

Shimadzu Journal

VOL. **07** **ISSUE 2**

Food Development and more...



Director's note

Dear Reader,

I am proud to announce the publication of the new issue of the Shimadzu Journal, which is focused on the topic of Food Development. In recent years, the interest in food safety and food development has increased significantly due to the effects of diet on health in both positive and negative ways. In particular, the development of functional foods has increased. In support of this changing dynamic, Shimadzu has applied resources to the development of applications and has conducted joint research with leading researchers around the world.

One such example is described in this issue through an interview with Professor Erich Leitner from Graz University in Austria, whose research is focused on research and development in the food market. He has been using Shimadzu's mass spectrometry technology for many years to study the quality of food and food contact materials. In addition, the application of the analysis of D / L Amino acids, developed by the joint research laboratory between Osaka University and Shimadzu Corporation, led by Professor Eiichiro Fukusaki, is introduced.

Shimadzu positions the healthcare field as a major growth market. We aim to achieve sustainable growth by developing technologies that contribute to a healthier society and support researchers studying the effects of food on lifestyles and aging. To realize this concept, we established the Healthcare R&D Center on June 5, 2019. This center includes a technology exhibition showcasing analytical instruments and medical imaging diagnostic technologies as well as a joint research laboratory. Our goal is for this center to serve as an incubator, encouraging dialogue and joint research with our customers.

We also held our second Global Innovation Summit in Kyoto on July 9 and 10 as a means of promoting global R&D and encouraging collaboration with our customers and colleagues. The slogan for the Global Innovation Summit 2019 was "Pioneering Partnership for Advanced Healthcare". 93 researchers from 21 countries participated, with many individuals delivering oral presentations or presenting their work in poster format. This sharing of research provided an excellent forum for the sharing of ideas among participants. We will continue to expand our collaboration through more events like this.

Shimadzu will continue to provide solutions in various fields with our partners. We hope that this journal will be a great help for all of you. Your generous feedback is always appreciated.

Yours Sincerely,



Shuzo Maruyama

General Manager, Analytical & Measurement Instruments Division



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Mineral oil (MO) residues in food raised public concern due to some elevated concentrations up to several thousand milligrams per kilogram food. The determination of MOSH and MOAH in food can be done by an automated LC-GC-FID system for routine analysis. Unfortunately, some food material like rice or chocolate contain natural occurring alkanes which can interfere and heavily disturb the analysis of the MOSH fraction. This study reports these interferences can be removed by flash chromatography on aluminium oxide columns.



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When the steric structure of amino acids was devised, it revealed that most amino acids could exist as mirror-image isotopes that are differentiated by an L-/D- notation into L-amino acids and D-amino acids. More recently, it is starting to appear as if some foods and particularly microorganism-associated fermented foods contain D-amino acids, and the function of these foods is gaining interest. In this article, the authors intend to explain a new analytical method for D-amino acids that uses a high-sensitivity triple quadrupole mass spectrometer and describe an example application of this method in fermented food analysis.



Food Development

Determination of garlic phenolic compounds using supercritical fluid extraction coupled to supercritical fluid chromatography/tandem mass spectrometry

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A rapid, simple and environmentally friendly supercritical fluid extraction and supercritical fluid chromatography coupled to mass spectrometry (SFE-SFC-MS/MS) method has been developed for the analysis of nine phenolic compounds in garlic. Through the optimization of the SFE parameters using response surface methodology (RSM), 15 phenolic compounds were successfully extracted at 50°C in 9 min with the addition of 30% methanol using a method that was automatic, efficient, green and prevented oxidation. Moreover, the operating conditions of the SFC-MS/MS were also optimized for the analysis of the SFE extracts. 15 phenolic compounds in the garlic showed good separation performance on a Shim-packUC-X Diol column, and 0.1 mM oxalic acid and 1 mM ammonium formate in methanol were chosen as the most suitable mobile phase components.



Shimadzu Selection

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These articles were selected by Shimadzu. Relating food analysis and development, they are from posters presented at ASMS 2019 and from application notes. They feature a variety of instruments we produce and include cutting-edge technologies. Please obtain the articles of your interest through the links on the titles.



Topics 1

Global Innovation Summit 2019

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Shimadzu Corporation held the "Shimadzu Global Innovation Summit 2019" from 9th-10th July. This year's event, on the theme of "Pioneering Partnerships for Advanced Healthcare", saw a diverse pool of 93 researchers from 21 countries travelling to Shimadzu's headquarters in Kyoto.



Topics 2

Food Safety seminar by SEG

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For several years, the companies Shimadzu and Merck have been offering food safety seminars that introduce a wide range of new applications, as well as tips and tricks for use in the food laboratory. In addition to the extensive lecture program, it provides an excellent platform for dialogue and exchange between experts in food production, control, and research & development.



New Products

MALDImini-1, GC-2010 Pro, UP Series, Multi-omics analysis package, Shim-pack Fast-OA

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Interview with Professor Erich Leitner



We interviewed Professor Erich Leitner from Graz University of Technology in Austria. Professor Erich Leitner is a leading expert on food quality. He is a major node in the local food network, having strong relations with local food producers and farmers in the region. Leitner has been using Shimadzu and mass spectrometers for over a decade to study food.

Professor Leitner, thank you very much for spending some time for this interview. At first, could you outline the research and let us know what discovery and achievement have been made so far?

The largest part of my work deals with the quality of food and food contact materials. This complex topic can be looked from different points of view. On one hand, we have the positive aspects as if the nice smell of a good meal or a fine glass of wine. On the other hand, we have the things neither consumer nor producers want to see in their products. These are off flavors or even things which might have a negative impact on human health like undesired residues from various sources (e.g.: mineral oil contamination).

Therefore, we try to measure by analytical methods what humans perceive during consumption of food. This task sounds quite simple, but there are molecules, which are perceived by humans at incredible low concentrations. In addition, the matrices are quite complex, so we are facing analytical challenges in terms of sensitivity and selectivity as well.



Why are you interested in this research? What is the goal?

I think I am one of the blessed persons who have the luck to combine job and hobby. I am obsessed about quality of food (however you may define it) and I definitely want to understand why a product is good or not. Finally, you are ending up in analytical chemistry trying to define the quality parameters. Therefore, the final goal for me should be to predict the quality of a product by analytical techniques. Nevertheless, it is very important for me to stress out that we still need humans for the final judgment, but analytical methods can greatly support this goal.

Could you tell us why you chose Shimadzu as your partner when you established this new lab?

I am in the business for over thirty years and I was looking for a cooperation partner ensuring a long-term relation. It is very important to me to have a good mutual trust, so the decision was for Shimadzu fulfilling these requirements for me.

How are our instruments helping you?

Shimadzu offers a wider range of instruments relevant for my work. After all the years I realized, that there is no machine existing which can handle all the different questions we have to answer. However, all the different Shimadzu instruments I am using show an incredible reliability and stability over years, handling hundreds or even thousands of measurements every month.

What are Shimadzu's strengths compared to other vendors (not limited to the instruments)?

I really like the fast response time to a request and the flexibility for single tailor made solutions. Some of my instruments have a unique configuration, which you cannot find somewhere else. In addition, Shimadzu helps me to realize my visions of instrumental set-ups, which are not from the normal assembly line and they are open for sometimes "crazy" discussions.



Finally, could you share any requests that you have with respect to analytical and measuring instrument vendors?

My advice to any instrument manufacturer is to listen to their customers. Every single customer is important!

It was significant to know what you think of us and our collaboration. We will strive to meet your request more than ever. Thank you very much.

Here are his recent publications:

2019

Flecker, T., Schicher, M., Leitner, E., Wagner, F.S. Residual solvent or intrinsically formed during production: analysing volatile compounds in unrefined vegetable oils using headspace gas chromatography coupled with mass spectrometry. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 36(7), pp. 996-100

A., Kopacic, S., Bauer, W., Leitner, E. Characterization of natural polymers as functional barriers for cellulose-based packaging materials Walzl, Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 36(6), pp. 976-988

Engleder, M., Strohmeier, G.A., Weber, H., Steinkellner G., Leitner E., Müller M., Mink D., Schürmann M., Gruber, K., Pichler, H. Evolving the Promiscuity of Elizabethkingia meningoseptica Oleate Hydratase for the Regio- and Stereoselective Hydration of Oleic Acid Derivatives. Angewandte Chemie - International Edition, 58(22), pp. 7480-7484

Kopacic, S., Walzl, A., Hirn, U., (...), Leitner, E., Bauer, W. Application of industrially produced chitosan in the surface treatment of fibre-based material: Effect of drying method and number of coating layers on mechanical and barrier properties Polymers, 10(11),1232

Engleder, M., Müller, M., Kaluzna, I., Mink D., Schürmann M., Leitner E., Pichler, H., Emmerstorfer-Augustin, A. Exploring castellaniella defragrans linalool (De)hydratase-isomerase for enzymatic hydration of alkenes Molecules, 24(11),2092

Velásquez-Hernández, M.D.J., Ricco, R., Carraro, F., Limpoco, F.T., Linares-Moreau, M., Leitner, E., Wiltsche, H., Rattenberger, J.d., Schröttner, H., Frühwirt, P., Stadler, E.M., Gescheidt, G., Amenitsch, H., Doonan, C.J, Falcaro, P. Degradation of ZIF-8 in phosphate buffered saline media. CrystEngComm, 21(31), pp. 4538-4544

2018

Kopacic, S., Walzl, A., Zankel, A., Leitner, E., Bauer, W. Alginate and chitosan as a functional barrier for paper-based packaging materials. Coatings, 8(7),235

Jurek, A., Leitner, E. Analytical determination of bisphenol a (BPA) and bisphenol analogues in paper products by LC-MS/MS. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 35(11), pp. 2256-2269

Determination of mineral oil residues in food Removing natural occurring alkanes



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Introduction

Mineral oil (MO) residues in food raised public concern due to some elevated concentrations up to several thousand milligrams per kilogram food^[1]. Due to the chemical structures two groups of MOs can be differentiated. Mineral oil saturated hydrocarbons (MOSH) consist of linear and branched alkanes, and alkyl-substituted cyclo-alkanes, whilst mineral oil aromatic hydrocarbons (MOAH) include mainly alkyl-substituted polyaromatic hydrocarbons. Technical grades of mineral contain aromatic hydrocarbons in a

concentration range from 15 - 35 %. The determination of MOSH and MOAH in food can be done by an automated LC-GC-FID system (liquid chromatography–gas chromatography–flame ionization detection) for routine analysis. Unfortunately, some food material like rice or chocolate contain natural occurring (odd numbered) alkanes in the range of C_{23} - C_{33} which can interfere and heavily disturb the analysis of the MOSH fraction. These interferences can be removed by flash chromatography on aluminium oxide columns^[2].

