. And then, the effect of the eluent pH on their retention was investigated. Under neutral conditions, the column showed higher affinity for Fab compared to Fc and was also found to retain both *κ* and *λ* Fab. Then, the separation of IgG fragments from digested IgG samples was performed by switching the mobile phase pH from 6.5 to 5.0 (Figure 1b), and the purity of the separated fragments was evaluated by reversed phase chromatography. As a result, three types of Fab were successfully purified with high purity and recovery, demonstrating that the Z34C mimetic column can be applied as a tag-free, comprehensive method for purifying both *κ* and *λ* Fab fragments.

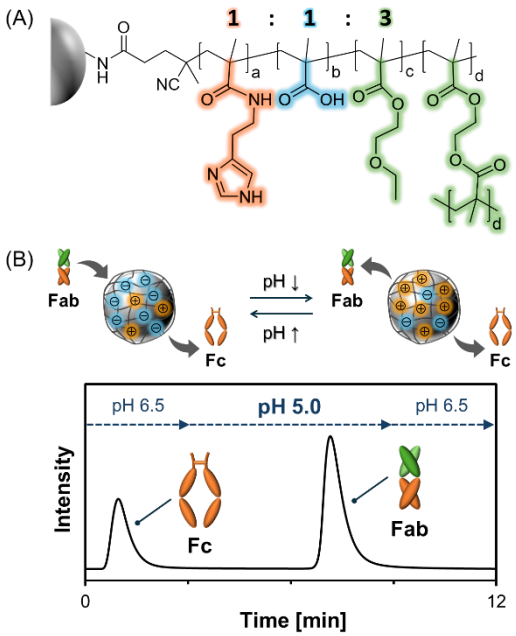

Figure 1 (a) Molecular structure of the Z34C mimetic polymer. (b) Principles of Fab purification.

Keywords: IgG fragment, purification, peptidomimetics, pH-responsive, polymer.

References

[1] G. Rodrigo, M. Gruvegård, J.M. Van Alstine, ***Antibodies***, 4 (3), 259-277, 2015. [2] K. Deura, A. Sakama, Y. Moriwaki, D. Citterio, Y. Hiruta, ***ACS Appl. Mater. Interfaces***, 16 (49), 67468–67476, 2024.

Development of alkali resistant reversed phase column packing material based on eggshell utilizing Layer-by-Layer self-assembly

Mai Kawamura*[a], Koichi Deura[a], Naoya Takahashi[a], Daniel Citterio[a], Yuki Hiruta[a]
[a] *Department of Applied Chemistry, Keio University, 3-14-1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223-8522, Japan.*

*Email: mai_k0214@keio.jp

Porous silica gel is commonly used as a column packing material for reversed-phase liquid chromatography (RPLC) due to its high separation capacity. However, silica gel has the disadvantage that surface silanol residues dissociate in alkaline mobile phases, leading to a decrease in separation efficiency. Therefore, there is a need to develop alkali-resistant column packing materials. We have previously developed alkali-resistant packing material, Eggshell-PMAcO based on eggshell modified with amphiphilic polymer polymaleic acid octadecene (PMAcO)[1]. A column packed with this material enabled the separation of basic compounds using alkaline mobile phase. However, compared to commercial silica gel columns, its retention capacity was relatively low due to the limited amounts of organic components modified on the surface of eggshell. In this study, Layer-by-layer (LbL) method was employed to develop eggshell based column with enhanced retention capacity. Eggshell powder was alternately immersed in anionic PMAcO and cationic polydiallyldimethylammoniumchloride (PDDA) electrolytes to form a multilayer organic membrane. Thermogravimetric analysis confirmed that the amount of organic modification was significantly increased by the LbL method. Scanning electron microscope images showed that the pores derived from the eggshell were retained. The retention of naphthalene was confirmed by HPLC measurements with an analytical column (φ4.6 mm x 150 mm), which showed that as the number of assembled layers increased from 1 to 7, the surface modification amount increased, resulting in stronger retention in reversed phase mode (Fig. 1). Furthermore, the symmetry factor was improved. Further evaluation of column performance associated with increased surface modification via the LbL method, as well as the separation behavior for various compounds, will be discussed in detail in the presentation.

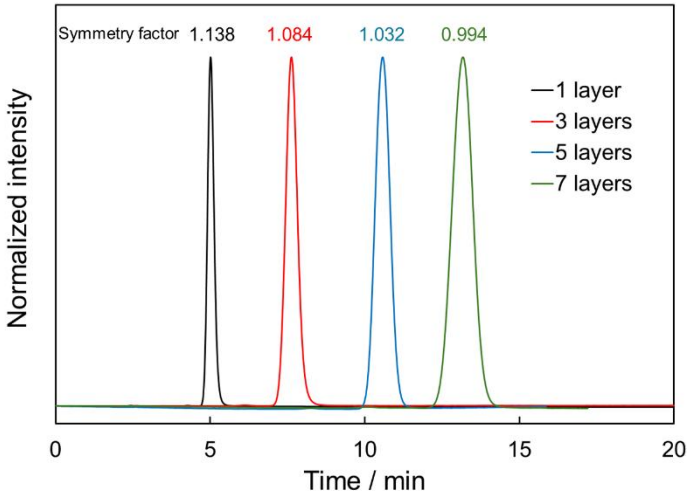

Fig. 1 Chromatograms of naphthalene. Analytical conditions, column temperature: 25 °C; mobile phase: water/methanol=50/50=(v/v); flow rate: 0.5 mL/min; detection: 254 nm; analytes: 0.5 mg/mL naphthalene, injection volume: 2 μL

Keywords: HPLC, Layer-by-layer self-assembly, eggshell, alkali resistant, polymer

References

[1] T. Yoshii, K. Nakano, T. Okuda, D. Citterio, Y. Hiruta, ***Anal. Methods***, 15, 1790-1796. 2023.

Automatic optimization of gradient profile using AI algorithms on functional food analysis with HPLC

Yoshiyuki Watabe*[a, b], Tetsuya Tanigawa[b], Shinichi Fujisaki[c] HIdetoshi Terada[c]
[a] *Shimadzu General Service, Inc., 1 Nishinokyo Kuwabara-cho、Nakagyo-ku, Kyoto, 604-8511 Japan*
[b] *Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29 Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606-8304, Japan.*
[c] *Shimadzu Corporation, 1 Nishinokyo Kuwabara-cho、Nakagyo-ku, Kyoto, 604-8511 Japan*

*Email: watabe.yoshiyuki@sgs.shimadzu.co.jp

Introduction

In general, HPLC method development, enormously time-consuming repeated analysis batch schedule creation is required as well as the chromatographic knowledge and experience in the process of optimizing analytical conditions. Consequently, labor savings through automated method development is paid attention. In this study, fifteen standards mixed solution of catechins, theaflavins, and gallic acid, which were typical functional food components, was employed as a simulated sample, and the AI algorithm equipped in l method development support software LabSolutions MD was utilized. Furthermore, the optimized method was applied to tea leaf analysis.

Experimental

Ten catechins, four theaflavins, and gallic acid, totally fifteen compounds were subjected to HPLC analysis. First, the gradient profile was optimized by LabSolutions MD using mixed standard solution. The optimized gradient profile was then applied to the analyses of six tea leaves. LabSolutions MD is equipped with a proprietary AI algorithm that automatically searches for the satisfactory conditions by alternately repeating gradient profile improvement then executes analysis under improved conditions. Gradient profiles were automatically searched with a minimum separation criterion of 1.5 for the standard mixture of fifteen compounds. The optimized method was applied to the quantitative analysis of extracts of tea leaves of different types and varieties.

Results and discussion

The gradient profile that met the criteria (minimum resolution of 1.5) were automatically searched, resulting in significant labor savings. Four green tea samples contained more catechins than those in black tea samples, and the highest concentration of functional component for all four green tea was epigallocatechin gallate, which is expected to inhibit elevated blood glucose in both varieties. In one of green tea samples, two methylated catechins were detected, which have been paid attention for their anti-allergic effects and ability to reduce hay fever. On the other hand, four types of theaflavins were detected in black tea samples. Although one tea sample and one black tea sample were "Benifuki" varieties, the comparison between the two suggests that catechins were converted to theaflavins during fermentation. Above mentioned detailed discussion can be done based to the HPLC results obtained under automatically created and optimized analytical conditions.

Keywords: HPLC, AI, artificial intelligence, automatic optimization, functional food

References

[1] E. Kanao, T. Morinaga, T. Kubo, T. Naito, T. Matsumoto, T. Sano, M. Yan, K. Otsuka, ***Chem. Sci.***, 11, 409-418, 2020. [2] E. Kanao, H. Osaki, T. Tanigawa, H. Takaya, T. Sano, J. Adachi, K. Otsuka, Y. Ishihama, T. Kubo, ***Anal. Chem.***, 95, 9304-9313, 2023. [3] T. Kubo, M. Yagishita, T. Tanigawa, S. K-Yamada, D. Nakajima, ***RSC Adv.*** 14, 12021-12029, 2024.

Identification of Isomerization in Tryptic Digested Proteins by LC-IM-TOFMS

Shinya Kitagawa*, Rio Suzumura, Reina Ogawa, Yoshinori Iiguni

Department of Engineering, Graduate School of Engineering, Nagoya Institute of Technology, Gokiso, Showa, Nagoya 466-8555, Japan.

*Email: kitagawa.shinya@nitech.ac.jp

Proteins are indispensable materials in biological systems for the structuring of cells and organisms, the catalyzing of metabolic reactions, and so on. In general, proteins in vivo are synthesized from L-amino acids and composed of L-amino acid residues. However, the existence of isomerized proteins containing isomerized D-amino acid and L/D-iso-form residues in vivo has been reported. Such proteins cause age-related diseases such as cataract and Alzheimer's disease. Therefore, the analysis of isomerized proteins is in biological systems is an essential and important task. Here, we focused on liquid chromatography-ion mobility-mass spectrometry (LC-IM-MS), in which the digested-peptides were separated in both LC and IM. In this study, we develop a non-target analysis method for isomeric peptides in LC-IM-TOFMS and the method was applied to tryptic digested proteins.

Synthetic peptides corresponding to the T12 peptide of egg white lysozyme (LZM) with L-, D-, L-iso-, and D-iso-Asp residues were used as the standard peptide sample. Egg white LZM was used as a protein sample. The LZM was dissolved in 50 mM acetate buffer (pH 4.0) and incubated at 60 °C for at least three days to accelerate isomerization. The LC-IM-TOFMS instruments of Synapt G2 HDMS and Cyclic IMS equipt with LC system (Waters) was also used.

First, LC-IM-TOFMS measurements were performed on the synthetic T12 peptide. High dimensional vectors were defined using the MS/MS spectrum intensities of each fragment ion via collision-induced dissociation and the area of the mobilogram with slicing. By the calculation of $\cos\theta$ values for the vectors, the identification and localization of isomerized Asp residues was achieved.

The tryptic digested LZM was then analyzed using the aforementioned method. The T12 peptides after the isomerization acceleration resulted in the separation in the time and arrival time planes. The calculation of $\cos\theta$ values revealed that the Asp4 residue in the T12 peptide had isomerized to L-iso-form. Additionally, isomerization of the Asp residues in T5 and T8 peptides was observed. The Asn residue in the -Asn-Gly- sequence generally results in isomerization following deamidation (*Anal. Chem.* 2006, 78 (18), 6645-6650). Isomerization of the Ans residue after the deamination was also investigated.

Keywords: isomerized protein, non-target, HPLC, ion mobility, mass spectrometry

This work was supported by JSPS KAKENHI Grant Numbers 25K01798.