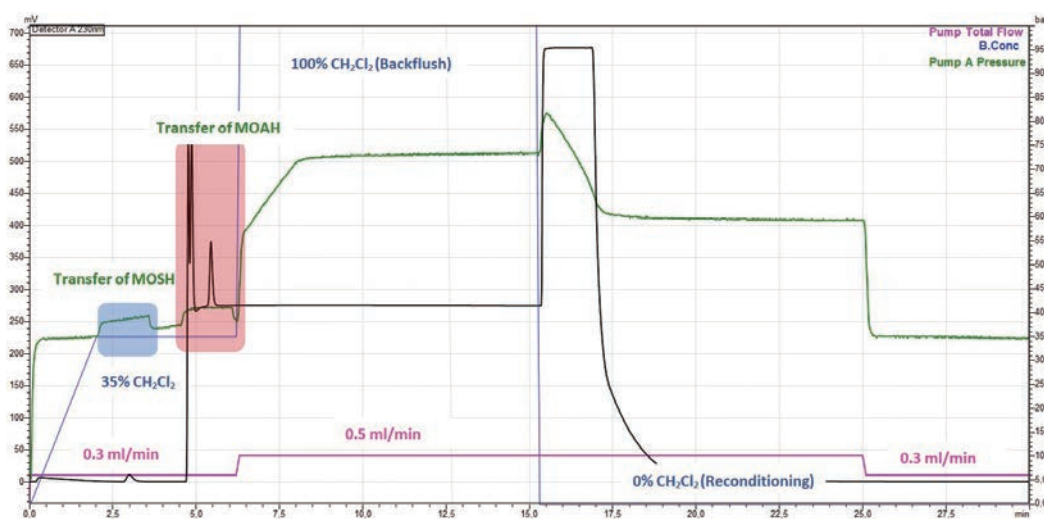


Fig. 1 Typical LC chromatogram (black: UV-signal, green: pump pressure, blue: CH_2Cl_2 concentration, purple: total flow)

Removing natural occurring alkanes with aluminium oxide (Alox)

It has been shown that activated aluminium oxide is feasible to retain long-chain alkanes with a chain length of $>C_{20}$, while iso-alkanes can pass nearly undisturbed. The retention was the biggest when using n-hexane as solvent and was destroyed under the presence of polar substances like water. Therefore polar interferences have to be removed e.g. by covering the alox with silica gel (which is not retaining alkanes).

The retention mechanism is relatively unknown and retention power of the alkanes on the alox is low: only about 1 mg of alkanes is retained by 30 g of alox. Furthermore, only the MOSH fraction elutes from the column, the MOAH is retained. Nevertheless, it can be a useful tool for decreasing of overloaded samples and to lower detection limits^[3,4].

System setup

The LC is directly connected to two high temperature GC columns with retention gaps which are installed in one GC oven. MOSH and MOAH fractions are separated on a silica gel column using a n-hexane / dichloromethane gradient. The interface between LC and GC is controlled by Axel Semrau LC-GC Chronect interface.

After transferring the MOSH fraction on column 1 and MOAH on column 2, the temperature programme is started and both fractions are separated simultaneously and detected by FID. Figure 1 shows a typical LC chromatogram with UV-signal in black, pump pressure in green, CH_2Cl_2 concentration in blue and total flow in purple. Figure 2 shows the LC-GC-FID system^[5].



Fig. 2 LC-GC online system

Sample preparation

Depending on the expected mineral oil concentration, 1 - 10 g of homogenized and finely ground samples were used. The samples were extracted in hexane, after the addition of an internal standard mixture (Restek MOSH / MOAH standard Cat.#:31070 containing 9 internal standards) at room temperature for 2 hours or overnight under occasionally shaking the flask. The glass columns with inserted filters were filled with 10 g of aluminium oxide (90, basic, 0.063 mm - 0.2 mm, activated for 16h at 500 °C) and 3 g of silica gel (60, 60 µm - 200 µm or 70 mesh-230 mesh respectively, activated for 16h at 40 °C). The columns were washed with 20 mL of n-hexane prior to loading the sample. The extracts were centrifuged, if necessary, concentrated to a volume of about 1 mL under a stream of nitrogen.

About 100 µL were taken and filled into an autosampler vial with micro insert for analysis of MOAH, the remaining samples was loaded onto the column. The MOSH fraction was eluted with 20 mL of n-hexane, concentrated in an automated solvent concentrator and transferred into a 2 mL autosampler vial. This was placed in the autosampler rack of the LC-GC system. Aliquots of 50 µL were injected into the LC and 450 µL were transferred directly on the pre-columns for the MOSH fraction only.

Mineral oil in rice – analytical result

Figure 3 shows two chromatograms of a MOSH fraction of a rice sample spiked with 5 mg/kg of a mineral oil. Before flash chromatography with alox n-alkanes in the range of C₂₅ - C₃₅ are present. "False positive" integration would give a MOSH value of 10.8 mg/kg.

After flash chromatography the n-alkanes were completely removed and data evaluation is possible without any interferences. The result for the MOSH value is 4.31 mg/kg this time. The MOAH value that was measured prior to flash chromatography gives a concentration of 0.64 mg/kg and results in a total MOAH recovery of 4.95 mg/kg of the spiked 5 mg/kg.

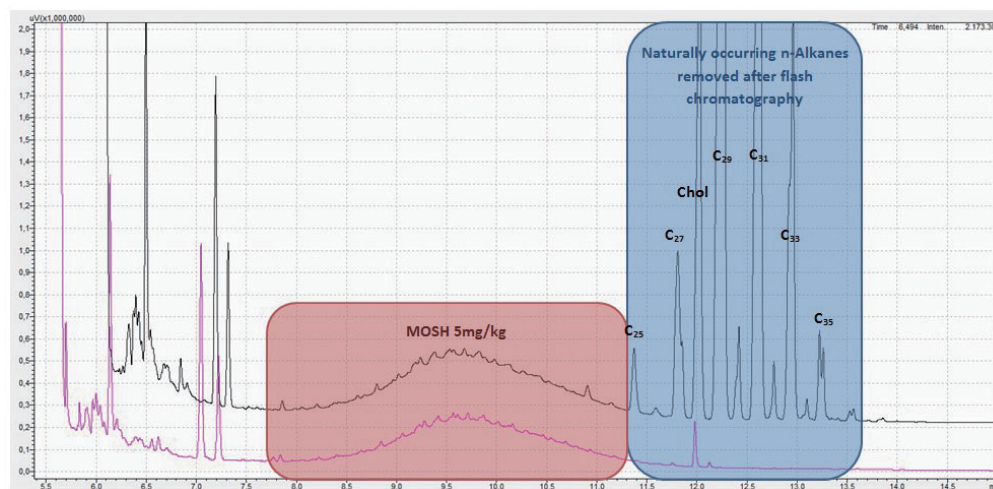


Fig. 3 Chromatograms of rice sample before and after flash chromatography with alox

Conclusion

Flash chromatography with aluminium oxide and silica gel gives an important tool for the removal of naturally occurring odd-numbered n-alkanes and allows an interference free analysis of complex food material such as rice or chocolate.

References

- [1] EFSA Panel on Contaminants in the Food Chain (CONTAM) Scientific Opinion on Mineral Oil Hydrocarbons in Food DOI: 10.2903/j.efsa.2012.2704
- [2] K. Fiselier, D. Fiorini, K. Grob, Activated aluminum oxide selectively retaining long chain n-alkanes. Part I, description of the retention properties, *Analytica Chimica Acta*. 634 (2008) 96-101. doi:10.1016/j.aca.2008.12.007.
- [3] K. Fiselier, D. Fiorini, K. Grob, Activated aluminum oxide selectively retaining long chain n-alkanes: Part II. Integration into an on-line high performance liquid chromatography–liquid chromatography–gas chromatography–flame ionization detection method to remove plant paraffins for the determination of mineral paraffins in foods and environmental samples, *Analytica Chimica Acta*. 634 (2008) 102-109. doi:10.1016/j.aca.2008.12.011.
- [4] Biedermann, M.; Grob, K. On-line coupled high performance liquid chromatography–gas chromatography for the analysis of contamination by mineral oil. Part 2: Migration from paperboard into dry foods: Interpretation of chromatograms. *Journal of Chromatography A* 2011, 1255, 76-99, doi:10.1016/j.chroma.2012.05.096.
- [5] Biedermann, M.; Grob, K. On-line coupled high performance liquid chromatography–gas chromatography for the analysis of contamination by mineral oil. Part 1: Method of analysis. *Journal of Chromatography A* 2011, 1255, 56-75, doi:10.1016/j.chroma.2012.05.095.

Development of a High Sensitivity Analytical Method for Quantitative Analysis of Trace Amounts of D-Amino Acids



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Abstract

When the steric structure of amino acids was devised, it revealed that most amino acids could exist as mirror-image isotopes (enantiomers) that are differentiated by an L/D- notation into L-amino acids (L-form) and D-amino acids (D-form). Almost all amino acids that occur in nature are L-amino acids, and D-amino acid function is an area that has not attracted much interest. More recently, it is starting to appear as if some foods and particularly microorganism-associated fermented foods contain D-amino acids, and the function of these foods is gaining interest. In this context, we have applied analytical technology that was developed by metabolomics to develop a new analytical method for D-amino acids that uses a high-sensitivity triple quadrupole mass spectrometer. In this article, we intend to explain this method and describe an example application of this method in fermented food analysis.

1-1. Current Research into D-Amino Acids

Of the 20 different constituent amino acids in proteins, all apart from glycine have one or two chiral centers consisting of four different functional groups or atoms bound to a single carbon atom, and a D- and L-form enantiomer for each of these chiral centers. By convention, the D/L- notation is commonly used to distinguish between these steric arrangements, where the D-form and L-form of an amino acid are mirror images (enantiomers) of one another with atom arrangements that cannot be superimposed (Fig. 1).

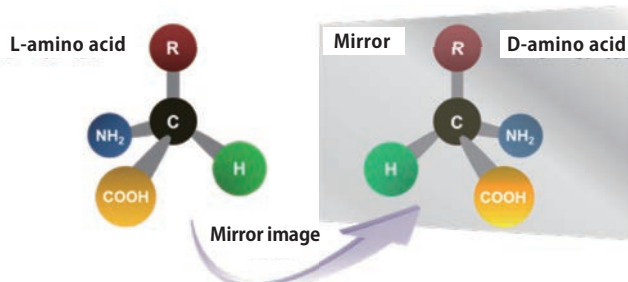


Fig. 1 Amino Acid Enantiomers R represents a side chain.

L-form amino acids account for the vast majority of amino acids in the natural world, and for many years, all living things on Earth were thought to be made up of only L-amino acids. However, from the mid-20th century, when the peptidoglycan cell wall layer of bacteria was reported to contain D-alanine and D-glutamic acid (Stevens et al. 1951), the realization grew that D-amino acids also existed alongside L-amino acids in the natural world. The relationship between D-amino acids and multiple different diseases has been a subject of recent discussion, with D-serine thought to affect the pathophysiology of several neurological diseases: Alzheimer's disease, schizophrenia, and amyotrophic lateral sclerosis (ALS) (Nishikawa 2011; Sasabe et al. 2007). A close relationship between D-serine and renal dysfunction has also been reported, and D-serine is expected to be used as a prognostic predictor in renal disease and as a new biomarker (Sasabe et al. 2014; Kimura et al. 2016).

As shown above, major developments in D-amino acid research have occurred in the field of medicine from a physiological perspective, though more recent D-amino acid research has been more expansive and even includes the field of food research. Reports have already shown the presence of D-amino acids in fruit and vegetable agricultural

produce (Brückner and Westhauser 1994; Gandolfi et al. 1994), in marine products (Abe et al. 2005), and in fermented foods. Reports on the presence of D-amino acids in fermented foods have also covered a wide variety of foods such as wine, beer, sake, yogurt, cheese, vinegar, soy sauce, and Japanese mirin, and microorganism involvement in fermentation and biochemical changes associated with ripening are understood to be part of the reason why various D-amino acids are produced (Friedman 1999; Mutaguchi et al. 2013; Inoue et al. 2016). Food function is broadly divided into a primary nutritional function, a secondary sensory (taste fulfillment) function, and a tertiary health function. The function of D-amino acids in food is often mentioned in terms of properties related to the secondary taste function and the taste qualities of each D-amino acid. The flavor enhancer L-glutamic acid monosodium is a good example of how amino acids play a major role as taste modifiers in food production and preparation, though the flavor and threshold levels of amino acids differ between L- and D-forms (Schiffman et al. 1981). So far, sensory evaluation and taste receptor analysis has demonstrated the L-form of almost all protein-constituent amino acids produces a bitter taste, while the D-form often produces a sweet taste (Kawai et al. 2012; Bassoli et al. 2014). Although only L-form amino acids are used as food additives, there is a move towards using some D-form amino acids as new taste substances, though in accordance with the Japanese Ministry of Health, Labour and Welfare regulations currently only allows the use of the D-form of alanine, threonine, methionine, and tryptophan. Furthermore, permission to use the D-form of these amino acids only extends to their use in a racemic mixture, hence the D-form can only be used as a food additive together with an equal amount of the L-form; this has led to a situation in which the unique taste of D-amino acids cannot be utilized to develop original foods.

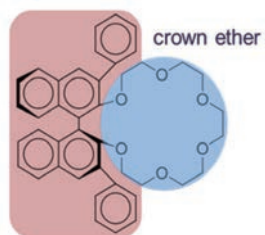
As mentioned above, D-amino acids exhibit unique biochemical functions associated with a variety of aspects of vital phenomena that are not exhibited by L-amino acids and have therefore been the subject of broad-ranging research including both fundamental and applied studies. Novel insights in medicine, food science, and other fields arise largely thanks to recent remarkable advances in analytical techniques, and the development of these techniques is still ongoing. There remains room for wide-ranging improvements in qualitative and quantitative aspects of analysis performance, and we look forward to even greater technological developments in the future.

1-2. Optical Resolution Technology for Amino Acids

Recently, our research laboratory developed a new analytical method for D-amino acid analysis (Konya et al. 2016). This method uses HPLC to separate chiral amino acids and time-of-flight mass spectrometry (TOFMS) for detection. Separation involves using a CROWNPAK CR-1 (+) column containing a crown ether with a binaphthyl skeleton as the chiral stationary phase. At present, the detailed separation mechanism has been unclear, but the presumed mechanism is shown below (Fig. 2). The amino group on a target amino acid is protonated by a very low pH mobile phase used for liquid chromatography (LC). An electrostatic attractive force acts between this protonated amino group and the crown ether of the stationary phase that retains the amino acid. Simultaneously, the crown ether is bound to a binaphthyl with an extremely bulky structure that causes hydrophobic retention but also varying steric hindrance depending on whether the amino acid is a D-form or L-form enantiomer. Electrostatic repulsion acting on the

protonated amino group of amino acids arising in the pores of stationary phase packing particles is also thought to play a role in separation. These interactions with the chiral selector and stationary phase are presumed to enable the separation of underivatized D- and L-amino acids where the selectivity of this separation is anticipated to arise through a mixed mode of reverse phase distribution, hydrophilic interaction, and electrostatic exclusion (Konya et al. 2018).

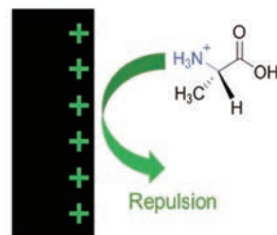
Effect of the Chiral Selector



binaphthyl

Binaphthyl causes hydrophobic retention and steric hindrance. Crown ether causes electrostatic attraction.

Effect of the Stationary Phase



Stationary phase

A significant portion of packing particle pore causes electrostatic repulsion

Fig. 2 Predicted Mechanism of D-Amino Acid Separation Occurring in Analytical Method Developed by Konya et al.

This HPLC- and TOFMS-based analytical method for chiral amino acids (the LC-TOFMS method) has three characteristic features. First, it does not require amino acid derivatization. This avoids a number of issues including unwanted side reactions resulting from derivatization. Second is the high throughput performance of the method. Apart from the secondary amine proline, this analytical method can completely separate all protein-constituent amino acids within 10 minutes. Considering numerous other reported methods of chiral amino acid analysis that require several hours or more the throughput performance provided by this separation deserves the descriptor “epoch-making.” Third is that this analytical method uses a mass spectrometer capable of high mass resolution. TOFMS provides accurate mass analysis that gives this analytical method excellent qualitative performance and the ability to differentiate between target amino acids of very similar molecular mass. Each of these features deserves special mention and marks a clear departure from previous analytical methods for D-amino acids. However, in terms of detection, TOFMS represents a fundamental bottleneck for improving the sensitivity and dynamic range of this method.

In this context, we have developed a new analytical method for D-amino acids that uses Shimadzu’s LCMS-8060 high-sensitivity quadrupole mass spectrometer. The ion-separating part of a quadrupole mass spectrometer contains four parallel metal rods (a quadrupole) to which both a high frequency voltage and direct voltage are applied in combination, thereby allowing only ions of a certain mass range to travel down the quadrupole. Quadrupole analysis is broadly divided into scan mode and SIM mode, where the later mode only detects ions of a certain mass and is used for target compound analysis. The ion-separating part of mass spectrometry / mass spectrometry (MS/MS) consists of two quadrupoles with a collision chamber located between the quadrupoles. The multiple reaction monitoring (MRM) mode, which is frequently used for MS/MS analysis, runs both quadrupoles in SIM mode and allows ions to be detected with high selectivity while simultaneously reducing the effect of background noise from contaminating ions, thereby allowing signals to be detected with relatively high sensitivity. As mentioned earlier, MS/MS also has a broad dynamic range and is therefore well-suited to comprehensive analysis of target compounds of substantially different concentrations and abundance ratios.

In constructing this analytical technology, we first fine-tuned MS/MS parameters to develop a method of analyzing trace amounts of D-amino acids with high sensitivity. We then compared the performance of this method with an existing method and evaluated the results. The findings of this investigation are presented below.

1-3. Development of a High-Sensitivity Analytical Method for Quantitative Analysis of Trace Amounts of D-Amino Acids

In using LC-MS/MS to develop a high-sensitivity quantitative analytical method for trace amounts of D-amino acids, we separated development into a stepwise investigation of the following three items: 1. analytical method development, 2. evaluation of the newly developed analytical method, and 3. method application in sample analysis. An overview of each of these three items is given below.

1. Analytical Method Development

For chiral amino acid separation, we attempted to apply the separation used in the LC-TOFMS method previously reported by Konya et al. (Konya et al. 2016). For detection, to achieve maximum sensitivity, we conducted a detailed investigation of each parameter affecting sensitivity. The structure of the MS/MS system used in this study is shown in the schematic drawing below (Fig. 3). After separation by LC, compounds are first ionized at the ion source (protonation reaction), then focused by a system of lenses, and enter the mass separation section before finally reaching the detector. Our first investigation determined the m/z of ions that travel through the two quadrupoles and voltage parameters, which effectively optimized the MRM transitions for MS/MS. Next, after adjusting the position of the ion source probe, we optimized the gas flowrate parameters. Multiple different gases are used to encourage ionization in the interface part of the ion source. We also optimized the voltage and heating parameters of the interface section.

2. Evaluation of the Newly Developed Analytical Method

The measurement range, linearity, repeatability, limit of detection, and lower limit of quantitation of the newly developed analytical method were evaluated using a serial dilution of an amino acid standard preparation. The linear range was evaluated by diluting the standard preparation across a concentration range of 0.005 to 100 nmol/mL, performing a linear regression analysis of the area obtained at each concentration, and calculating the correlation coefficient. Repeatability was evaluated based on the relative standard deviation of detection peak areas obtained by repeating analysis $N = 3$ times. Limits of detection were calculated based on the signal-to-noise ratio of the mass spectrometer.

3. Method Application in Sample Analysis

This part of development involved verifying whether the analytical method we developed could be used not only to analyze a standard solution but also to analyze samples containing contaminating materials. Black vinegar has been analyzed in a previous report of D-amino acid detection and was chosen as the sample.

We analyzed the same sample with both previous LC-TOFMS method and our newly developed analytical method and compared the results.