Retention Behavior for Aromatic Compounds with Polyimide Fine Filaments as a Stationary Phase in Reversed-Phase Liquid Chromatography

Koki Nakagami*[a], Ayato Yamaguchi[a], Sota Nakamura[a], Ikuo Ueta[b], Yoshihiro Saito[a]
[a] *Department of Applied Chemistry and Life Science, Toyohashi University of Technology, 1-1 Hibarigaoka, Tempaku-cho, Toyohashi 441-8580, Japan.*
[b] *Department of Applied Chemistry, University of Yamanashi, 4-3-11 Takeda, Kofu 400-8511, Japan.*

*Email: nakagami@chem.tut.ac.jp

HPLC is a popular and also an effective analytical method for the separation of various types of samples complex mixtures. In order to achieve a successful separation, an appropriate choice of the stationary phase from many types of phases developed for modern HPLC separation is particularly important. Recently, polymer-based stationary phases have been introduced as the separation media in chromatographic analysis because of several advantageous features of polymer materials, such as good chemical-resistance, heat-resistance and excellent mechanical strength. Our research group reported the applications of fibrous polymer materials as the separation media in chromatographic methods or the extraction media for sample preparation techniques. As an example, fibrous polyimide was applied to a separation medium in gas chromatography, and a good separation performance was observed. On the basis of the result, the fibrous polyimide enabled the applications as a stationary phase in HPLC method, where the retention behavior of typical aromatic compounds on the polyimide filaments phase has been studied.

Fibrous polyimide-packed column was prepared by longitudinally packing bundles of polyimide filaments (approximately 52800 filaments) into a stainless-steel tube (4.6 mm i.d., 250 mm length), and the column was installed to a typical HPLC system equipped with a UV/Vis detector. As the mobile phase, a mixture of methanol/water was used, and the flow-rate was typically set at 0.50 mL/min.

The retention tendency was studied for aromatic compounds having various alkyl groups on the polyimide-packed column. On typical nonpolar stationary phase, such as octadecylsilica (ODS) phase, a good correlation between the logarithm of retention factors (ln k) of the analytes and the corresponding hydrophobicity (log P) was obtained. The results showed that the ODS phase has a retention mechanism based on hydrophobic interaction. On the polyimide-fiber phase, however, a different trend was observed for the retention of the analytes with short alkyl functionalities in the molecular structure, when compared to the data obtained on conventional ODS phases. The results clearly demonstrated a possibility of the polyimide filaments as a novel stationary phase having a unique selectivity for various aromatic compounds in HPLC.

Keywords: HPLC, stationary phase, polyimide filament, retention behavior

Development and Separation characteristic evaluation of β-cyclodextrin modified monolithic silica capillary column

Takashi Yukiyama*, Hiromi Takano, Hideyuki Otsuki
SHINWA CHEMICAL INDUSTRIES LTD., 50-2 Kagekatsu-cho, Fushimi-ku, Kyoto 612-8307 JAPAN.

*Email: t-yukiyama@shinwa-cpc.co.jp

1. Introduction

The ULTRON AF-HILIC-CD for HILIC column with β-cyclodextrin modified on the silica gel surface has a molecular recognition for the structural isomers. However, the separation power was sometime insufficient due to its weak hydrophilic interactions. Therefore, we had inspired the idea of a novel HILIC column that modifying β-cyclodextrin onto the ULTRON HF-MBS which is a high-resolution capillary column composed monolithic silica with a hierarchical porous structure. In this presentation, we will introduce the characteristic evaluation for the novel HILIC column (Prototype HF-CD) under development and examples of glycan separation.

2. Experimental method

For preparate the Prototype HF-CD, we had prepared a β-Cyclodextrin derivatives has silane reactive groups and diluted that with pyridine. Subsequently, the derivatives solution was passed through the HF-MBS (0.1 mmI.D. x 750 mmL) to react between with the silanol groups on the silica surface. Finally, organic solvents and water had been passed thoroughly through the column for washing, the Prototype HF-CD was obtained.

3. Results and observations

Fig. 1 shows the α(2dG/3dG)-α(α/β) plot to evaluate based on the characterization method for HILIC stationary phases [1] for 34 columns including in the Prototype HF-CD, the HF-MBS, SIL columns and the AF-HILIC-CD.

As results of characteristic evaluation HILIC stationary phase of the ULTRON HF-MBS and the Prototype HF-CD, they showed a tendency to have high molecular structure recognition ability similar to that of the particle type, and the β-cyclodextrin-modified type was more obvious.

That high recognition ability of the Prototype HF-CD was demonstrated in the separation examples 2-AB-labeled glucose homopolymer ladder, Cyclic oligosaccharides and IgG N-Linked Glycans, there were good separations. These chromatograms will be show in the presentation.

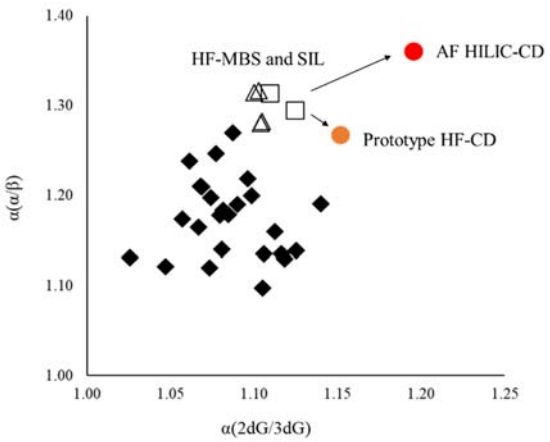

Fig.1 The α(2dG/3dG)-α(α/β) plot for 34 HILIC columns.

Keywords: HILIC, β-cyclodextrin, monolithic silica capillary column, molecular structure recognition, Glycan separation

References

[1] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, ***J. Chromatogr. A*** 35, 5903-5919, 2011.

Design of Experiments-based optimization of acylcarnitines electrospray ionization process

Julia Jacyna-Gębala*[a], Małgorzata Waszczuk-Jankowska[a], Julia Białkowska[a], Wiktoria Struck-Lewicka[a], Renata Wawrzyniak[a], Michał Jan Markuszewski[a], Danuta Siluk[a]

[a] *Medical University of Gdańsk – Department of Biopharmacy and Pharmacodynamics, Al. Gen. J. Hallera 107, 90-416 Gdańsk, Poland.*

*Email: julia.jacyna-gebala@gumed.edu.pl

Carnitines play a key role in cellular energy metabolism. Acylcarnitines facilitate the transport of fatty acids to mitochondria for their β-oxidation - one of the key cellular processes related to energy metabolism. Carnitine compounds have also been identified as potential biomarkers of cardiovascular diseases. That is why, the aim of this study was to develop a quantitative method for determination of selected medium- and long-chain acylcarnitines in human plasma with the use of HPLC-ESI-QqQ-MS/MS system.

Isolating acylcarnitines from plasma requires the use of an appropriate sample preparation method in order to concentrate the sample and purify the matrix from interfering substances. At the same time, it is necessary to find optimal ionization parameters' settings, so that the resulting method will enable the determination of analytes at the highest possible sensitivity. To achieve this goal in the most efficient way, Design of Experiments methodology was used, which allows for finding the optimal settings of the method parameters in order to obtain the highest possible signal intensity while maintaining the highest repeatability of analyses. Parameters' ranges (capillary voltage: 3000–4500V, nebulizer pressure: 30–50psi, drying gas flow rate: 6–12L/min and drying gas temperature: 280–350°C) were selected based on the literature review and capabilities of apparatus used. Box–Behnken design was chosen because it allows reliable determination of the response surface for studied design space (along with optimal settings) with relatively little experimental work and reagent consumption.

Based on multivariate regression analysis separate models for each acylcarnitine were built. Such an approach enabled prediction of influence of tested parameters on individual response. The most significant variables in the built models were the flow rate of drying gas and its temperature. Although nebulizer pressure and capillary voltage were not found statistically significant, those factors were found to interact with other parameters and affect ionization process. Models built for individual metabolites allowed to conclude all studied compounds would benefit from similar conditions.

With the use of maximize desirability function and assuming that all the discussed compounds are equally important a compromise setting was achieved. The exact suggested parameters settings for positive ionization mode would be gas temperature 350 °C and flow rate of 12L/min. However, overall desirability values hardly changed in higher ranges for studied factors. For example, decrease in gas temperature from 350 °C to 340 °C or 330 °C only slightly reduced the fit (from 0.998 to 0.996 or 0.969).

Keywords: acylcarnitines, ionization, design of experiments, optimization, ESI

Acknowledgments

The project was founded by the Medical Research Agency under the National Plan for Rehabilitation and Enhancement of Immunity (Component D: Efficiency, accessibility and quality of the health care system, Investment D3.1.1: Comprehensive development of research in medical and health sciences (no 2024/ABM/03/KPO/KPOD.07.07-IW.07-0075/24-00)).

Exploration of suitable columns for EPSA measurement using supercritical fluid chromatography and expansion of target compounds

Shotaro Hirota[a], Yusuke Masuda[a], Yasuhiro Funada[a], and Ryo Kubota[a]
[a] *Shimadzu Corporation, Analytical & Measuring Instruments Division, Solutions COE, 1 Nishinokyo kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan.*

*Email: hirota.shotaro.o6i@shimadzu.co.jp

Abstract body (up to 350 words)

The polar surface area (PSA) is an indicator that represents the area of the polar portion of a molecule's surface, and it is important for evaluating cell membrane permeability in drug structure optimization. PSA, similar to the octanol/water partition coefficient (logP), indicates the polarity and lipophilicity of a molecule. It has been found that there is a good correlation between PSA and experimental data related to membrane permeability. For example, compounds with a very large PSA (over 140 Å^2) are empirically known to have low membrane permeability [1].

Since the membrane permeability of drug candidates is directly related to their ability to reach the target site, rapid evaluation at an early stage of development is essential. This evaluation can facilitate the selection of candidate compounds and streamline the development process.

In previous studies, a method for deriving the experimental polar surface area (EPSA) using supercritical fluid chromatography (SFC) has been established [2]. EPSA is calculated based on the retention time of analytes in a calibrated system using compounds with known EPSA values.

In this study, the correlation between retention time and EPSA value was evaluated using various stationary phase columns with different retention selectivity. Good correlation between retention time and EPSA was observed in several polar stationary phases. By optimizing the analytical conditions and using compounds with known polar surface area, it was shown that it is possible to estimate the polar surface area of small molecule drugs and peptide drugs.

By using this new EPSA calculation method using SFC, we can eliminate and promote candidate compounds in the drug discovery phase and streamline the drug development process.

Keywords: HPLC, SFC, EPSA

References

[1] Daniel F. VeberStephen R. JohnsonHung-Yuan ChengBrian R. SmithKeith W. WardKenneth D. Kopple, ***J. Med. Chem.,*** 45, 12, 2615–2623, 2002 [2] Yue-Ting Wang, Edward Price, Mei Feng, Jason Hulen, Stella Doktor, David M. Stresser, Estelle M.Maes, Qin C. Ji, and Gary J. Jenkins, ***J. Med. Chem.***, 2024,67,13765−13777

Estimation of Surface Area of Gold Nanoparticles Through the Adsorption Amount of Cysteine by Capillary Zone Electrophoresis

Toshio Takayanagi*[a], Minamo Seto[b], Hitoshi Mizuguchi[a], Hirotaka Okabe[c], Naoki Matsuda[c]

[a] *Graduate School of Technology, Industrial and Social Sciences, Tokushima University, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan.*
[b] *Graduate School of Science and Technology for Innovation, Tokushima University, 2-1 Minamijosanjima-cho, Tokushima 770-8506, Japan.*
[c] *Sensing System Research Center, National Institute of Advanced Industrial Science and Technology, 807-1 Shukumachi, Tosu 841-0052, Japan.*

*Email: toshio.takayanagi@tokushima-u.ac.jp

Abstract

The surface area of gold nanoparticles (AuNPs) was estimated through the adsorption of cysteine onto the AuNPs. The AuNPs used were synthesized by a solution plasma process and were polycrystalline. Capillary zone electrophoresis (CZE) was employed to monitor the cysteine adsorption. An AuNP dispersed solution with cysteine adsorption was directly introduced into the CZE system, and a broad peak was obtained in the electropherograms with the dispersed AuNPs. Shot signal for the aggregated AuNPs was not detected in the electropherograms, indicating that the dispersion of the AuNPs was maintained even after the cysteine adsorption. The effective electrophoretic mobility of the AuNPs was reduced by the adsorption of cysteine to the AuNP surface. Any peak attributed to the cysteine was not detected at low concentrations of the cysteine, and the added cysteine was mostly adsorbed onto the AuNPs. An additional peak attributed to the residual cysteine was detected at higher concentrations of cysteine from 100 μmol L^{-1}, and a linear relationship was obtained between the concentration of cysteine and the peak area of the cysteine with an x-intercept at 86 μmol L^{-1}. This concentration represents the saturation point of surface adsorption onto the AuNPs. The surface area of the AuNP dispersed solution was estimated from the adsorption amount of cysteine and the cross-section area of a cysteine molecule, resulting in an estimated surface area of 0.078 m^2 mL^{-1}. The estimated surface area was also verified through the adsorption of glutathione onto the AuNP.