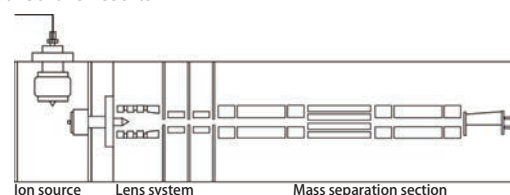


Fig. 3 Schematic Drawing Showing the Structure of the Triple Quadrupole Mass Spectrometer

1-3-1. Analytical Method Development

In developing our high-sensitivity LC/MS-MS analytical method for D-amino acids, we first optimized MRM transitions for target amino acids then verified conditions for chiral amino acid separation. LC separation conditions were investigated based on the conditions used in the already-reported LC-TOFMS method (Konya et al. 2016). As described in detail earlier, the LC-TOFMS method uses a column (CROWNPAK CR-I (+)) with a chiral selector consisting of a crown ether bound to a binaphthyl skeleton. Interaction between the stationary phase crown ether and the target amino acid and also steric hindrance caused by the binaphthyl skeleton are presumed to have a substantial effect on optical resolution of D- and L-amino acids (Fig. 4). The LC-TOFMS method achieves optical resolution of almost all target D- and L-amino acids with very high resolving power (Fig. 4). In contrast with the LC-TOFMS method, we found the MS/MS of the newly developed method failed to differentiate between L-glutamine and D-lysine and recognized them as the same compound. This failure was attributed to the amino acids being eluted at similar retention times and having the same MRM transitions. Because TOFMS can obtain accurate mass data on compounds, the LC-TOFMS method can achieve separation by MS even when LC has difficulty achieving separation. By contrast, the mass resolving ability of MS/MS used in

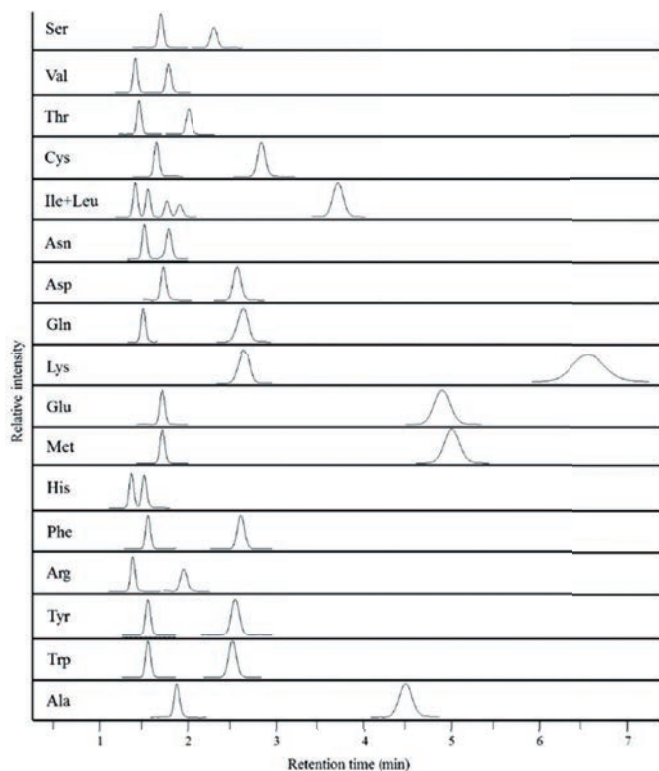


Fig. 4 Chromatogram of 18 Protein-Constituent Amino Acids

All compounds in the 1 nmol/mL D-/L-amino acid standard solution were eluted within 8 minutes. Analysis with the CROWNPAK CR-I (+) column eluted the D-form of each amino acid before its L-form. The D- and L-forms of isoleucine and leucine were eluted in the order of D-isoleucine + D-allo-isoleucine (coelution), D-leucine, L-allo-isoleucine, L-isoleucine, and L-leucine.

the newly developed method cannot discern a difference between the mass of L-glutamine (monoisotopic mass of 146.069) and D-lysine (monoisotopic mass of 146.106). To resolve this problem, we investigated using the CROWNPAK CR-I (-) column with a structure complementary to the CROWNPAK CR-I (+). The binaphthyls used in CROWNPAK CR-I (+) and CROWNPAK CR-I (-) are axis chiral enantiomers (CR-I (+): (S)-18-crown-6-ether, CR-I (-): (R)-18-crown-6-ether) (Fig. 5). Although we await detailed elucidation of the mechanism of separation, D- and L-amino acids are eluted in opposite orders by the two columns. That is, using the CR-I (+) column the new method elutes respective D-amino acids first, and using the CR-I (-) column the new method elutes respective L-amino acids first. Based on the above, using CR-I (-) in addition to CR-I (+) allowed the newly developed method to recognize L-glutamine and D-lysine as single peaks rather than a coeluted peak (Fig. 6). Similarly, using both columns allowed D-isoleucine and D-allo-isoleucine to be recognized as single peaks rather than a coeluted peak. Also, by monitoring for peaks only 0.75 minutes either side of the predicted retention time, we reduced the number of MRM transitions that needed to be collected per analysis and were able to configure analysis to achieve even better repeatability.

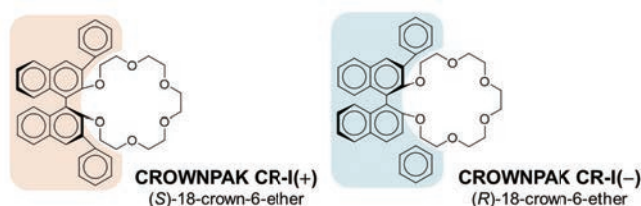


Fig. 5 Structure of the Chiral Selectors in CROWNPAK CR-I (+) and CROWNPAK CR-I (-)

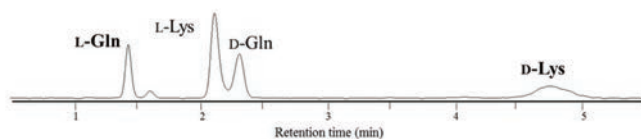


Fig. 6 Extracted Ion Chromatogram of Glutamine and Lysine Obtained Using CROWNPAK CR-I (-)

Next, we adjusted the position of the electrospray probe at the ion source. Ionization efficiency and mobile phase desolvation during target component ionization are largely dependent on probe position, hence probe position is considered to have a bearing on sensitivity improvements and a recent report has even investigated sensitivity and positional parameters for the ion source in detail (Janusson et al. 2015). The sensitivity of the newly developed method improved dramatically when the probe was moved from its default position further away from the area where ions enter the mass spectrometer. This improvement was particularly apparent for glycine and alanine, which are relatively low-molecular-weight amino acids. Lastly, we optimized MS interface gas parameters (nebulizer gas, heating gas, and drying gas), heating parameters (desolvation line temperature and heat block temperature), and interface voltage. We consider the two major features of Shimadzu's LCMS-8060 are that it allows this type of detailed investigation of MS parameters and that its user interface is easy to use.

1-3-2. Evaluation of the Newly Developed Analytical Method

The performance of the newly developed method of chiral amino acid analysis using the optimized LC-MS/MS (the LC-MS/MS method) was evaluated by examining measurement range, linearity, repeatability, limit of detection, and lower limit of quantitation (Table 1). This evaluation involved using the newly developed method to analyze a serial dilution of an amino acid standard solution (0.005, 0.01, 0.05, 0.5, 1, 5, 50, and 100 nmol/mL), dividing the area data for each amino acid by the peak area of an internal standard, and correcting for injection errors, etc. that can arise during analysis. In this study, each validated item was evaluated based on a variety of criteria including validation guidelines for analytical method development established by the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). The measurement range was determined by considering the accuracy and trueness of analytical values at either extremity and taking the maximum linear range for D-methionine, D-aspartic acid, D-glutamine, and D-glutamic acid (0.005–100 nmol/mL). This produced a high correlation coefficient of 0.99 or above for the respective measurement ranges of each target amino acid and demonstrated the LC-MS/MS method is capable of quantitative analysis of D-amino acids across a wide dynamic range. Repeatability was evaluated based on the relative standard deviation (RSD) of the area of each peak obtained after analyzing an amino acid standard solution three times in succession. A mean RSD of 8.1% was obtained for all target amino acids at 0.05 nmol/mL other from D-alanine, and for D-alanine at 0.5 nmol/mL the RSD was 15.9%. An RSD of not more than 20% for any peak area showed the LC-MS/MS method achieves good repeatability. Limits of detection were taken as the target compound concentration where the detected peak (signal intensity) was three (or more) times the noise, and limits of quantitation were taken as the target compound concentration where the detected peak (signal intensity) was 10 (or more) times the noise. Noise was calculated from the standard deviation of signals at measurement points in sections before and after target compound detection. Limits of detection differed by amino acid in the concentration range 0.005–0.5 nmol/mL and D-amino acids were detectable on the order of several pmol, showing that this method gives favorable results when used for the analysis of trace amounts of target compounds in samples.

Table 1 Performance Evaluation of the New Method

	Range (nmol/mL)	r	RSD (%)	LOD (nmol/mL)	LOQ (nmol/mL)
D-alanine	0.5-100	0.9979	15.9	0.01	0.05
D-arginine	0.005-50	0.9997	4.7	0.005	0.005
D-asparagine	0.01-50	0.9983	0.7	0.005	0.01
D-aspartic acid	0.01-100	0.9934	18.2	0.005	0.01
D-cysteine	0.01-100	0.9982	7.1	0.005	0.005
D-glutamine	0.005-50	0.9998	10.3	0.005	0.005
D-glutamic acid	0.005-50	1.0000	7.3	0.005	0.005
D-histidine	0.005-50	0.9997	9.0	0.005	0.005
D-isoleucine	0.01-50	0.9998	5.8	0.005	0.005
D-leucine	0.01-50	0.9996	11.5	0.005	0.01
D-lysine	0.01-100	0.9994	5.8	0.005	0.01
D-methionine	0.005-100	0.9975	6.6	0.005	0.005
D-phenylalanine	0.05-50	0.9996	18.7	0.005	0.05
D-serine	0.05-100	0.9952	12.5	0.005	0.5
D-threonine	0.05-100	0.9970	11.7	0.005	0.01
D-tryptophan	0.005-50	0.9993	2.9	0.005	0.005
D-tyrosine	0.005-50	0.9987	4.3	0.005	0.05
D-valine	0.01-50	0.9999	2.1	0.005	0.01

r: correlation coefficient, RSD: relative standard deviation, LOD: limit of detection, LOQ: limit of quantitation

1-3-3. Method Application in Sample Analysis

To evaluate the usefulness of the LC-MS/MS method for chiral amino acid analysis, the method was used for quantitative analysis of D-amino acids in food. Three different types of commercially available black vinegar were used as samples. Reports have revealed that vinegar contains several different D-amino acids (Tani et al. 2009; Miyoshi et al. 2014), and a number of different bacteria including lactic acid bacteria are reported to play a major role in D-amino acid formation in vinegar (Mutaguchi et al. 2013). First, the three different black vinegars underwent pretreatment. The pretreatment method reported by Konya et al. (2017), which has been thoroughly examined for recovery rates and effect on LC-MS analysis, was used for this D-amino acid analysis. Quantitative analysis of D-amino acids was performed using a calibration curve created from peak areas obtained by analyzing a serial dilution of an amino acid standard solution. The serial dilutions used in quantitative analysis underwent the same pretreatment as vinegar samples and were corrected for extraction errors. All vials intended for analysis also contained a fixed concentration of DL-alanine-2,3,3,3-d4 internal standard, and each data point was corrected based on the measured result for D-alanine-2,3,3,3-d4 to account for injection and ionization errors. Target D-amino acids were analyzed simultaneously using CROWNPAK CR-I (+), and CROWNPAK CR-I (-) was used to detect D-lysine and to identify the peaks of D-isoleucine and D-allo-isoleucine.

The LC-MS/MS method detected and quantified 14 D-amino acids in the vinegar samples (D-alanine, D-serine, D-valine, D-threonine, D-allo-isoleucine, D-leucine, D-asparagine, D-glutamic acid, D-methionine, D-histidine, D-phenylalanine, D-arginine, D-tyrosine, and D-lysine). Similar D-amino acids were detected in all three vinegars, though D-amino acid concentrations differed in each vinegar. Next, the same samples were analyzed again within 24 hours by the LC-TOFMS method. To allow a comparison of results, the separation conditions used in LC and other analytical conditions were consistent across both the LC-TOFMS method and the LC-MS/MS method. The LC-TOFMS method detected and quantified 13 D-amino acids (D-alanine, D-serine, D-valine, D-threonine, D-allo-isoleucine, D-leucine, D-asparagine, D-glutamic acid, D-methionine, D-histidine, D-phenylalanine, D-arginine, and D-tyrosine) in all three vinegars. The quantitative D-amino acid results obtained by the LC-MS/MS method and LC-TOFMS method were very similar (Fig. 7). This indicates the LC-MS/MS method can be used as an alternative to the existing LC-TOFMS method. Furthermore, the LC-TOFMS method did not detect D-lysine in any vinegar or D-valine in vinegar C, but the LC-MS/MS method both detected and quantified D-lysine in all vinegars and D-valine in vinegar C. The above findings demonstrate the LC-MS/MS method is superior to the LC-TOFMS method in terms of quantitation and could be a powerful method for quantitative analysis of trace D-amino acids in samples.

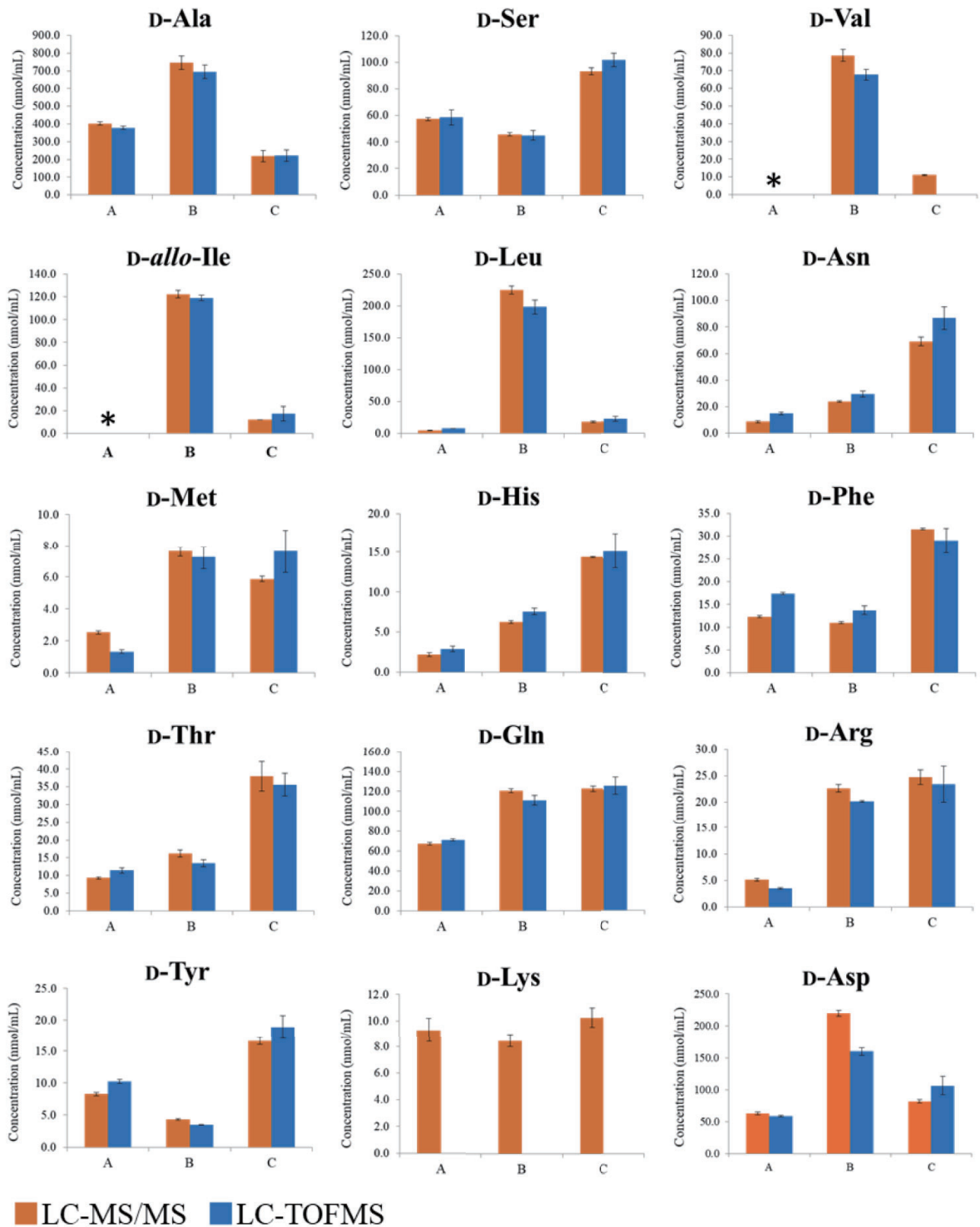


Fig. 7 Quantitative Results for D-Amino Acids in Black Vinegars Measured by LC-TOFMS and LC-MS/MS

The measured concentration of each D-amino acid in vinegars A, B, and C is shown in orange for the LC-MS/MS method and in blue for the LC-TOFMS method (N = 3). The asterisks (*) show when a peak was detected but the concentration was below the limit of quantitation.

1-4. Conclusion

In this study, we developed an LC-MS/MS analytical method for high-sensitivity quantitative analysis of trace amounts of D-amino acids.

First, the MRM transitions of MS/MS were optimized, then various parameters were investigated in detail including interface probe position, temperature, gas flowrate, and voltage, then the sensitivity of target amino acid detection was improved. We encountered an issue during separation in which some amino acids were coeluted preventing recognition of their individual peaks, but using a column with a complementary structure alongside the original column allowed analysis of all target amino acids. Next, we verified the measurement range, linearity, repeatability, limit of detection, and lower limit of quantitation of the newly developed method, and evaluated its performance in quantitative analysis of trace amounts of D-amino acids. We also performed quantitative analysis of a sample (black vinegar) for D-amino acids using the newly developed method. Comparing results with an existing LC-TOFMS method revealed a very similar D-amino acid profile, and also revealed that some D-amino acids were only quantified by the LC-MS/MS method, demonstrating a significant advantage of this method in terms of detection sensitivity.

Although a number of past reports have used LC-MS/MS to analyze trace amounts of D-amino acids, a distinguishing feature of the LC-MS/MS method we describe is that it requires no derivatization and is capable of high-throughput and high-sensitivity simultaneous analysis of chiral amino acids. The ability to process many samples in a short period of time represents a major advantage for this analytical method.