Keywords: Gold nanoparticles, surface area, cysteine, adsorption amount, CZE

Towards building a foundation model for automated high-performance liquid chromatography (HPLC) analysis and design

Stephen Wu,[1,2*] Yoshihiro Hayashi[1,2,3], Ryo Yoshida[1,2,3], Hikaru Takaya[4] and Takuya Kubo[5]

[1] *The Institute of Statistical Mathematics, Research Organization of Information and Systems, Tachikawa, Tokyo 190-8562, Japan*
[2] *The Graduate Institute for Advanced Studies, SOKENDAI, Tachikawa, Tokyo 190-8562, Japan*
[3] *Advanced General Intelligence for Science Program (AGIS), RIKEN-TRIP, Wako, Saitama 351-0198, Japan*
[4] *Department of Life & Health Sciences, Teikyo University of Science, Adachi, Tokyo 120-0045, Japan*
[5] *Graduate School of Life and Environmental Science, Kyoto Prefectural University, Sakyo, Kyoto 606-8522, Japan*
**stewu@ism.ac.jp*

High-performance liquid chromatography (HPLC) is a critical analytical technique widely employed for chemical separations, yet current method development remains labor-intensive, empirical, and often highly dependent on operator expertise. A central challenge in HPLC analysis and design is accurately predicting the retention time of molecules under specific chromatographic conditions. Recent machine learning approaches have leveraged large retention-time databases from a single standardized column and mobile-phase configuration to build initial models [1], subsequently fine-tuning them for different chromatographic setups to predict their separation capabilities [2]. However, such fine-tuning approaches still demand substantial training data to achieve practical accuracy [3], and the criteria for successful adaptation remain unclear. Foundation models—large, versatile machine learning models pre-trained on extensive datasets, such as GPT for language tasks or AlphaFold for protein structure predictions—present a promising alternative due to their robust generalization and efficient adaptation to related tasks with minimal additional data. To realize a general foundation model for HPLC retention-time prediction, we investigate critical factors influencing efficient model adaptation across various chromatographic conditions. Specifically, we explore scaling laws—previously validated in polymer property prediction [4]—and assess the impact of explicitly incorporating mechanistically relevant chromatographic features, such as particle size and column dimensions. Our approach aims to establish foundational principles guiding efficient, accurate, and broadly generalizable predictions, significantly reducing the experimental workload associated with developing and optimizing HPLC methodologies.

[1] X. Domingo-Almenara et al., Nat. Comm. **10**, 5811 (2019).
[2] Q. Yang, H. Ji, H. Lu, and Z. Zhang, Anal. Chem. **93(4)**, 2200–2206 (2021).
[3] R. Ju et al., Anal. Chem. **93(47)**, 15651–15658 (2021).
[4] S. Minami et al., npj Comput. Mater. *accepted* (2025).

The evaluation of a small-capacity polypropylene vial that achieves low bleed and low adsorption

Tomoha Somano*[a], Kosuke Namiki [b], Yuki Sato [b], Yusuke Osaka [a]
[a] *Shimadzu Corporation, 1, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan.*
[b] *Shimadzu GLC Ltd., 5-20-8 Asakusabashi, Taito-ku, Tokyo 111-0053, CS Tower 5F, Japan.*

*Email: shirouchi.tomoha.u7z@shimadzu.co.jp

Many peptides exhibit hydrophobic and ionic adsorption, and the adsorption to vials can impact analytical accuracy. Vials with surface coatings designed to suppress adsorption may sometimes show bleed from the vial, which can compromise the quantitativeness of the analytes. In this context, we have prepared a small-capacity polypropylene (PP) vial, Prototype A, with a special coating on the vial surface that suppresses bleed as well as hydrophobic and ionic adsorption. In this presentation, we will report the results of evaluating the adsorption properties and bleed of various PP vials, including Prototype A, with respect to peptides.

In the evaluation of bleed, we compared Prototype A with other PP vials. The encapsulated solvents included water and a mixture of water and organic solvents. The LC system was the Nexera (Shimadzu), and the mass spectrometer used was the LCMS-2050 (Shimadzu). For the evaluation of adsorption, we compared Prototype A with other PP vials and glass vials. The analytes were peptides, and LC/MS analysis was conducted. The analytical column used was the Shim-pack Scepter series (Shimadzu). The LC system was the Nexera (Shimadzu), and the mass spectrometer used was the LCMS-8060RX (Shimadzu).

In the evaluation of bleed, it was found that Prototype A exhibited bleed levels comparable to or lower than those of other companies' PP vials that claim low adsorption. In the adsorption evaluation, when comparing the peak area values of peptides, many of the other company's vials and glass vials showed no detectable peaks, while Prototype A showed detectable peaks. It is believed that the hydrophilic regions of the peptides adsorbed onto the glass vials due to ion exchange reactions, whereas the hydrophobic regions of the peptides adsorbed onto the other company's PP vials due to hydrophobic interactions. On the other hand, Prototype A is coated with a non-ionic hydrophilic group on the vial surface, which is thought to suppress both ion exchange reactions and hydrophobic interactions, achieving low adsorption. Based on these results, it can be stated that the small-capacity PP vial Prototype A successfully combines peptide adsorption suppression and low bleed through the hydrophilization treatment of the vial surface. (350 words)

Keywords: HPLC, adsorption, peptide, vial, bleed

Application study of online trap column for fast sample cleanup

Keiko Yamabe*[a], Daiki Fujimura[a], Yusuke Osaka[a]
[a] *Shimadzu Corporation, 1, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 6048511, Japan.*

*Email: yamabe@shimadzu.co.jp

Abstract body

In LCMS analysis of biological samples, sample preparation such as protein removal is required to exclude the effects of matrix components. Protein removal methods include denaturation with acids or organic solvents, removal by filters, as well as solid-phase extraction methods and column-switching LC systems.

A reversed-phase chromatography column coated with methylcellulose on the silica gel outer surface is a pretreatment trap column used in column-switching LC systems, which can remove plasma proteins and concentrate small molecule compounds. It demonstrates high reproducibility and durability in small molecule analysis of drugs in plasma. [1-2]

If this protein elimination ability can be used in the pretreatment of other protein-containing samples in the same way, a wide variety of sample preparation can be further speeded up. Therefore, we made prototypes of high-pressure columns (2 mm i.d.×30 mmL.) filled with similar coated silica gel to evaluate protein removal performance.

When the main components of milk protein were injected separately and the eluate from the column was monitored by UV detection, rapid protein elution from the column was confirmed. By increasing the flow rate of the mobile phase, the pretreatment time could be greatly reduced, and it was found that high-pressure columns are effective for efficient protein removal. In addition, a longer length of the pretreatment column increases the pressure but is advantageous for the capture of small molecules. The test sample was injected into the pretreatment column and the elution peak in the gradient after the pretreatment process was monitored by UV to confirm the retention behaviour. A recovery test with the addition of caffeine to a coffee beverage containing milk found that caffeine was captured in the pretreatment column without co-eluting with protein.

In the measurement of coffee beverages by automatic pretreatment using a column-switching system, each quantitative value was equivalent to that of corresponding offline protein removal sample, but the samples were not diluted, allowing for high-sensitivity measurements or low-volume sample injections. It also provided the effect of cleaning up coexisting ingredients.

Keywords: HPLC

References

[1] E. Yamamoto, K. Murata, Y. Ishihama, N. Asakawa, ***Anal. Sci.*** 17, 1155-1159, 2001 [2] S. Kawano, H. Murakita, E. Yamamoto, N. Asakawa, ***J. Chromatogr. B*** 792, 49-54, 2003.

Automated scale-up workflow from analytical to Preparative SFC

Hidetoshi Terada*[a], Yusuke Masuda[a], Ryo Kubota[a]
[a] *Shimadzu Corporation, 1, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto, 6048511, Japan*.

*Email: h-terada@shimadzu.co.jp

In drug discovery and development laboratories, the synthesis, screening, and purification of target compounds are essential processes. During synthesis, impurities often accompany the main compound, making purification critical for precise analyses. Preparative reversed phase liquid chromatography (RPLC) and supercritical fluid chromatography (SFC) are commonly used methods for purification. However, these methods require chromatographic expertise and significant time investment, which hinders their widespread adoption. Thus, enhancing the efficiency of these techniques is crucial for researchers, particularly for medicinal chemists.

SFC offers advantages such as improved separation through appropriate column selection, yielding high purity products. It also reduces solvent consumption and increases productivity for preparative purification. However, developing effective preparative SFC methods remains challenging, as non-experts often perform these tasks, leading to less frequent use compared to RPLC.

This study investigates an automated scale-up workflow utilizing SFC-MS. The workflow features an automated function that recommends optimal purification columns through systematic screening and generates ideal preparative conditions based on analytical SFC-MS results. We employed three types of columns with varying retention selectivity for separating low molecular weight pharmaceuticals, enabling effective purification of diverse compounds.
An algorithm for calculating preparative gradient conditions from retention times obtained in screening analysis was developed using seven test compounds for each column.

We will present the details of the algorithm for calculating preparative gradient conditions from retention time obtained in screening analysis and, examples of its application to the SFC preparation of small molecule pharmaceuticals containing impurities.

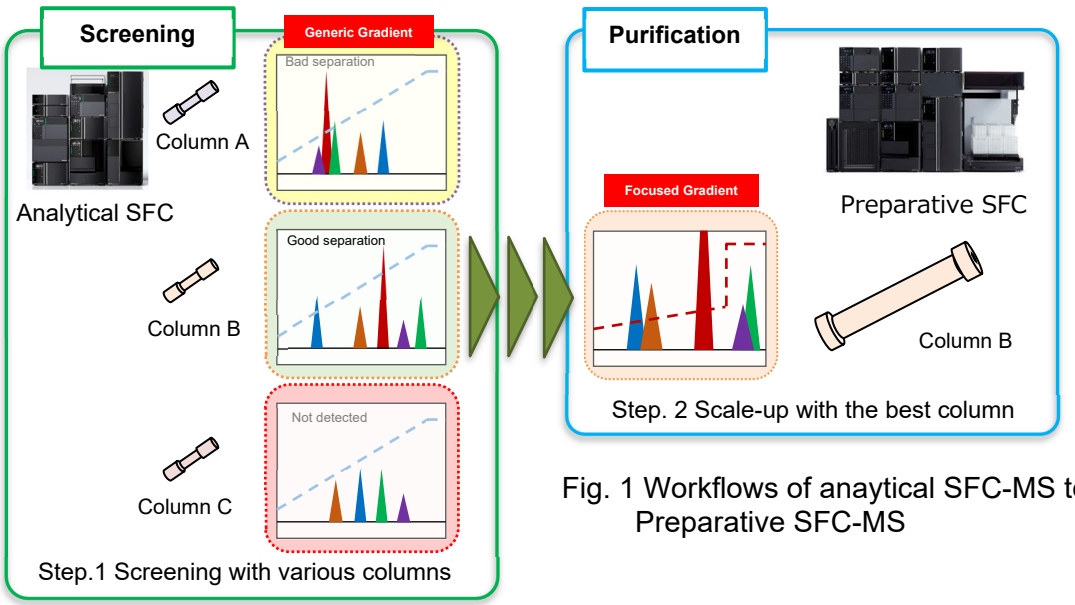

Fig. 1 Workflows of anaytical SFC-MS to Preparative SFC-MS

Keywords: SFC, purification, scale-up

PRIMARY SUTRUCTURAL ANALYSIS OF PEPTIDES WITH MODIFIED AMINO ACIDS AND CYCLIC PEPTIDES WITH DISULFIDE BONDS

Miho Akagi*[a], Tomoko Kuriki[a], Kumiko Yamaguchi[a] and Hidetoshi Terada[a]
[a] *Shimadzu Corporation, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto, 604-8511, Japan*

*Email: akagi.miho.7ey@shimadzu.co.jp

Traditionally, the majority of pharmaceuticals have been small molecules produced by chemical synthesis. In recent years, the number of macromolecular drugs (e.g., antibody drugs and biopharmaceuticals) that use proteins as active ingredients have been increasing along with the development of biotechnology. In addition, as a new type of pharmaceutical product, middle molecular drugs, which combine the advantages of both small molecular drugs and antibody drugs, have been attracting attention. Peptide therapeutics are one of these new classes of middle molecular drugs. Peptide therapeutics can be manufactured by chemical synthesis like small molecular drugs, which is relatively inexpensive and can be produced in large quantities. They can easily penetrate cell membranes and act on the intercellular targets because they are also smaller in molecular size than macromolecular drugs. A peptide drug is designed to be pharmaceutically effective by well-considered its amino acid composition, sequence, and steric structure.