References

- Abe, H., Yoshikawa, N., Sarower, M.G., Okada, S.: Physiological Function and Metabolism of Free D-Alanine in Aquatic Animals. *Biol. Pharm. Bull.*, 28, 1571–1577 Available at: <http://joi.jlc.jst.go.jp/JST.JSTAGE/bpb/28.1571?from=CrossRef> [Accessed September 28, 2018].(2005).
- Bassoli, A., Borgonovo, G., Caremoli, F., Mancuso, G.: The taste of D- and L-amino acids: In vitro binding assays with cloned human bitter (TAS2Rs) and sweet (TAS1R2/TAS1R3) receptors. *Food Chem.*, 150, 27–33 Available at: <https://www.sciencedirect.com/science/article/pii/S0308814613015550?via%3DIuhub> [Accessed September 28, 2018].(2014).
- Brückner, H., Westhauser, T.: Chromatographic determination of D-amino acids as native constituents of vegetables and fruits. *Chromatographia*, 39, 419–426 Available at: <http://link.springer.com/10.1007/BF02278756> [Accessed December 11, 2017].(1994).
- Friedman, M.: Chemistry, Nutrition, and Microbiology of d-Amino Acids. Available at: <https://pubs.acs.org/doi/full/10.1021/jf990080u> [Accessed September 28, 2018].(1999).
- Gandolfi, I., Palla, G., Marchelli, R., Dossena, A., Puelli, S., Salvadori, C.: D-alanine in Fruit Juices: A Molecular Marker of Bacterial Activity, Heat Treatments and Shelf-life. *J. Food Sci.*, 59, 152–154 Available at: <http://doi.wiley.com/10.1111/j.1365-2621.1994.tb06921.x> [Accessed September 28, 2018].(1994).
- Inoue, Y., Katsumata, T., Watanabe, H., Hayase, F.: Mechanisms of d-amino acid formation during maturation of sweet rice wine (mirin). *Food Sci. Technol. Res.*, 22, 679–686 Available at: https://www.jstage.jst.go.jp/article/fstr/22/5/22_679/_article [Accessed September 28, 2018].(2016).
- Janusson, E., Hesketh, A. V., Bamford, K.L., Hatlelid, K., Higgins, R., McIndoe, J.S.: Spatial effects on electrospray ionization response. *Int. J. Mass Spectrom.*, 388, 1–8 Available at: <http://www.sciencedirect.com/science/article/pii/S1387380615002195> [Accessed May 18, 2017].(2015).
- Kawai, M., Sekine-Hayakawa, Y., Okiyama, A., Ninomiya, Y.: Gustatory sensation of l- and d-amino acids in humans. *Amino Acids*, 43, 2349–2358 Available at: <http://link.springer.com/10.1007/s00726-012-1315-x> [Accessed September 28, 2018].(2012).
- Kimura, T., Hamase, K., Miyoshi, Y., Yamamoto, R., Yasuda, K., Mita, M., Rakugi, H., Hayashi, T., Isaka, Y.: Chiral amino acid metabolomics for novel biomarker screening in the prognosis of chronic kidney disease. *Sci. Rep.*, 6, 26137 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/27188851> [Accessed November 28, 2016].(2016).
- Konya, Y., Bamba, T., Fukusaki, E.: Extra-facile chiral separation of amino acid enantiomers by LC-TOFMS analysis. *J. Biosci. Bioeng.*, 121, 349–353 Available at: <https://www.sciencedirect.com/science/article/pii/S1389172315002662> [Accessed October 9, 2018].(2016).
- Konya, Y., Taniguchi, M., Fukusaki, E.: Novel high-throughput and widely-targeted liquid chromatography–time-of-flight mass spectrometry method for d-amino acids in foods. *J. Biosci. Bioeng.*, 123, 126–133 (2017).
- Konya, Y., Taniguchi, M., Furuno, M., Nakano, Y., Tanaka, N., Fukusaki, E.: Mechanistic study on the high-selectivity enantioseparation of amino acids using a chiral crown ether-bonded stationary phase and acidic, highly organic mobile phase by liquid chromatography/time-of-flight mass spectrometry. *J. Chromatogr. A*, 1578, 35–44 Available at: <https://www.sciencedirect.com/science/article/pii/S0021967318312494> [Accessed October 29, 2018].(2018).
- Miyoshi, Y., Nagano, M., Ishigo, S., Ito, Y., Hashiguchi, K., Hishida, N., Mita, M., Lindner, W., Hamase, K.: Chiral amino acid analysis of Japanese traditional Kurozu and the developmental changes during earthenware jar fermentation processes. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, 966, 187–92 Available at: <http://www.sciencedirect.com/science/article/pii/S1570023214000464> [Accessed February 19, 2016].(2014).
- Mutaguchi, Y., Ohmori, T., Akano, H., Doi, K., Ohshima, T.: Distribution of D-amino acids in vinegars and involvement of lactic acid bacteria in the production of D-amino acids. *Springerplus*, 2, 691 Available at: <http://springerplus.springeropen.com/articles/10.1186/2193-1801-2-691> [Accessed February 23, 2016].(2013).
- Nishikawa, T.: Analysis of free D-serine in mammals and its biological relevance. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, 879, 3169–3183 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21992750> [Accessed January 9, 2016].(2011).
- Sasabe, J., Chiba, T., Yamada, M., Okamoto, K., Nishimoto, I., Matsuoka, M., Aiso, S.: D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. *EMBO J.*, 26, 4149–59 Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2230675&to=lmcentrez&rendertype=abstract> [Accessed December 4, 2015].(2007).
- Sasabe, J., Suzuki, M., Miyoshi, Y., Tojo, Y., Okamura, C., Ito, S., Konno, R., Mita, M., Hamase, K., Aiso, S.: Ischemic Acute Kidney Injury Perturbs Homeostasis of Serine Enantiomers in the Body Fluid in Mice: Early Detection of Renal Dysfunction Using the Ratio of Serine Enantiomers L. R. James, ed. *PLoS One*, 9, e86504 Available at: <http://dx.plos.org/10.1371/journal.pone.0086504> [Accessed September 26, 2018].(2014).
- Schiffman, S.S., Sennewald, K., Gagnon, J.: Comparison of taste qualities and thresholds of D- and L-amino acids. *Physiol. Behav.*, 27, 51–59 Available at: <http://www.sciencedirect.com/science/article/pii/0031938481902985> [Accessed December 11, 2017].(1981).
- Stevens, C.M., Halpern, P.E., Gigger, R.P.: Occurrence of D-amino acids in some natural materials. *J. Biol. Chem.*, 190, 705–10 Available at: <http://www.ncbi.nlm.nih.gov/pubmed/14841221> [Accessed September 26, 2018].(1951).
- Tani, Y., Itoyama, Y., Nishi, K., Wada, C., Shoda, Y., Satomura, T., Sakuraba, H., Ohshima, T., Hhayashi, Y., YABUTANI, T., MOTONAKA, J.: An amperometric D-amino acid biosensor prepared with a thermostable D-proline dehydrogenase and a carbon nanotube-ionic liquid gel. *Anal. Sci.*, 25, 919–923 Available at: <http://joi.jlc.jst.go.jp/JST.JSTAGE/analsci/25.919?from=CrossRef> [Accessed October 22, 2018].(2009).

Determination of garlic phenolic compounds using supercritical fluid extraction coupled to supercritical fluid chromatography/tandem mass spectrometry



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Introduction

Garlic (*Allium sativum* L.) has been widely cultivated and consumed throughout the world as an important vegetable [1,2]. The physiological functions of garlic are likely due to its bioactive composition, such as organosulfur compounds, vitamins, saponins, alkaloids and flavonoids [3,4]. Since phenolic compounds have significant bioactivity and play vital roles in food, a simple, accurate, and green environmental technique for analyzing phenolic compounds is needed. Conventional sample preparation, such as traditional solvents, ultrasound, microwaves, ultrahigh pressure and enzymes have all been applied to extract of phenolic compounds from plants or foods [5,6], despite the complex composition and unstable structure of phenolic compounds. Recently, supercritical fluid extraction (SFE) has been widely applied in the extraction or separation of bioactive components, due to the powerful physicochemical properties of supercritical carbon dioxide (SC-CO₂), which is the most commonly employed supercritical fluid [7]. SC-CO₂ can also be employed as the mobile phase for chromatographic analysis, which is known as supercritical fluid chromatography (SFC). SFC demonstrates an outstanding isolation capacity for compounds with a wide range of polarities when using a mixture of SC-CO₂ and organic solvents as the mobile phase [8]. In this study, an off-line SFE-SFC-MS/MS method has been established for the analysis of phenolic compounds in garlic using a Shimadzu Nexera-UC system (Shimadzu, Japan); the extraction of phenolic compounds was first performed using an automatic SFE process, and then the extracted component was analyzed by SFC-MS/MS. Finally, the method was validated and employed for the measurement of the phenolic compounds present in garlic.

Optimization of SFC-MS/MS condition

SFC columns with different stationary phases show differences in both selectivity and polarity. Thus, four SFC columns with the same dimensions and particle sizes (4.6 mm I.D. x 150 mm L., 3 μm), (a) Shim-pack UC-X Sil; (b) Shim-pack UC-X RP; (c) Shim-pack UC-X NH₂; (d) Shim-pack UC-X Diol were compared in terms of their separation efficacy of the target phenolic compounds (Fig. 1). All of the target compounds had nearly no retention in the Sil column and eluted completely before 0.5 min, perhaps because the Sil column comprised high-pure silica gel without a bonded phase. Silanol groups on the surface of the silica gel are mainly weakly acidic, which could have strong retention for basic compounds, but weak retention for acidic compounds [9]. The results in Fig. 1 show the poor resolution of the target compounds using the RP column, with the major compounds eluting in 1–4 min, indicating that the interactions between the stationary phase in the RP column and the phenolic compounds were not strong enough to produce a satisfactory effect. In contrast, all of the retention behaviors of the phenolic compounds in the NH₂ column were probably due to the strong interactions between the hydroxyl groups in the phenolic compounds and the amino-propyl groups of the NH₂ column. Compounds 8, 9, and 11 did not elute from the NH₂ column within 12 min; compound 7 exhibited a broad and poor peak shape; and compounds 1, 2, 3, 4, 5 emerged with tailing peaks. It is clear that the Diol column presented the best resolution for the 11

phenolic compounds, with all of the compounds exhibiting satisfactory separation effects, gradient times, and perfect peak shapes. The diol group bonded in the Diol column provides a polar and neutral character to the column and offers appropriate interaction with the hydroxyl group in the phenolic compounds. The elution order observed was (1) ferulic acid, (2) p-coumaric acid, (3) naringenin, (4) apigenin, (5) protocatechuic acid, (7) isorhamnetin, (8) luteolin, (9) phthalic acid, and (11) quercetin. The Shim-pack UC-X Diol column was selected as the most suitable chromatographic column for the separation of the phenolic compounds.

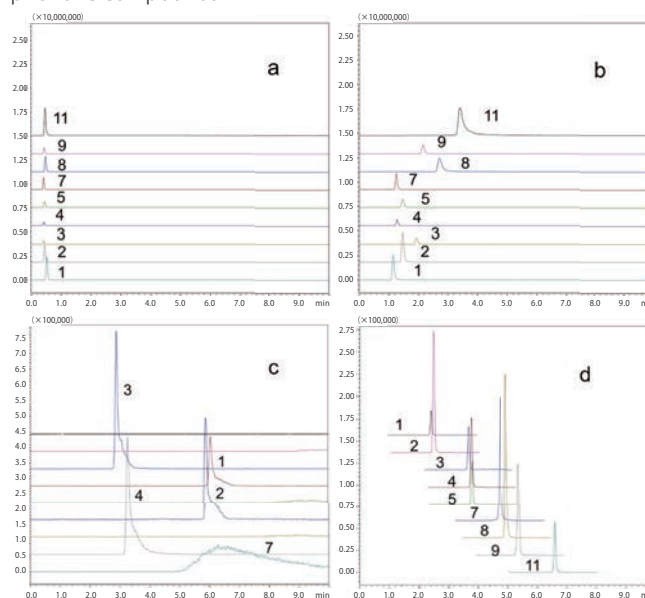


Fig. 1 Comparison of separation effects among four different columns.

Optimization of off-line SFE conditions

The extraction time, temperature, and volume ratio of methanol in the solvent were determined to be the most important extraction factors and were investigated based on the total peak areas of 11 target compounds. The results of the 20 experiments performed under different conditions were used to fit a quadratic regression model. More prominent variations in peak areas were observed along with the temperature and volume ratio of methanol in the solvent over time. An increasing trend of the peak area was exhibited when the temperature changed from 30°C to 50°C. Increasing the temperature of the off-line SFE leads to a decrease in the solvent density and the increase of the vapor pressure, and the former causes a lower solubility and the latter causes an opposing effect; thus, a balance point should be determined between the density and the vapor pressure of the extraction solvent [10]. In this work, the enhancement of the peak area followed by the increasing temperature was possibly attributed to the effect of the

vapor pressure, which was in agreement with the report by Bimakr et al^[1]. On the other hand, the increase in temperature could also induce faster molecular desorption and diffusion and contribute to the extraction efficiency. In summary, the optimization results indicated that extracting the 15 phenolic compounds at 50°C in 9 min with the addition of 30% methanol was the best set of off-line SFE conditions. In addition, subcritical phase could be eventually reached for SFE with these optimized factors, it is necessary to extract polar compounds in samples, especially for phenolic compounds.

Method validation

Sample pretreatment of offline SFE is shown in Fig 2. The pretreatment process is rather simple. The developed off-line SFE-SFC-MS/MS method for phenolic compounds was validated. Each standard solution of the phenolic compounds with the concentrations of 5, 10, 20, 50, 100, 200, 500 and 1000 ng/mL was mixed with 0.5 g garlic and extracted by SFE. A total of 11 phenolic compounds was screened, presented acceptable linearity ($R^2 > 0.99$) over the concentration range of 0.02–8 µg/g. The LODs of the 11 phenolic compounds varied from 0.6 to 12 ng/g, and the scope of the LOQs ranged from 2 to 40 ng/g.