For structural analysis of peptides, it is common to use mass spectrometers and search engines using genome databases as in protein analysis. But some types of peptides are difficult to analyze using this method. The experimental result may differ from the mass number from genome database when the amino acid sequence of a peptide with modified amino acids is analyzed by a mass spectrometer, resulting in unreliable experimental data. In addition, it is very complicated and difficult to identify amino acid sequences using mass spectrometers without using databases.

We report peptide analysis using a protein sequencer with Edman degradation and a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer, MALDI-TOF MS, to identify the amino acid sequence of a peptide containing a modified amino acid, or the steric structure of a peptide with two disulfide bonds.

Keywords: protein sequencer, HPLC, peptides, PTH-amino acids

References

Multidimensional Chiral HPLC Analysis of Lysine and Its Metabolites in Human Urine

Reiko Koga*[a], Akari Matsuo[a], Masashi Mita[b], Hideyuki Yoshida[a], Hitoshi Nohta[a], Kenji Hamase[c]

[a] *Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan.*
[b] *KAGAMI Inc., 7-7-15 Saito-asagi, Ibaraki, Osaka, 567-0085, Japan.*
[c] *Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.*

*Email: rkoga@fukuoka-u.ac.jp

Lysine (Lys) is an essential amino acid metabolized in mammals *via* the pipecolate and saccharopine pathways to pipecolic acid (PA) and 2-aminoadipic acid (2-AAA), respectively. These compounds are known to exist in both D- and L-forms owing to their chiral structures. Recent findings have revealed the presence of D-amino acids in mammals, highlighting their potential physiological roles and utility as biomarkers. However, due to the extremely low abundance of D-amino acids and interference from various coexisting biological substances, studies distinguishing their enantiomers *in vivo* are limited. Therefore, highly selective analytical methods are crucial for the precise evaluation of trace amounts of amino acid enantiomers in biological samples.

In this study, we developed a multidimensional HPLC system combining reversed-phase, anion-exchange, and enantioselective separations for the selective analysis of Lys, PA, and 2-AAA enantiomers in human urine. Urine samples were diluted with water and derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) at 60 °C for 2 min. The reaction was terminated by the addition of an aqueous trifluoroacetic acid solution, and an aliquot was subjected to the multidimensional HPLC. Fluorescence detection of NBD-labeled amino acids was performed at 530 nm with excitation at 470 nm.

NBD-labeled amino acids were first separated by a reversed-phase column (Singularity RP18, 1.0 × 250 mm), then purified using an anion-exchange column (Singularity AX, 1.5 × 250 mm), and finally resolved on an enantioselective column (Singularity CSP-001S, 1.5 × 250 mm) for enantiomer separation. This system enables baseline separation for all target amino acids, with resolution values exceeding 2.5. In human urine samples, all target enantiomers were detected, with %D values of 6.8 % for Lys, 72.1 % for PA, and 1.3 % for 2-AAA. Additionally, slight fluctuations in the %D value of 2-AAA were observed over the course of the day, whereas the %D values of Lys and PA remained relatively stable throughout the day. These results demonstrate the applicability of multidimensional HPLC with pre-column fluorescence derivatization for trace-level chiral analysis in complex biological matrices and support further investigations into the physiological significance of the enantiomeric distribution of lysine and its metabolites.

Keywords: Multidimensional HPLC, amino acids, enantiomer separation

Advanced Strategies for High-Efficiency Extraction, Ultra-Sensitive Detection of PFAS from Groundwater with Novel Adsorbents Using Fluorous Affinity

Xin Geng*, Takuya Kubo

Graduate School of Life and Environmental Science, Kyoto Prefectural University, 1-5 Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan.

*Email: xgeng@kpu.ac.jp

Abstract

Per- and polyfluoroalkyl substances (PFAS) are widely used synthetic chemicals persisting in the environment and have been frequently detected in groundwater, raising concerns over their potential health impacts. However, accurate enrichment and separation of PFAS, particularly short- to medium-chain compounds, remain analytically challenging due to their high polarity and diverse chemical structures [1]. In Japan, recent nationwide surveys have identified multiple PFAS-contaminated regions, highlighting the urgent need for analytical methods with broad applicability and high selectivity.

Following our prior investigation on fluorous affinity and fluorinated stationary phases in high performance liquid chromatography (HPLC) [2], we aimed to improve PFAS retention by increasing the fluorine surface density on silica supports. A high-density fluorinated silica gel was synthesized using trimethoxy(1H,1H,2H-heptadecafluorodecyl) silane (F18-C10-silane) under optimized grafting conditions. FTIR analysis indicated intensified and broadened absorption around ~1200 cm^{-1}, consistent with successful high-density grafting of perfluoroalkyl chains. BET analysis showed reduced surface area (from 324.5 m^2/g to 16.1 m^2/g) and pore size (to 9.5 nm), indicating partial pore filling. These results suggested that dense fluorinated grafting alters surface properties, forming a microenvironment conducive to F–F interactions and PFAS selectivity. The HPLC column packed with the modified material exhibited improving retention for mono- to hexa-fluorinated aromatic PFAS. Compared to both non-fluorinated C10-silica gel and our previous fluorinated phases, the new stationary phase enhanced retention strength. It also enabled efficient separation of perfluorinated esters (C5–C13) using purely organic mobile phases. These esters showed substantial retention on the new stationary phase, while non-fluorinated analogues exhibited almost no retention. Notably, this selective separation was achieved without aqueous modifiers, underscoring the material's strong fluorous affinity and enhanced fluorophilic interactions enabled by increased surface fluorination.

These findings demonstrate that fluorinated surfaces can serve as effective platforms for capturing a wide range of PFAS. Current efforts aim to optimize mobile phase conditions for real groundwater samples and support comprehensive PFAS analysis for future in vitro and in vivo toxicity studies. This approach may help connect environmental monitoring with health risk evaluation, supporting effective PFAS risk management.

Keywords: HPLC, PFAS, stationary phase, fluorous affinity, separation performance

References

[1] E.R. Pezoulas, B. Tajdini, Y. Ko, A.A. Uliana, R. Giovine, H. Furukawa, H. Vatankhah, J. Börgel, K.C. Kim, C. Bellona, J.R. Long, **J. Am. Chem. Soc.** 147 (25) 21832-21843, 2025.
[2] A. Tadano, Y. Watabe, T. Tanigawa, S. Konishi-Yamada, T. Kubo, **J. Sep. Sci.** 47 (16), 2024.

A NOVEL PIM SAMPLING PROBE FOR ELECTRIC FIELD-ENHANCED DRUG EXTRACTION FROM BIOLOGICAL FLUID

Thien Hee Liew[1,2], Woei Jye Lau[3,4], Pei Sean Goh[3,4], Muhammad Firdaus Omar[5], Michael C. Breadmore[6], Ahmad Fauzi Ismail[3,4], Hong Heng See[1,2]

1. Centre for sustainable nanomaterials, Ibnu Sina Institute for Scientific and Industrial Research, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.
2. Department of Chemistry, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.
3. Faculty of Chemical and Energy Engineering, Universiti Teknologi Malaysia, Skudai 81310, Johor, Malaysia.
4. Advanced Membrane Technology Research Centre (AMTEC), Universiti Teknologi Malaysia, 81310, UTM Johor Bahru, Johor, Malaysia.
5. Department of Physic, Faculty of Science, Universiti Teknologi Malaysia, 81310 UTM Johor Bahru, Johor, Malaysia.
6. Australian Centre for Research on Separation Science (ACROSS), School of Natural Sciences, University of Tasmania, Hobart, Tasmania, 7001, Australia

Corresponding author: t.hee1998@graduate.utm.my

Abstract

The analysis of biological fluids in clinical laboratories remains a critical yet challenging task, primarily due to the complexity of sample preparation and the potential for analyte loss during multi-step protocols. To overcome these limitations, we present a novel, low-cost, and disposable sampling probe that enables solvent-free, electric field-driven extraction of drug analytes directly from biological matrices. This innovative probe integrates a polymer inclusion membrane (PIM) as both the extraction and storage medium, streamlining the entire sample pretreatment process. By leveraging electrophoretic transport, the system facilitates efficient analyte isolation, concentration, and retention within the PIM. The proposed approach significantly reduces handling steps, enhances analyte recovery, and offers a practical solution for point-of-care or high-throughput clinical analysis [1]. The sampling probe comprises a non-conductive glass rod, a copper wire, and a PIM fabricated at the end of the glass rod. The glass rod is immersed in a homogeneous membrane solution containing an optimized composition of cellulose triacetate (CTA) as the base polymer, 2-nitrophenyl octyl ether (2-NPOE) as the plasticizer, and 1-butyl-3-methylimidazolium trifluoromethylsulfonate ([BMIM] [Otf]) as the ionic carrier, resulting in a 90 µm-thick PIM on the sampling probe [2]. The developed probe electrokinetically extracted ofloxacin from human serum, and human plasma. The practicability and reliability of the electric field enhanced-extraction were evaluated by HPLC-FLD to quantify the desorption of extracted ofloxacin. Under optimized conditions, a quantification limit of 20-200 ng/ml was achieved for the two biological fluid.

Keywords: Polymer inclusion membrane (PIM), Sample preparation, Electrokinetic extraction

References

1. Nanthasurasak, P., et al., *In-Transit Electroextraction of Small-Molecule Pharmaceuticals from Blood.* Angewandte Chemie International Edition, 2019. **58**(12): p. 3790-3794.

2. Tey, H.Y., M.C. Breadmore, and H.H. See, *Electrokinetic Extraction of Doxorubicin from Biological Fluids by Polymer Inclusion Membrane Sampling Probe.* Analytical Chemistry, 2023. **95**(4): p. 2134-2139.

LC-MS-Based Comprehensive Lipidomic Analysis of Plant-derived Exosome-Like Nanoparticles from Flower Petals

Hikaru R. Takaya*[a], Mizuki Yoshihara[a], Ayaka Sato[a], Yoshihiro Sasaki[b], Masanao Yoshimoto[c], Hidenori Takahashi[d]

[a] *Department of Health and Life Sciences, Teikyo University of Science, 2-2-1 Adachi, Tokyo 120-0045, Japan.*

[b] *Graduate School of Engineering, Kyoto University, Katsura, Nshikyo, Kyoto 615-8510, Japan.*

[c] *Antimicrobial Technology, 329 Nishiura, Kamitoba, Minami, Kyoto 601-8145, Japan*

[d] *MS Business Unit, Life Science Business Department, Analytical & Measuring Instruments Division, Shimadzu, 1 Kuwabara, Nakagyo, Kyoto 604-8511, Japan.*

*Email: takayahikaru@ntu.ac.jp

Recently, plant-derived exosome-like nanoparticles (PELNs) have been in the spotlight as sustainable and biocompatible nanocarriers for pharmaceuticals and cosmetics. They boast benefits such as cost-effective production, stable supply, resistance to gastrointestinal digestion, and favorable skin and blood-brain barrier permeability. However, detailed knowledge of their lipid composition remains limited. In this study, apoplast-derived exosome-like nanoparticles from various fresh flower petals, such as rose (RANa: Rose Apoplast-derived Nanoparticles), were isolated using SEC after sequential filtration, without tissue disruption or ultracentrifugation. Characterization of the particles by NTA and TEM revealed particles with an average diameter of 170–220 nm at concentrations of up to 1.2×10^9 particles/mL.

Chloroform-free lipid extraction was performed for RANa, and comprehensive lipidomic analyses were conducted using LC-ESI-OAD-Q-TOF (Shimadzu LCMS-9050) and LC-ESI-QqQ-MS/MS (Shimadzu LCMS-8045). LC separation was performed using an Imtakt Unison UK-C18 MF for LCMS-9050-based non-targeted lipidomic analysis; a Shim-pack Velox C18 for LCMS-8045-based TG MRM analysis; and a Phenomenex Kinetex C8 for LCMS-8045-based phospholipid MRM analysis. The oxygen attachment dissociation (OAD) technique enabled non-targeted lipidomic analysis with precise localization of double bonds. This allowed identification of lipids, including ceramides and other species not detectable by Shimadzu MRM library methods, with data processed and annotated using MS-DIAL. In contrast, for targeted lipid quantitation using LCMS-8045, a triple quadrupole system of was operated under ESI conditions, employing multi reaction monitoring (MRM) based on a pre-validated lipid library. The triglyceride (TG) and phospholipid MRM libraries enable quantitation across 60 registered TGs and major phospholipid classes, including lysophospholipids, phospholipids, and sphingomyelins. OAD-based analysis revealed a total of 528 lipid species were contained in RANa, with the composition was dominated by unidentified lipids (54%), with TG (17%), DG (11%), Cer (12%), phospholipids (1%), and others (6%). The proportion of neutral lipids versus ceramides (40%) was higher than typically observed in PELNs. TG and DG species were rich in unsaturated fatty acids, while ceramides displayed structural diversity such as Cer-HS, Cer-NS, AHexCer, and Hex2Cer.

These findings suggest that RANa exhibits characteristics of solid lipid nanoparticles (SLN) or nanostructured lipid carriers (NLC), with stability for over two months at room temperature and potential benefits such as barrier improvement, antioxidant activity, and enhanced penetration. This study provides the first detailed lipidomic profile of rose petal-derived PELNs and supports their potential application as high-value cosmetic and pharmaceutical ingredients. In this presentation, we will also compare the lipid profiles obtained from OAD-based non-targeted analysis and MRM library-based targeted quantitation to highlight their complementary strengths and differences.

Keywords: LC-MS, lipidomics, PELNs, flower-derived extracellular vesicles, rose

Development of molecularly imprinted polymers recognizing folic acid in aqueous systems for the separation of folic acid-modified liposomes

Kosuke Tsukada* Takuya Kubo

Graduate school of Life and Environmental Science,Kyoto Prefectural University 1-5 Shimogamo Hangi-cho, Sakyou-ku, Kyoto 606-8522, Japan

In this study, we develop a molecularly imprinted polymer (MIP) that recognizes folic acid in aqueous condition for the separation of folic acid-modified liposomes.

Liposomes are nanoscale vesicles composed of biocompatible phospholipids and are commonly used as drug carriers. They can efficiently encapsulate and deliver both hydrophilic / hydrophobic drugs. According to these properties, folic acid-modified liposomes have attracted attention as a means of enabling targeted delivery to cancer cells that overexpress folate receptors. Although folate receptors are present in normal cells such as the placenta and intestines, they are localized at the apical surface of polarized epithelial cells, making them difficult to access from ligands in the bloodstream. In contrast, they are often exposed on the cell membrane of cancer cells. By exploiting this characteristic, liposomes modified with folic acid on their surface can selectively and efficiently deliver drugs to cancer cells. However, multifunctional liposomes require multiple processes, such as the introduction of imaging molecules and modification of target ligands, leading to complex manufacturing processes. As a result, batch-to-batch variability is likely to occur, making it difficult to uniformly control surface properties and particle size thereby limiting reproducibility and efficacy *in vivo*. These manufacturing challenges hinder scale-up and are one of the major factors preventing targeted liposome formulations from reaching clinical application.

This study aims to address the challenges of ensuring uniformity in particle surface properties and particle size by applying molecular imprinting technology to develop separation equipment that can selectively separate liposomes based on the recognition of folic acid. To achieve the objective, we focus on molecularly imprinted polymer (MIP). During polymerization for MIPs, template molecules are incorporated into the crosslinked polymer to construct selective binding sites. However, liposomes are structurally unstable in organic solvents and may cause structural damage. Therefore, in this study, we are optimizing the conditions for preparing MIPs that can selectively recognize and separate folic acid under aqueous conditions, with a view to applying them to liposomes.

Temperature Responsive Mixed Mode Chromatography for Effective Separation of Ionic Biomolecules and Proteins

Kenichi Nagase*[a,b], Sakiko Kitazawa[b], Maria Watanabe[b], Fumihiko Zen[b]
Hideko Kanazawa[b]

[a] *Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima, 734-8553, Japan.*
[b] *Faculty of Pharmacy, Keio University, 1-5-3 Sibakoen, Minato-ku, Tokyo, 105-8512, Japan.*

*Email: nagase@hiroshima-u.ac.jp

Temperature-responsive chromatography have been investigating for analysis and separation of the various types of biomolecules [1]. We developed two types of temperature-responsive mixed-mode chromatography for the effective separation of ionic biomolecules and proteins. The temperature-responsive mixed-mode chromatography column was prepared by packing poly(*N*-isopropylacrylamide) (PNIPAAm)-modified beads and poly(2-acrylamido-2-methylpropane sulfonic acid) (PAMPS)-modified beads in various compositions (Fig.1A). The PNIPAAm-modified silica beads and PAMPS-modified silica beads were synthesized via surface-initiated atom transfer radical polymerization of NIPAAm and AMPS, respectively. The elution behavior of cold remedy medicines and monoamines from the prepared mixed-mode column was observed. The effective separation of these analytes was achieved by altering the composition of the PNIPAAm and PAMPS beads. Additionally, we developed another type of mixed-mode column using a mixed polymer brush composed of PNIPAAm and poly(*N,N*-dimethylaminopropyl acrylamide) (PDMAPAAm) as the ligand in the stationary phase (Fig.1B). The elution behavior of various acidic proteins from the prepared beads was also observed. Several proteins were adsorbed onto the column at elevated temperatures because of the enhanced electrostatic interaction of exposed PDMAPAAm and the increased hydrophobic interaction resulting from the dehydration of PNIPAAm. Using the developed mixed-mode column, a mixture of proteins was separated using a step temperature gradient. These results indicate that the developed temperature-responsive mixed-mode chromatography is a useful separation tool for ionic biomolecules and proteins.

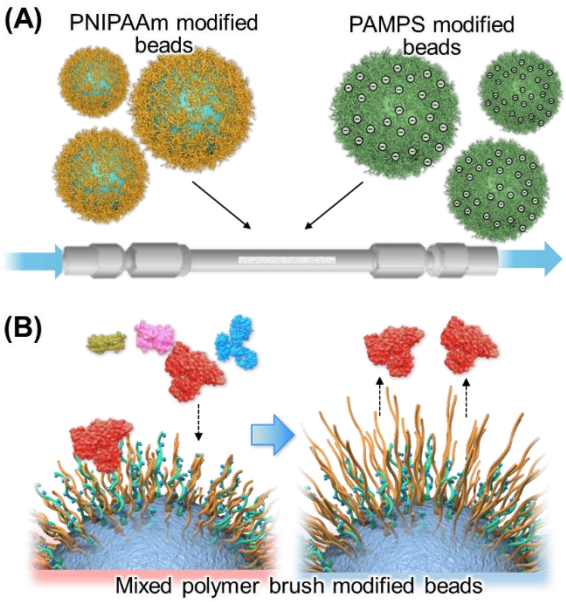

Fig.1 Temperature-responsive mixed mode chromatography using (A) mixture of two types of beads and (B) mixed polymer brush modified beads.

Keywords: Mixed mode column, Bioseparation, Electrostatic interaction, Protein Separation

References

[1] K. Nagase, H. Kanazawa, ***Anal Chim Acta***, 1138, 191, 2020.

A Rapid and Efficient Screening Method Development of Secondary Metabolites using LC-Raman

Kana Matsuoka*[a, b], Masahiro Ando[c], Takuji Nakashima[c, d], Shunnosuke Suwa[a], Haruko Takeyama[a, c, e]

[a] *Graduate School of Advanced Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan.*
[b] *Shimadzu Corporation, 1, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan.*
[c] *Research Organization for Nano and Life Innovation, Waseda University, 513 Wasedatsurumaki-cho, Shinjuku-ku, Tokyo 162-0041, Japan.*
[d] *Department of Field Sciences, University of Human Environments, 9-12 Dogohimata, Matsuyama, Ehime 790-0825, Japan.*
[e] *Institute for Advanced Research of Biosystem Dynamics, Graduate School of Advanced Science and Engineering, Waseda Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan.*

*Email: matsuoka.kana.u4k@shimadzu.co.jp

Natural products have been played a key role in drug discovery due to their unique structures and diverse bioactivities [1]. However, owing to the complexity of their resources such as the microbial cultures and plant extracts including plenty of bioactive compounds, screenings for natural products have been time-consuming and laborious [2]. Although recent utilization of liquid chromatography-mass spectrometry (LC/MS), nuclear magnetic resonance (NMR) and genome techniques to the efficiency improvement of screenings, rapid screening and structural characterization remains challenging [3].

Raman spectroscopy is a non-destructive and non-contact analytical technique that enables rapid characterization of chemical structures. In addition, it works in aqueous environments. Therefore, liquid chromatography-Raman (LC-Raman) spectroscopy, Raman spectroscopy combined with sample separation by liquid chromatography (LC), has the higher potential to facilitate the compounds in the complex biological materials to be characterized. In this study, we especially introduce the screening for nitrogen-containing compounds in actinomycete cultures since they have a potential to be various drugs.

The secondary metabolites of actinomycetes isolated and cultured from soybean roots were used for the screening. First, the strains containing nitrogen-containing compounds were selected by Raman spectra of their chloroform extracts. Second, LC-Raman screening of the selected strains was conducted to identify the nitrogen-containing compounds using Raman spectra database. In this screening, after the separation by LC, the extracts were fractionated on well plates at every constant interval and dried to obtain LC-Raman spectra.

The compound eluted at the retention time of 8 minutes in the *Streptomyces* sp. extract was confirmed a nitrogen-containing compound exhibiting the Raman bands between 3100 and 3500 cm^{-1} corresponding to NH stretching. The LC-Raman spectra of the compound agreed well with that of nocardamine [4], a bioactive compound. Raman and LC-Raman spectroscopy realized the rapid identification and characterization of the compounds by providing their structure information in the early stages of the screening.

Keywords: HPLC, Raman spectroscopy, Screening, Secondary metabolites

References

[1] D. J. Newman, G. M. Cragg, ***J. Nat. Prod.***, 83, 770-883, 2020. [2] J. Berdy, ***J. Antibiot.***, 58, 1-26, 2005. [3] S. P. Gaudencio, F. Pereira, ***Nat. Prod. Rep.***, 32, 779-810, 2015. [4] C. Deboer, A. Dietz, ***J. Antibiot.***, 29, 1182-1188, 1976.