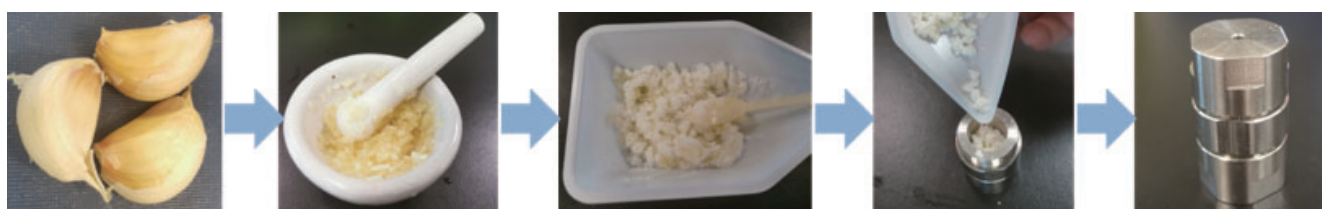


Fig. 2 Sample pretreatment of offline SFE

Conclusions

In this study, a rapid, simple and environmentally friendly off-line SFE-SFC-MS/MS method has been developed for the analysis of phenolic compounds in garlic. After the careful optimization of the SFE parameters, phenolic compounds were successfully extracted in less than 13 min. Besides, a matrix matched standard was employed for removing matrix effects and phenolic compounds showed acceptable selectivity, linearity, sensitivity, recovery and precision by the validation data. The constructed method achieved its practical application through the analysis of phenolic compounds in garlic from different origins in China. In general, this is the first specific study of the SFE extraction of phenolic compounds from garlic followed by SFC-MS/MS, which can be considered a potential method for the analysis of the phenolic compounds in garlic and may be useful in expanding the application of the Shimadzu Nexera-UC system in other fields, such as plant samples, animal samples and other food samples.

References

- [1] B. Bremer, K. Bremer, M. Chase, Angiosperm phylogeny group, an update of the Angiosperm phylogeny group classification for the orders and families of flowering plants: APG III, *Bot. J. Linn. Soc.* 161 (2009) 105–121.
- [2] W.H.H. Gunther, Garlic and other alliums à the lore and the science, *Sulfur Rep.* 34 (2010) 208.
- [3] J.S. Yang, L.F. Kok, Y.H. Lin, T.C. Kuo, J.L. Yang, C.C. Lin, G.W. Chen, W.W. Huang, H.C. Ho, J.G. Chung, Diallyl disulfide inhibits WEHI-3 leukemia cells in vivo, *Anticancer Res.* 26 (2006) 219–225.
- [4] A. Borkowska, N. Knap, Antosiewicz J, Diallyl trisulfide is more cytotoxic to prostate cancer cells PC-3 than to noncancerous epithelial cell line PNT1A: a possible role of p66Shc signaling axis, *Nutr. Cancer* 65 (2013) 711–717.
- [5] D.P. Makris, J.T. Rossiter, An investigation on structural aspects influencing product formation in enzymic and chemical oxidation of

Garlic samples were spiked with mixed phenolic compound standards at 0.8 µg/g, 1 µg/g and 2 µg/g, which were used to assess the accuracy and precision of this method. The recoveries at the 3 spiked concentrations were 63–131.5%, 71.8–109.5% and 64.1–110.4%, respectively. Except ferulic acid and quercetin, which were spiked at the level of 0.8 µg/g, the accuracies of most of the target analytes were acceptable for the general analytical method. Meanwhile, most RSD values for the evaluation of precision (repeatability of the recovery) were lower than 10%.

Real sample analysis

Finally, our established method was used to measure 11 phenolic compounds in garlic from the four main producing areas of China. The detection results indicate that ferulic acid was detected in all four origins (Cangshan, Jinxiang, Pizhou, and Dali) at relatively high levels, from 0.47 to 0.86 µg/g, while different types of phenolic compounds were found in samples collected from the different locations. All of these results suggest that this off-line SFE-SFC-MS/MS method could be adapted for the qualitative and quantitative analysis of the phenolic compounds in garlic.

- quercetin and related flavonols, *Food Chem.* 77 (2002) 177–185.
- [6] C.L. Silva, N. Haesenb, J.S. Câmara, A new and improved strategy combining a dispersive-solid phase extraction-based multiclass method with ultrahigh pressure liquid chromatography for analysis of lowmolecular weight polyphenols in vegetables, *J. Chromatogr. A* 1260 (2012) 154–163.
- [7] Y. Huang, T. Zhang, H. Zhou, Y. Feng, C. Fan, W. Chen, J. Crommen, Z. Jiang, Fast separation of triterpenoid saponins using supercritical fluid chromatography coupled with single quadrupole mass spectrometry, *J. Pharm. Biomed. Anal.* 121 (2016) 22–29.
- [8] A. Chafer, T. Fornari, A. Berna, R.R. Stateva, Solubility of quercetin in supercritical CO₂+ ethanol as a modifier: measurements and thermodynamic modeling, *J. Supercrit. Fluids* 32 (2004) 89–96.
- [9] J. Nawrocki, The silanol group and its role in liquid chromatography, *J. Chromatogr. A* 779 (1997) 29–71.
- [10] M.M.R.D. Melo, A.J.D. Silvestre, C.M. Silva, Supercritical fluid extraction of vegetable matrices: Applications, trends and future perspectives of a convincing green technology, *J. Supercrit. Fluids* 92 (2014) 115–176.
- [11] M. Bimakr, R.A. Rahman, F.S. Taip, A. Ganjloo, L.M. Salleh, J. Selamat, A. Hamid, I.S.M. Zaidul, Comparison of different extraction methods for the extraction of major bioactive flavonoid compounds from spearmint (*Mentha spicata* L.) leaves, *Food. Bioprod. Process.* 89 (2011) 67–72.

Shimadzu Selection

These articles were selected by Shimadzu. Relating food analysis and development, they are from posters presented at ASMS 2019 and from application notes. They feature a variety of instruments we produce and include cutting-edge technologies. Please obtain the articles of your interest through the links on the titles.



Selection 1 Food QAQC

Classification and Visualization of Beer Quality Using GC-MS and GC-FID

In this poster, by metabolomics profiling using GC-MS and GC-FID, we introduce a new approach towards classification and visualization of beer quality to identify how specific components influence taste from plant to plant.



Selection 2 Food Safety

LC-MS/MS Method for Sensitive Detection and Quantitation of 8 Water-Soluble vitamins in Infant Milk Powder

Here, we present a LC-MS/MS method for simultaneous determination of 8 water-soluble B vitamins in infant formula with 5 isotope labelled internal standards, with a simple sample extraction procedure.



Selection 3 Food QAQC

Discrimination of Soybean Oil and Olive Oil by Benchtop Linear MALDI-TOF

In this study, we attempt to analyze edible oil using a novel benchtop linear MALDI-TOF. The result showed that soybean oil and olive oil were distinguished successfully, which indicates that benchtop linear MALDI-TOF can be used for edible oil analysis.



Selection 4 Food Safety

Off-flavor System of Shimadzu Analyzes the Odor Components in Edible Oil

The edible oil will produce different odors during the production or storage process, and it directly affects the flavor quality and edible value of the food. In this poster, a simple and fast method of analyzing 150 kinds of odor substances was established for rapid screening of odor components in edible oil with GCMS-TQ8040.



Selection 5 Food Fraud

Qualitative and Quantitative Analysis of Pork in Beef Food with LC-MS/MS

Food fraud is a problem on global scale, especially the meat fraud. The ELISA method often results in false positive or false negative results, and the PCR method is not so accurate for DNA being prone to degradation. The characteristic peptides multiple reaction monitoring (MRM) method by LC-MS/MS is a good choice for the qualitative and quantitative analysis of meat authentication.



Selection 6 Food Safety

Analysis of Odor Components in Fish by Shimadzu Off-Flavor System

In this paper, the Shimadzu GCMS-TQ8050 triple quadrupole GC/MS and AOC-6000 multi-function autosampler were combined with the Off-flavor database to determine the odor components in three kinds of fish meat.



Selection 7 Food Safety

Study of a Method for Coping with Matrix Effects in Pesticide Residue Analysis Using GC/MS/MS

One of the issues cited in the analysis of pesticide residues in food using GC/MS/MS is the matrix effect that causes abnormal recovery rates. In this article, we report on the results of studying a method for coping with matrix effects when PEG300, and a combination of AP, were used.



Selection 8 Food QAQC

Analysis of Volatile Sulfur Compounds in Beer Using Nexis™ SCD-2030

Volatile sulfur compounds in beer can be generated during production and storage, and it gives beer their distinct aroma and flavor characteristics and can have a significant impact on product quality. Analysis of sulfur compounds with high sensitivity is required for accurate quality control and product development.



Selection 9 Food Analysis

High Sensitivity Analysis of Coffee Aroma Components Using the SPME Arrow

The SPME Arrow method was developed as a new sample introduction option for the AOC-6000 Multifunctional Autosampler to address the shortcoming of the conventional sampling methods. This article presents the results of analyzing coffee aroma components using the SPME Arrow.



Selection 10 Cleaning validation

Cleaning Validation of Food Production Equipment by TOC and TN Measurement

This application news introduces an example in which the effect of cleaning was confirmed by measuring the TOC/TN of the rinse water when cleaning a container in which vinegar had been introduced, using Shimadzu TOC-LCPH and TNM-L.



Selection 11 Food Analysis

Micro-Volume Measurement of Food Dye and Amino Acid Compound – Application of TrayCell and NanoStick –

In this report, the micro-volume measurements of food dyes and amino acid compounds using the TrayCell (Hellma Analytics) and the Nano Stick (SINCO Inc.) which are microvolume cells and need sample quantities of only a few micro liters for a measurement and the UV-1900 UV-VIS spectrophotometer are demonstrated.



Selection 12 Food QAQC

Identification Testing of Health Food Ingredients Using FTIR: Support for GMP

Recently in Asian countries, GMP is now imposed by law even on health foods. This article introduces an identification of L-glutamic acid, an ingredient in health foods, using the LabSolutions software which controls Shimadzu's FTIR instruments.



Selection 13 Food Analysis

Measurement of Purine Content in Foods Using HPLC

Among purines in the body, about 20 % are from the foods we eat while the remaining 80 % are endogenous. This article introduces analyses of purine content in foods using the "Prominence-i" integrated high performance liquid chromatograph (hereinafter, Prominence-i).



Selection 14 Food Analysis

Monitoring Organic Acids during Fermentation with Shim-pack(TM) Fast-OA High-Speed Organic Acid Analytical Column

Organic acids are attracting attention not only as taste and flavor components in food, but also as raw materials for pharmaceuticals and chemical products, and have been analyzed in various fields. Here we report a case of monitoring organic acid content during fermentation using an ion exclusion column, Shim-pack Fast-OA.