Functional Polymer Modified Interfaces for Cell Separation

Kazutaka Nishikawa[a], Reona Kamidoi[a], Kenichi Nagase*[a,b]

[a] *School of Pharmaceutical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima, 734-8553, Japan.*
[b] *Graduate School of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima City, Hiroshima, 734-8553, Japan.*

*Email: nagase@hiroshima-u.ac.jp

Regenerative medicine, which involves cell transplantation for the treatment of intractable diseases, has garnered significant attention. Current research focuses on temperature-responsive polymer-modified cell culture dishes and cell separation materials, where polymer-modified interfaces regulate cell adhesion for applications involving cell sheets and separations [1]. This study examined polymer-modified interfaces for the temperature-dependent control of cell adhesion (Fig. 1). Air plasma was applied to a glass substrate (24 mm × 50 mm, thickness 0.02 mm) to activate its surface, followed by humidification at 60% for 2 h. Chloromethylphenyl ethyl trimethoxysilane (CPTMS), an initiator for atom transfer radical polymerization (ATRP), was dissolved in toluene and reacted with the glass substrate. Functional polymers were grafted onto the glass surface via ATRP, utilizing $CuCl_2$ as a catalyst, tris[2-(dimethylamino)ethyl]amine (Me_6TREN) as a ligand, and ascorbic acid as the reducing agent. The polymer chain length was modulated by altering the monomer concentrations. The state of the polymer modification was assessed through contact angle measurements, and the molecular weight was determined using gel permeation chromatography (GPC). NIH/3T3 cells were seeded at 37°C on polymer-modified glass within a 35 mm dish and incubated. Subsequently, the temperature was reduced to 20°C to facilitate cell detachment. Variations in the contact angle after each modification step confirmed the success of the reactions. The molecular weight was controllable by adjusting the monomer concentration. Under optimal polymer chain length conditions, cells adhered at 37°C and detached at 20°C (Fig. 2). At 37°C, polymer dehydration induces hydrophobicity and cell adhesion, whereas rehydration at lower temperatures results in cell detachment from the surface. Alterations in the composition of the culture medium also influenced cell adhesion behavior, suggesting that both polymer state modification and medium composition can effectively regulate cell adhesion and detachment.

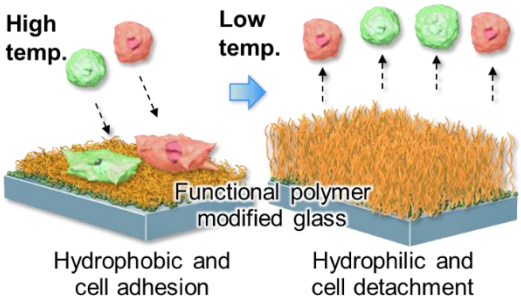

Fig.1 Temperature-modulated cell adhesion and detachment using functional polymer modified interfaces.

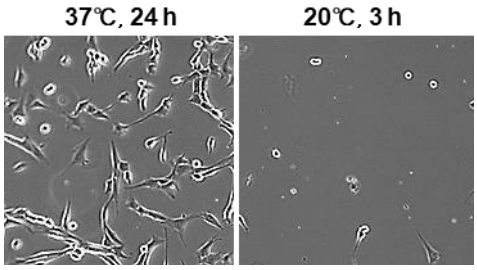

Fig.2 Cell adhesion and detachment on functional polymer modified interfaces.

Keywords: Functional Interface, Cell separation, Temperature-responsive chromatography

References

[1] K. Nagase, ***Adv. Colloids Interfaces Sci.***, 1138, 191, 2020.

Evaluation of the behavior for fluorous affinity using HPLC

Shotaro Sasahara* Takuya Kubo

Graduate School of Life and Environmental Science, Kyoto Prefectural University, 1-5 Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan.

*Email: s822310011@kpu.ac.jp

Per- and poly fluoroalkyl substance (PFAS) are resistant to hydrolysis, photodegradation, microbial degradation, and metabolism, and are known as forever chemicals. Among these compounds, perfluorooctanoic acid (PFOA) and perfluorooctane sulfonic acid (PFOS) in particular have been used in a variety of applications in the past, and due to their resistance to degradation, they have been cited as causing environmental pollution and adverse effects on human health. They are likely to dissolve in water due to water solubility and are anticipated to persist in the environment for extended periods because of their high chemical stability, water, non-volatility, and non-biodegradability. To prevent environmental pollutions of PFAS, the development of selective separation mechanisms for PFAS at the disposal stage is necessary.

On the other hand, there are still unresolved aspects regarding the behavior of fluorine. As an example of fluorine behavior, it is known that organic fluorine compounds are not mixed with water layers and/or organic layers but instead mix with other organic fluorine compounds. Meanwhile, in high performance liquid chromatography (HPLC), organic fluorine compounds can be separated using a fluorine-modified column under reverse-phase conditions. In this separation, the fluorous affinity may contributes and the detailed mechanism has not evaluated [1]. In this study, we develop a new fluorine-modified column to elucidate the behavior of unresolved fluorous affinity (fluorine-fluorine interaction). We are also aiming to further improve the separation efficiency of fluorinated compounds using the newly developed column.

References

[1] A. Tadano, Y. Watabe, T. Tanigawa, S. Konishi-Yamada, T. Kubo, Evaluation of fluorous affinity using fluoroalkyl-modified silica gel and selective separation of poly-fluoroalkyl substances in organic solvents. ***J Sep Sci.***, 47:2400121, 2022.

Development and Optimization of a Novel Silica-Titania Monolithic Stationary Phase for Capillary Liquid Chromatography

Mitsuki Kuriyama*[a], Tsugufumi Matsuyama [a, b], Lee Wah Lim[a, b]
[a] *Graduate School of Natural Science and Technology, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan.*
[b] *Department of Chemistry and Biomolecular Science*, *Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan.*

*Email: kuriyama.mitsuki.f1@s.gifu-u.ac.jp

Silica monoliths possess a dual-pore structure consisting of mesopores and macropores. This unique structure, characterized by high porosity and large specific surface area, enables high-efficiency separations at high flow rates. However, silica-based monoliths have a major drawback of low resistance to basic conditions. Due to its low resistance to basic conditions, the monolithic structure may collapse when basic compounds as mobile phase or sample are used [1, 2, 3].

To improve this drawback, we aimed to develop a basic-resistant silica-based monolithic column by introducing titanium, *i.e.* a material that has high pH stability, during the monolith synthesis process. In addition, capillary columns can contribute to reducing solvent waste and enable the use of expensive or novel mobile phase additives, thereby expanding the range of mobile phase compositions and reducing environmental burden [3, 4].

In this study, titanium tetraisopropoxide, *L*-lactic acid, and water were mixed at room temperature and stirred until a clear solution. We defined the resulting solvent as “titania”. A separate precursor solution was prepared by mixing 0.01 M acetic acid, polyethylene glycol (Mw = 10,000), urea, titania as well as tetramethoxysilane, and was stirred under ice-cooling. This mixed solution was filled into a pretreated capillary (0.320 mm I.D. × 0.450 mm O.D.), and both the capillary and remaining solution in the vial were heated *via* the sol–gel method. After heating, the white powder formed in the vial was also packed into the capillary.

Under various combinations, the effect of heating conditions during the sol–gel method on the phase separation behavior of the silica–titania monolith inside the capillary was evaluated. The basic-resistance of the white powder formed in the vial was tested using Tris–HCl and sodium carbonate buffer solutions, proving its durability of up to pH 11. Furthermore, chromatographic evaluation of the packed columns using various acetonitrile (ACN) concentrations revealed that the column could be operated under both reversed-phase as well as hydrophilic interaction liquid chromatography (HILIC) separation modes.

Keywords: Capillary LC, monolithic stationary phase, silica monolith, titania, sol-gel method

References

[1] S. Miyazaki, S. Ota, T. Kubo, K. Morisato, K. Nakanishi, M. Ohira, N. Tanaka, ***CHROMATOGRAPHY***, 32, 87-94, 2011. [2] T. Umemura, N. Kojima, Y. Ueki, ***BUNSEKI KAGAKU***, 57, 517-529, 2008. [3] Jérôme Randon, Jean-François Guerrin, Jean-Louis Rocca, ***Chromatography A****,* 1214, 183-186, 2008. [4] T. Takeuchi, Developments in Capillary Liquid Chromatography, The 15th Annual Meeting of the Society for Chromatographic Sciences, Kitasato University, November 11-12, 2004.

Development of a separation platform for biopharmaceuticals using a spongy-like polymer

Eisuke Kanao*[a,b], Tetsuya Tanigawa[a,c], Shunsuke Tanaka[a],
Takuya Kubo[c], Yasushi Ishihama[a,b]

[a] *Graduate School of Pharmaceutical Sciences, Kyoto University, 46-29, Yoshidashimoadachicho, Sakyo-ku, Kyoto 606-8501, Japan.*
[b] *National Institutes of Biomedical Innovation, Health and Nutrition, 7-6-8, Saito-Asagi, Ibaraki City, Osaka 567-0085, Japan.*
[c] *Graduate School of Life and Environmental Science, Kyoto Prefectural University, 1-5 Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan.*

*Email: kanao.eisuke.7s@kyoto-u.ac.jp

Abstract body

Recently, the focus of drug development has shifted from small-molecule compounds to macromolecular biopharmaceuticals and is further expanding to therapies that leverage extracellular particles and even whole cells. As therapeutic modalities diversify and grow more complex, separation and purification still account for roughly half of manufacturing costs, making faster development and cost reduction pressing issues for end users. In parallel, MS-based proteomics is indispensable for the analysis of these biotherapeutics, yet there is a strong demand for sample-preparation techniques that can rapidly process minute samples. Against this backdrop, we have begun developing an ultraporous, sponge-type monolithic polymer (“sponge monolith”) with high flexibility and flow-through characteristics.

The sponge monolith is produced by melt-kneading thermoplastic polymers to yield a flexible, porous separation matrix. It features very large through-pores (～μm) that provide exceptionally high permeability compared with conventional media, and it is composed of bioinert polymers. The matrix surface carries reactive epoxide groups, enabling facile immobilization of diverse protein ligands and functional moieties. Furthermore, by adding functional microparticles—such as hydrophobic chromatographic beads or titanium dioxide—during melt-kneading, hybrid materials with enhanced performance can be fabricated. Using this material, we achieved high-throughput separation and purification of antibodies and extracellular particles such as viruses and exosomes, as well as whole cells [1-6]. The sponge monolith has also been validated as an effective sample-preparation medium for low-input omics analyses, including proteomics [7]. In this presentation, we describe the performance of the sponge monolith and discuss its future prospects as a universal separation platform for the life sciences and pharmaceutical development.

Keywords: Liquid chromatography, spongy-like polymer, biopharmaceuticals, proteomics.

References

[1] K. Kubota, T. Kubo, T. Tanigawa, T. Naito, K. Otsuka, ***Sci. Rep.***, 7, 178, 2017. [2] E. Kanao, S. Wada, H. Nishida, T. Kubo, T. Tanigawa, K. Imami, A. Shimoda, K. Umezaki, Y. Sasaki, K. Akiyoshi, K. Otsuka, J. Adachi, Y. Ishihama, ***Anal. Chem.***, 94, 18025-18033, 2022. [3] T. Kubo, E. Kanao, K. Ishida, S. Minami, T. Tanigawa, R. Mizuta, Y. Sasaki, K. Otsuka, T. Kobayashi, ***Anal. Chem.***, 95, 13185-13190, 2023. [4] E. Kanao, K. Ishida, R. Mizuta, Y. Li, K. Imami, T. Tanigawa, Y. Sasaki, K. Akiyoshi, J. Adachi, K. Otsuka, Y. Ishihama, T. Kubo, ***Anal. Chem.***, 95, 14502-14510, 2023. [5] K. Tsuji, T. Tanigawa, Y. Tsutsumi, T. Kubo, N. Kaji, ***Talanta Open***, 9, 100321, 2024. [6] R. Mizuta, E. Kanao, K. Ukyo, S. Kuwada, S. Sawada, Y. Ishihama, K. Akiyoshi, Y. Sasaki, ***Nano Lett.***, 24, 12907-12914, 2024.[7] E. Kanao, S. Tanaka, A. Tomioka, K. Ogata, T. Tanigawa, T. Kubo, Y. Ishihama, ***Anal. Chem.***, 96, 20390-20397, 2024.