A highlight of Shimadzu's global efforts to catalyze collaboration: Looking back at the Shimadzu Global Innovation Summit 2019



Shimadzu Corporation held the "Shimadzu Global Innovation Summit 2019" from 9th-10th July. This year's event, on the theme of "Pioneering Partnerships for Advanced Healthcare", saw a diverse pool of 93 researchers from 21 countries travelling to Shimadzu's headquarters in Kyoto. The event featured an engaging schedule, including a poster session and conference-style seminars, all of which facilitated lively exchanges amongst the participants.



The Global Innovation Summit has become a key part of Shimadzu's vision as a company with a strong focus on the value of global cooperation.

This year marked the second Global Innovation Summit, opened as before by the CEO of Shimadzu, Teruhisa Ueda: "We want to make this event a stage for global research development, a place where free discussion and the establishment of new connections can lead to true cooperation with the goal of the betterment of our society."

The Advanced Healthcare field, past and future

Following Dr. Ueda's introductory remarks, Shuzo Maruyama, the General Manager of Shimadzu's Analytical & Measuring Instruments Division, took to the stage. He introduced multiple examples of collaborations that have led to success, including groundbreaking research for the early detection of Alzheimer's via blood plasma analysis, achieved through collaboration with Prof. Colin L. Masters from Melbourne University; an adrenal venous (AVS) support system for primary aldosteronism developed in cooperation with Prof. Kei Takase from Tohoku University; and a cancer photoimmunotherapy method developed together with Hisataka Kobayashi from the National Cancer Institute (NCI). In showcasing this exciting research, Mr. Maruyama underscored his expectation for even more successful collaborations in the future.



Showcasing successful collaborations and their results

The first day continued with the introduction of researchers who met at the "Shimadzu Global Innovation Summit 2017", and their successful collaboration over the past years. One such example was between Prof. Kevin Schug (University of Texas at Arlington), who specializes in environmental research, and Prof. Alex J. Rai (Columbia University), whose research is focused on pathology and cell biology.



Upon meeting at the 2017 Summit, they soon commenced a collaborative research project. As Prof. Schug explained, this research aims to “to develop methodology for intact protein quantitation using liquid chromatography – triple quadrupole mass spectrometry.” In their presentation, the two professors introduced their research and talked about their collaboration in detail.

Prof. Schug also mentioned the Shimadzu technology that aided in their research: “Shimadzu instrumentation is an essential part of our analytical chemistry research. Shimadzu consistently offers new instrumentation, which pushes the boundaries of what can be measured. As one example, the variable collision gas pressure in the LCMS-8050 and LCMS-8060 instruments is essential to being able to fragment large biomolecules in a multiple reaction monitoring-type experiment.”

In addition, Prof. Schug underlined what they wish to achieve with their research: “We can contribute to society and the well-being of humankind by helping clinicians better assess cancer diseases, as well as to potentially help the pharmaceutical industry develop new treatments.”



In addition, numerous other researchers introduced exciting developments in their research that proposed new synergies between analytical science and medical imaging technology.

A poster session brimming with innovation

Following the initial round of speeches, the event moved on to the poster session, in which 57 guests and 15 Shimadzu employees showcased their research. The open layout of the session allowed for lively discussion between researchers from diverse fields. After voting by all participants, a panel of judges determined 6 award winners, who presented their research on the main stage the following day.

One award winner was Dr. Silvia Giordano (Mario Negri Institute Milan), who in recent years has started a collaborative project with Shimadzu at its Innovation Center in Germany, with the goal of rapid and objective tumor identification. In her research, she uses Shimadzu’s DPiMS-2020 (Direct Probe Ionization Mass Spectrometer), which was developed to assist fast analysis of pathology specimens. It was created together with Prof. Sen Takeda of the Faculty of Medicine at the University of Yamanashi as part of a Japan Science and Technology Agency (JST) program for the development of advanced measurement and analysis systems. The implementation of this technology into the clinical field is expected to reduce the surgical burden of cancer patients. Dr. Giordano has coupled this technology with Artificial Intelligence to obtain accurate and reproducible results.



Another award winner, Dr. Adam Noah (Yale School of Medicine) has focused his unique research on one of the most fundamental social interactions we make: eye contact. In this research, Dr. Noah made use of Shimadzu’s functional spectral neuroimaging technology. Specifically, he seeks to understand the neurological basis of this interaction. To this end, he began a project investigating the role of direct eye contact in the modulation of activity in the temporal-parietal junction as well as neural synchrony between interacting partners in the face and socially-sensitive areas of the brain during direct eye gaze.



Shimadzu hopes that these kinds of collaborations aimed at research breakthroughs in Advanced Healthcare were accelerated even further at this year’s summit. The exchange of ideas between researchers from differing backgrounds, and the innovation this has the potential to give rise to, is exactly in line with Shimadzu’s vision of Advanced Healthcare.

For more details, visit at:
<https://www.shimadzu.com/an/gis/index.html>

A highlight of Shimadzu's global efforts to catalyze collaboration: Looking back at the Shimadzu Global Innovation Summit 2019

Uwe Oppermann, uo@shimadzu.eu



Lecture presentation at Shimadzu Europa GmbH in Duisburg, Germany

By 2050, the world population will have grown to over nine billion people. This development will be one of the major challenges for mankind: how to ensure the supply of safe food and clean water? how to increase agricultural yields? and how to develop of additional food sources? Experts estimate that general food production will have to be doubled compared to today.

How can the safety and quality of food be guaranteed in the future? There are many laws and regulations within the EU and beyond the borders of Europe. Analytical instrumentation has become an indispensable tool for monitoring the safety and quality of food and beverages as well as consumer goods.

It detects residues from fertilizers, and pesticides, or emissions that enter groundwater, lakes, and rivers - and migrate from there into the food chain. Furthermore, it contributes to secure supply and manufacturing chains in food production, starting with raw materials and ingredients, their treatment, processing and all the way to packaging. Each ingredient or material could contain prohibited or dangerous substances and pollutants.

Food analysis is challenging due to complex matrices. Moreover, the challenges for analytics and laboratory staff are constantly increasing, for instance, through qualitative and quantitative methods with ultra-fast detection in the ultra-trace range.

New applications, tips, and tricks for the food laboratory

For several years, the companies Shimadzu and Merck have been offering seminars on this subject. They have introduced new applications, as well as tips and tricks for use in the food laboratory. These training forums are aiming at all analytical chemists responsible for food monitoring and analysis. In addition to the extensive lecture program, they provide an excellent platform for dialogue and exchange between experts in food production, control, and research & development.



Training forums in seven European cities

The idea for this event was launched in 2016 and implemented in the same year at the Shimadzu European headquarters in Duisburg, and at the Merck corporate headquarters in Darmstadt. After four years, the user seminars on food analysis have become so popular that in 2019, a total of seven events will take place at European locations. Alongside Leipzig, Darmstadt, and Paris in the first half of 2019, Vienna, Rotterdam, Bologna, and Milton Keynes are on the calendar for the second half of the year.

Asking their opinions about the seminars, our customers have given us a very positive feedback, as they benefit from the expert knowledge of the speakers and will be able after the seminar to implement the new applications including sample preparation as well as the system parameter sets directly in their local laboratory workflow. Customers are really happy when taking the opportunity talking with Shimadzu/ Merck specialists as well as invited speakers and getting solutions for their actual analytical challenges. On the other hand, the product/ and market specialists are gathering important requests from user side and implementing such important information in new product/ application development. So in the end this is a true win-win-situation for all parties involved.



The following is an overview of the lectures. In the second half of the year 2019, guest speakers will complement the agenda with current topics.

Coupling of LC-GC (MS) for MCPD, cholesterol, and PAH determination

Dr. Stephan Schröder, Shimadzu Germany

Ionic liquids as GC capillary column phase—unique selectivity & applications, incl. examination of water and aqueous samples

Klaus Buckendahl, Merck/Sigma-Aldrich Chemistry

The suitable quality of ultrapure water for your application in the Food & Bev laboratory

Ekkehart Berndt, Merck

What's in my grocery bag? Latest LC/LCMS technology for food analysis

Dr. Klaus Bollig, Shimadzu Germany

Modern HPLC phases from Merck for robust and cost-effective methods in food analysis

Dr. Martin Finkbeiner, Merck

Use of atomic spectroscopy for the determination of heavy metals in food and food packaging

Uwe Oppermann, Shimadzu Europa

Sample preparation of high-fat and complex matrices using solid-phase extraction & QuEChERS for pesticide analysis

Dr. Martin Finkbeiner, Merck

Filtration and particle monitoring in the F&B laboratory & sample preparation for analytical instrumentation

Uwe Wagner, Merck

Size matters—particle size distribution in the food industry

Sascha Hupach, Shimadzu Germany



New Products

MALDImini-1 (MALDI Digital Ion Trap Mass Spectrometer)

Towards new possibilities with
the compact MALDImini-1



[Click here>>](#)

Despite its light and compact shape, The MALDImini-1 is capable of achieving MS³ analysis, making it suitable for a large number of applications. With its simple configuration and compact size, it is possible to install the MALDImini-1 in places where mass analysis devices could not previously be used.

Features

- The MALDI-MS that can be installed anywhere
- Begin taking measurements quickly without fuss
- MSⁿ analysis is possible over a wide mass range, even with microquantity samples

GC-2010 Pro (Gas Chromatography) The Proven Solution



[Click here>>](#)

The GC-2010 Pro represents a new generation in Routine GC. Fast oven heating and cooling reduces the analysis time and allow high sample throughput. Based on GC-2010 Plus technology, it combines easy operation with efficient analysis and excellence in sensitivity and precision.

Features

- Two analytical lines can be operated simultaneously for different applications
- Responding to the ever increasing demands for trace level analysis, all detectors of GC-2010 Pro realize high sensitivity in miniaturization design.
- Simultaneously install up to three injection units and up to three detectors.

Note: This product is only available in Europe, Southeast Asia and China.

UP Series (Top-Loading Balances)

Superior Response
Significantly Increases
Weighing Efficiency



[Click here>>](#)

UP Series incorporates cutting edge digital control technology, and their response time when measuring trace quantities has been shortened to approximately 1/9th the time for previous models. This is expected to significantly improve the efficiency of the weighing process.

Features

- The display response time for weighing minute quantities (from about ten times the minimum display value) is about one second
- Using the highly impact-resistant UniBloc sensor minimizes downtime due to malfunctions
- The STABLO-AP ionizer eliminates static electricity effects to provide highly reliable measurements without any time-consuming steps

Multi-omics analysis package

(LC/MS,
GC/MS Data Analysis Software)



Shim-pack Fast-OA (High-speed Organic Acid Analysis Column)





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