Liquid Chromatographic Separation of H/D Isotopologues Enabled by Aromatic π Interactions

Xiaoting LI*[a], Takuya KUBO[a]
[a] *Graduate School of Life and Environmental Science, Kyoto Prefectural University, 1-5 Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan.*

*Email: xli@kpu.ac.jp

Abstract

Deuterium (D), an isotope of hydrogen (H) containing an additional neutron, is approximately twice as heavy as ordinary hydrogen. Although deuterated and hydrogenated molecules exhibit similar chemical properties, they can be readily distinguished by mass spectrometry (MS) and nuclear magnetic resonance (NMR) due to the deuterium isotope effect. This phenomenon is widely investigated in organic synthesis, biosynthesis, and pharmaceutical development; however, efficient chromatographic separation of hydrogen/deuterium isotopologues remains limited. Liquid chromatography (LC), based on analyte partitioning between stationary and mobile phases, is highly sensitive to weak intermolecular interactions. Our previous studies demonstrated fullerene-modified stationary phases capable of enhancing π-π stacking, spherical recognition, and dipole-induced dipole interactions; however, the isotope effect in LC was not systematically examined.

In this work, we developed a graphene nanosheet-decorated silica stationary phase to harness graphene's strong π interactions for isotopologue separation. Graphene dispersion was prepared by sonicating graphite flakes in dichlorobenzene (DCB), settling for one week, and centrifuging to obtain the supernatant. Amino-functionalized silica (SiO_2·NH_2) was reacted with 4-azido-2,3,5,6-tetrafluorophenyl succinate (PFPA·NHS) in toluene under dark conditions, followed by microwave-assisted decoration with graphene nanosheets. As shown in Figure 1, scanning electron microscopy confirmed uniform graphene coverage on the silica particles. When packed into an LC column and operated under normal-phase conditions, the material achieved clear separation of hydrogenated and deuterated benzenes. Benzene exhibited longer retention than Benzene-D6, consistent with stronger π-π interactions between graphene and protiated aromatics.

This study demonstrates a graphene-based stationary phase as an effective platform for investigating isotope effects and achieving isotopologue separation in LC. The findings provide new insight into isotope-surface interactions and offer potential applications in analytical chemistry, isotope labeling studies, and pharmaceutical analysis.

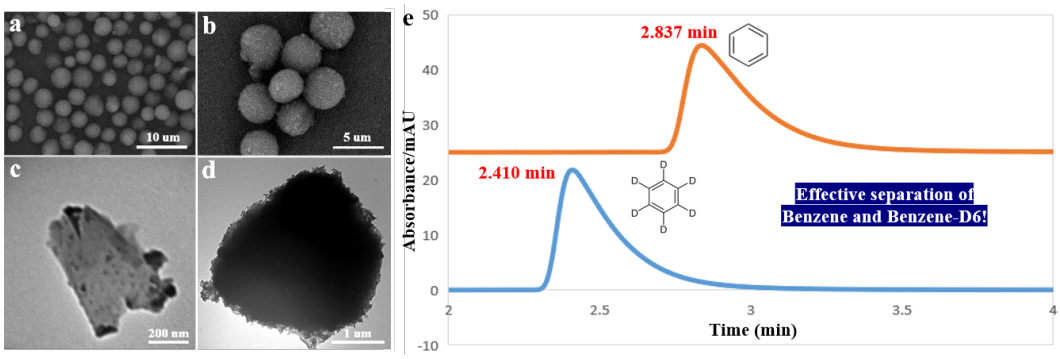

Figure 1. SEM images of (a) SiO_2·NH_2 and (b) graphene-decorated SiO_2·NH_2-PFPA·NHS; TEM images of (c) graphene nanosheets and (d) graphene-decorated SiO_2·NH_2-PFPA·NHS; (e) chromatograms of benzene and benzene-D6 with hexane as the mobile phase.

Keywords: HPLC, H/D isotopologues, graphene, π-π interactions, microwave

Development of Dendrimer-type Monolithic Capillary Stationary Phases for Mixed-mode Chromatography

Koki Abe*[a], Tsugufumi Matsuyama[a, b], Lee Wah Lim[a, b]
[a] *Graduate School of Natural Science and Technology, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.*
[b] *Faculty of Engineering, Gifu University, 1-1 Yanagido, Gifu 501-1193, Japan.*

*Email: abe.koki.i9@s.gifu-u.ac.jp

Monolithic stationary phases offer several advantages, including high fluid permeability, high mass transfer and their ease of preparation in a fused silica capillary [1]. Mixed-mode chromatography combines multiple separation modes to allow for simple and efficient separation of complex or practical samples. Dendrimers are molecules with a structure in which side chains regularly branch off from a core, and the number of functional groups at the ends increases exponentially with the number of branches (*i.e.*, generations) [2]. In this study, dendrimer-type monolithic stationary phases were synthesized by repeatedly reacting 1,4-butanedioldiglycidyl ether (BDDE) and ammonia on the surface of monolithic stationary phases. Anion exchange chromatography, reversed-phase chromatography, and hydrophilic interaction chromatography (HILIC) were performed to evaluate the mixed-mode function of each generation.

To obtain anchoring sites for the chemical grafting of the polymer at the inner wall of a fused silica capillary (0.32 mm I.D.), 3-(trimethoxysilyl) propyl methacrylate was reacted with silanol groups inside the capillary. Glycidyl methacrylate and ethylene dimethacrylate were dissolved in a porogen consisting of a mixture of ethanol, 1,4-butanediol, and pure water. 2,2'-azobisisobutyronitrile was dissolved in the mixture and subsequently sonicated for 5 min. The resulting mixture was subsequently introduced into the pretreated capillary and sealed at both ends. The capillary was immersed in a water bath at 60˚C for 24 h to allow for polymerization. Subsequently, the stationary phase was reacted with ammonia in the water bath at 80˚C for 1 h. Here we call the resulting stationary phase 0th generation. Subsequently, the stationary phase was reacted with BDDE in the water bath at 80˚C for 3 h and reacted with ammonia in the same way to obtain 1st generation. To obtain higher generations of the stationary phase, the reactions with BDDE and ammonia were repeated.

The retention times of polycyclic aromatic hydrocarbons decreased with increasing generations of the dendrimer-type stationary phases. This result indicates that the hydrophobicity of the stationary phases decreases as the generation of dendrimer-type stationary phases increases. The retention times of nucleobases were small values in each generation, while these of inorganic anions increased with increasing generations of dendrimer-type stationary phases.

Keywords: dendrimer, organic polymer monolith, mixed-mode chromatography, stationary phase

References

[1] T. Umemura, N. Kojima, Y. Ueki, ***BUNSEKI KAGAKU***, 57, 517-529, 2008. [2] I. Kawase, L.W. Lim, T. Takeuchi, ***Chromatography***, 38, 9-14, 2017.

Examination of Analytical Conditions for Synthetic Peptides

Daiki Fujimura*[a], Yusuke Osaka[a]
[a] *Shimadzu Corporation, 1, Nishinokyo Kuwabara-cho, Nakagyo-ku, Kyoto 604-8511, Japan.*

*Email: d-fuji@shimadzu.co.jp

In recent years, medium-molecule drugs have gained attention as promising new therapeutics. Peptide drugs, such as insulin and GLP-1, are representative examples of medium-molecule drugs. Peptides used in peptide drugs exhibit various properties depending on their amino acid composition, sequence, and conformation. Liquid chromatography is commonly employed for the structural analysis and quantification of peptides; however, challenges such as adsorption and carryover often arise during peptide analysis. In this study, we investigated analytical conditions with a focus on addressing these issues in columns and vials.

Semaglutide, used as the evaluation sample, was purchased from AA Blocks. A stock solution of 1 mg/mL was prepared by dissolving 5 mg of Semaglutide in 5 mL of 1% formic acid/acetonitrile (75:25, v/v). This stock solution was utilized for column evaluation, while a diluted solution of 0.0001 mg/mL was prepared for vial adsorption evaluation.

To evaluate adsorption effects on column materials, analyses were performed using three types of Shim-pack Scepter C18-120 columns (Stainless Steel type, PEEK-lining type, and bioinert coating type) from Shimadzu. The LC system used was Nexera (Shimadzu). For vial adsorption evaluation, TORAST-H PP (Shimadzu GLC) vials were compared with two other types of low-adsorption vials from different vendors.

In the column adsorption evaluation, the PEEK-lining type and bioinert coating type columns demonstrated similar peak area intensities. In contrast, the Stainless Steel type column exhibited a 10% lower peak area intensity compared to the PEEK-lining type and bioinert coating type columns, suggesting adsorption on the metal surface.

For vial adsorption evaluation, changes in peak area intensity were measured from the time the sample was placed in the vial to 2 hours later. Among the three vials tested, the peak area of vendor A's vial decreased by 25% over 2 hours, vendor B's vial decreased by 48%, and the TORAST-H PP vial showed only a 9% decrease. These results confirmed that the degree of adsorption varies depending on the vial type.

In the poster presentation, we will also discuss the evaluation of carryover effects on columns and equipment. (335 words)

Keywords: HPLC, peptide, GLP-1, adsorption

Development of Novel Organic Reaction Field for The Compounds with Catechol Structure using Triptycene Based Polymer

Mai Sasaki*[a], Takuya Kubo[a,b]

[a] Graduate School of Engineering, Kyoto University, Katsura, Nishikyo-ku, Kyoto 615-8510, Japan

[b] Graduate School of Life and Environmental Science, Kyoto Prefectural University, Shimogamo Hangi-cho, Sakyo-ku, Kyoto 606-8522, Japan.

*Email: sasaki.mai.64m@st.kyoto-u.ac.jp

Triptycene has characteristic paddle-wheel structure and the unique space surrounded by aromatic rings, called the internal free volume (IFV), which is known to be suitable for adsorption of specific molecules. Although triptycene-based polymer has been used as adsorbent materials by taking advantage of this space, the molecular recognition ability in the IFV has not been clarified in detail and there are few examples of the applications utilizing this property.

In organic synthesis, it is common to introduce the protecting group to prevent unwanted side reactions. However, the protecting step gives complicated reactions and decreases the yield. On the other hand, in our previous study, we used triptycene-based polymer as the stationary phase to evaluate the specific interactions between the aromatic rings in polymer and analytes, and we found that triptycene-based polymer exhibited selective adsorption toward catechol. Therefore, we consider the idea of applying triptycene-based polymer as a solid-phase material for the reaction involving the compounds with catechol structure.

In this study, triptycene-based polymers were synthesized by the solvent knitting method [1] and packed into the HPLC column to investigate adsorption characteristics and elution conditions by HPLC measurement. As a result, compounds with catechol structure were selectively adsorbed onto the polymer briefly. The compounds were strongly retained when acetonitrile was used as the mobile phase and easily eluted by adding formic acid. Furthermore, we employed dopamine in an acetylation reaction with and without the polymer. As a result, monoacetylated compounds were obtained in the presence of the triptycene-based polymer, whereas triacetylated compounds were mainly produced in the control experiment without the polymer.

References

[1] A. Giri, S. Biswas, MD. W. Hussain, T. K. Dutta, and A. Patra., ***ACS Appl. Mater. Interfaces*** 2022, 14, 5, 7369–7381.

Development of Multi-Dimensional HPLC Systems for the Analysis of Fermentation Related D-Amino Acids in Food/Beverage Samples

Pattraporn Chobpradit[a,b], Naho Kondo[a], Takeyuki Akita[a], Chiharu Ishii[a], Masashi Mita[c], Chadin Kulsing[b], Kenji Hamase*[a]

[a] *Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.*
[b] *Department of Chemistry, Faculty of Science, Chulalongkorn University, 254 Wang Mai, Pathum Wan, Bangkok 10330, Thailand.*
[c] *KAGAMI, Inc., 7-7-15 Saito-asagi, Ibraraki, Osaka 567-0085, Japan.*

*Email: hamase@phar.kyushu-u.ac.jp

Amino acids (AAs) play key roles in nutrition, adulteration, and quality assessment of natural products. Although L-enantiomers of AAs are found in foods and beverages as the predominant isomers, their D-form antipodes are also present in some of the edible materials. Specifically, the D-form of alanine (Ala), aspartic acid (Asp), glutamic acid (Glu) and serine (Ser) have been reported at relatively high levels (compared with those of other D-amino acids) in fermented products, and are considered to be associated with physiological functions relevant to human health through the daily diet. However, the amounts of these D-AAs are low in most cases, and are often accompanied by complex interferences present in the real-world samples, making their accurate determination challenging. To address this limitation, multi-dimensional HPLC systems coupled with fluorescence detection has been developed to achieve highly selective and sensitive analysis, and applied to the determination of D-amino acids in food/beverage samples.

The sample preparation procedure for 4 chiral amino acids (8 enantiomers) involved dilution of the sample in an aqueous solution, followed by pre-column fluorescence derivatization. To the diluted sample, Na-borate buffer (pH 8.0) and an acetonitrile (MeCN) solution of 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) were added. After heated at 60 °C for 2 min, 0.2 % aqueous trifluoroacetic acid (TFA) is added, and subsequently introduced into the multi-dimensional HPLC system. Fluorescence detection was performed with excitation and emission wavelengths of 470 nm and 530 nm, respectively.

For the development of the two-dimensional HPLC system, 7 Pirkle-type columns (Singularity CSP-003S, -013S, -403S, -603S, -001S, -019S, -021S, 1.5 mm × 250 mm) were tested for separating D-AAs and their L-counterparts in the second-dimension. As a result, the Singularity CSP-603 column (having *N*-(3,5-dinitrophenylaminocarbonyl)-L-diphenylalanine in the chiral recognition site and connected to the aminopropyl silica via a γ-aminobutyric acid linker) exhibited the most effective separation performances. Using mixed solutions of MeCN and methanol (50:50, v/v) containing 0.15–0.60 % formic acid, separation factors of 1.54–2.35 and resolutions of 4.44–11.22 were obtained for Ala, Asp, Glu and Ser enantiomers. Integrating a reversed-phase column (Singularity RP18, 1.0 mm × 250 mm) and the Singularity CSP-603S column in the first and second dimensions, a two-dimensional HPLC system was developed and several liquor samples were analyzed. As a result, D-Ala, D-Asp, D-Glu and D-Ser were detected and relatively high levels of D-AAs were found in 2 Thai wines produced from mangosteen and Syrah grape. Further investigations on their origins and physiological significance are expected.

Keywords: Multi-dimensional HPLC, amino acid, chiral separation, fermented foods

Development of a Two-Dimensional Chiral LC-MS/MS System for the Determination of Alanine, Aspartic Acid and Serine Residues in Proteins Exposed to Stress Conditions

Kaito Murata [a], Chiharu Ishii[a], Masashi Mita[b], Takeyuki Akita[a], Tadashi Ueda[a], Kenji Hamase*[a]

[a] *Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.*
[b] *KAGAMI, Inc., 7-7-15 Saito-asagi, Ibaraki, Osaka 567-0085, Japan.*

*Email: hamase@phar.kyushu-u.ac.jp

Recently, the presence of D-aspartic acid (Asp) and D-serine (Ser) residues is clarified in the proteins under age-related disease conditions along with the progress of analytical techniques. The isomerized amino acid residues have also been found in proteins exposed to various stresses conditions, drawing attention to the environment surrounding the proteins as a factor of epimerization. However, the conditions and mechanisms of isomerization remain mostly unclear, and determination of the D-amino acid residues under various degradation conditions is expected. In the present study, chiral analysis of amino acid residues was performed using our analytical platform combining the separation by a two-dimensional LC system and detection by a tandem mass spectrometer in the proteins stored under high temperature and basic pH conditions.

Protein samples were hydrolyzed in the gas phase with 6 M ^{2}HCl/^2H_2O. After the hydrolysates were dried up, the residues were dissolved in water to obtain amino acid aqueous solutions. The amino acids were reacted with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F)/acetonitrile, then the reaction was terminated by adding an aqueous solution containing 0.2 % trifluoroacetic acid. A portion of the reaction mixture was analyzed by a two-dimensional LC-MS/MS system.

In the first dimension, the target amino acids were separated as their NBD derivatives in approximately 100 min using a microbore ODS column (Singularity RP18, 1.0 x 250 mm). In the second dimension, a narrowbore Pirkle-type column (Singularity CSP-013S having L-diphenylalanine in the chiral selector, 1.5 x 250 mm) was used, and practically sufficient chiral separations (Rs≥2.44) were obtained for all target NBD-amino acids. The method was applied to analyze D-amino acid residues in the proteins stored under various stress conditions. When ovalbumin (OVA) was dissolved in K/Na phosphate buffer (pH 7.4) and stored at 37 ºC for 2 weeks, a clear D-form peak was observed only for Ser (%D = 3.0). In case of OVA dissolved in K/Na borate buffer (pH 9.6) and stored at 37 ºC for 2 weeks, the %D of Asp and Ser increased up to 1.2 and 10.8. On the other hand, high %D values (1.3 for alanine, 15.3 for Asp and 30.3 for Ser) were observed when OVA was stored at pH 9.6 and 60 ºC for 2 weeks. These results indicate that isomerization of amino acid residues occurs when proteins are exposed to high temperature and basic pH conditions, and further investigations to clarify isomerization mechanisms and biological significance are expected.

Keywords: Enantiomer separation, D-amino acid residues, protein degradation, 2D LC-MS/MS

Development of a Two-Dimensional HPLC System for the Determination of Alanylalanine Stereoisomers in the Plasma of Mice with Renal Dysfunction

Fuga Watanabe[a], Yuri Nagata[a], Chiharu Ishii[a], Masashi Mita[b], Takeyuki Akita[a], Jumpei Sasabe[c], Kenji Hamase*[a]

[a] *Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.*
[b] *KAGAMI, Inc., 7-7-15 Saito-asagi, Ibaraki, Osaka 567-0085, Japan.*
[c] *Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.*

*Email: hamase@phar.kyushu-u.ac.jp

By the recent advances in analytical techniques, the presence of several D-amino acids and peptides containing D-amino acids has been demonstrated in mammals including humans. Especially, D-alanine (Ala) has been found in various mammalian species and reported to be associated with renal dysfunction. Additionally, the presence of dipeptides containing D-Ala in real-world biological samples has also been reported. Notably, the dipeptide AlaAla, composed of two Ala residues, has been shown to exist as its DD-form in the bacterial peptidoglycan. Therefore, chiral analysis of AlaAla stereoisomers and elucidation of their contents in mammals (the host of microflora) were expected. For analyzing trace levels of stereoisomers with similar physicochemical properties in complicated biological matrices, two-dimensional (2D) HPLC combining reversed-phase and chiral separations is an effective methodology. In the present study, a 2D-HPLC system has been developed, and applied to the enantioselective determination of Ala and AlaAla amounts in the plasma of mice with renal dysfunction.

The plasma samples were deproteinized by adding 20 volumes of methanol (MeOH), and a portion of the obtained supernatant was dried under reduced pressure. The residue was re-dissolved in water, then amino acids and dipeptides were reacted with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F). The reaction mixtures were subjected to the 2D-HPLC system with fluorescence detectors (Ex. 470 nm, Em. 530 nm).

For the first dimension, an ODS column (Singularity RP18, 1.0 x 250 mm) was used and the concentration of acetonitrile (MeCN) in the mobile phase and column temperature were examined. By using 7 % MeCN 0.01 % trifluoroacetic acid in water (50 ºC), the two NBD-AlaAla diastereomers and NBD-Ala were sufficiently separated ($Rs \geq 2.21$). These 3 targets were also separated from other proteinogenic amino acids. For the second dimension, 4 enantioselective columns having different amino acids (Singularity CSP-001S having L-leucine, 008S having L-2-naphthylalanine, 013S having L-diphenylalanine and 019S having L-Ala, 1.5 x 250 mm) were tested. As a result, the Singularity CSP-013S column showed the superior separations for NBD-D/L-Ala, NBD-DD/LL-AlaAla and NBD-DL/LD-AlaAla. By using the mixtures of MeCN/MeOH (25/75, v/v) containing 0.015–0.1 % formic acid as the mobile phases, and target analytes were enantioseparated within 40 min ($Rs \geq 2.29$). The developed 2D-HPLC system was applied to the analysis of plasma samples obtained from mice with renal dysfunction. As a result, D/L-Ala and DD-AlaAla were clearly observed, and the amounts of DD-AlaAla of mice with renal dysfunction were higher than those in control mice. Further investigations of Ala and AlaAla stereoisomers to clarify their tissue distributions and associations with disease are in progress.

Keywords: AlaAla, stereoisomer, 2D-HPLC, chiral separation, renal dysfunction

Three-Dimensional HPLC Analysis of Glutamic Acid Enantiomers in Mouse Testis and Related Tissues

Kazuki Kubo[a], Mai Oyaide[a], Chiharu Ishii[a], Masashi Mita[b], Takeyuki Akita[a], Jumpei Sasabe[c], Kenji Hamase*[a]

[a] *Graduate School of Pharmaceutical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan.*
[b] *KAGAMI, Inc., 7-7-15 Saito-asagi, Ibaraki, Osaka 567-0085, Japan.*
[c] *Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan.*

*Email: hamase@phar.kyushu-u.ac.jp

Recent progress of analytical methods enables the determination of D-amino acids (enantiomers of widely observed L-amino acids) in higher animals including mammals. Among them, D-glutamic acid (Glu) was reported to be localized in the testis of some animals (mouse and Japanese kuruma prawn) and considered to be involved in the reproduction system. Although the determination of D-Glu in the testis and related tissues is expected, the amounts of D-Glu in mammalian tissues are extremely low and interfered with uncountable intrinsic substances. Therefore, a highly selective and sensitive analytical method is essential and in the present study, a rapid three-dimensional (3D) HPLC system has been developed and applied to the determination of Glu enantiomers in mouse testis and related tissues.

Mouse tissues were deproteinized with methanol (MeOH), and a portion of the supernatant was dried under reduced pressure. The residue was dissolved in water and amino acids were derivatized with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) under alkaline conditions (pH 8.0). The mixture was heated at 60 °C for 2 min, and 0.2 % trifluoroacetic acid (TFA) in water was added to terminate the reaction. NBD-amino acids were detected by their fluorescence emission at 530 nm with excitation at 470 nm.

For the development of the 3D-HPLC system, the stationary and mobile phases were examined. As the stationary phases for the first and second dimensions, two reversed-phase columns with different retention characteristics, Singularity RP18 (1.0 × 150 mm) and Sunniest Biphenyl (1.0 × 150 mm), were tested. To solve the mobile phase compatibility issues, the retention of a stationary phase used in the second dimension must be stronger than that in the first dimension under the same mobile phase conditions. Adopting 12.5 % MeCN 0.05 % TFA aqueous solution as a mobile phase, NBD-Glu was eluted at about 20 min using both Singularity RP18 and Sunniest Biphenyl columns. On the other hand, adopting 20 % MeOH 0.05 % TFA aqueous solution as a mobile phase, NBD-Glu was eluted at 20 min using a Singularity RP18 column and at 35 min using a Sunniest Biphenyl column. Therefore, a Singularity RP18 column (20 % MeOH 0.05 % TFA in water) was selected for the first dimension, and a Sunniest Biphenyl column (12.5 % MeCN 0.05 % TFA in water) was selected for the second dimension. For the third dimension, a Singularity CSP-013S column (1.5 × 150 mm) was selected as the stationary phase, and a mixture of MeCN and MeOH (25/75, v/v) containing 0.4 % formic acid was used as the mobile phase (*Rs* = 2.65 for Glu enantiomers, separated within 20 min). By using this 3D-HPLC system, determination of Glu enantiomers was performed in the mouse testis and related tissues. In the testis, the %D value was 0.15 and a distinct peak of the D-form was observed. On the other hand, in the related tissues (caput epididymis, cauda epididymis, vas deferens and seminal vesicle), the amounts of D-Glu were trace. Further studies to evaluate the amounts of D-Glu in the testis and related tissues of various animal species, and to clarify the biological significance are ongoing.

Keywords: 3D-HPLC, amino acid, enantiomer separation, D-Glu, testis