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Shimadzu Journal

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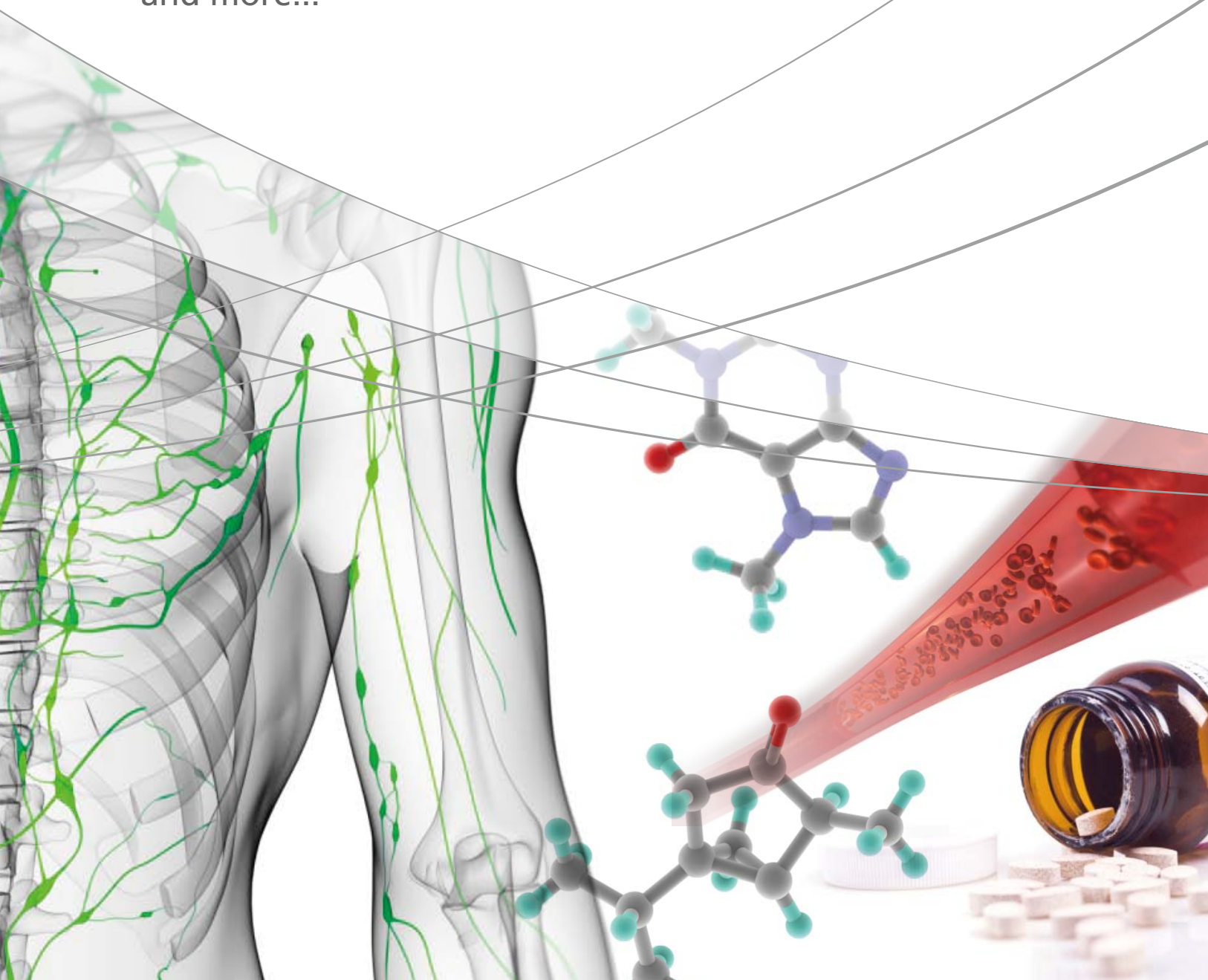
Pharmaceuticals and Human Science
and more...



Director's note

Dear Reader,

Welcome to the second issue of ***Shimadzu Journal***, in which we introduce collaborative research projects as well as industry-specific technical reports and applications. As reflected in our brand statement, "Excellence in Science," this journal exemplifies our desire to work with leading researchers around the globe and address customers' requirements by offering superior, world-class technologies.

The Analytical and Measuring Instruments Division continues to develop and manufacture analytical instruments that deliver results faster and more efficiently than ever before to meet the needs of researchers, scientists, and manufacturers around the globe. These state-of-the-art solutions are used in a wide variety of fields that impact the health and lives of people.

Currently, pharmaceuticals, life science, and food safety are the main fields of our concern. Collaborating with researchers is vital to us as a manufacturer in order to develop new solutions that contribute to society. For that reason, the Shimadzu Journal introduces various collaborative research projects and the potential impact they may have on society.

For this issue, we have focused on pharmaceutical analysis. It contains information on three collaborations. One is with Professor van Breemen of University of Illinois, Chicago, USA, who is studying medicinal properties of natural resources, such as lycopene in tomatoes, with advanced mass spectrometry. An interview with Professor van Breemen provides details on his current studies. The second and third collaborations are with Japanese pharmaceutical companies developing new technologies for the Nexera X2 series, Shimadzu's flagship liquid chromatograph. In addition, information on other applicable topics, as well as the latest news and applications are included.

We will always strive to develop the highest technology and seek valuable solutions that meet the needs of customers throughout the world. We wish to be a good partner for you, and hope this Journal provides valuable insight and information that benefits you and your colleagues.

Yours Sincerely,

Teruhisa UEDA, PhD.

General Manager, Analytical & Measurement Instruments Division

CONTENT

Focused on Pharmaceuticals and Human Science

Professor Richard B. van Breemen, University of Illinois, Chicago, USA

We interviewed Professor Richard B. van Breemen who is studying medicinal properties of natural products at the University of Illinois, College of Pharmacy, in Chicago, using advanced mass spectrometry. Recently he found that lycopene (the red carotenoid in tomatoes) could reduce risk of prostate cancer. Shimadzu has been supporting Prof. van Breemen's laboratory for six years, by providing Shimadzu's state-of-the-art mass spectrometry such as IT-TOF, LCMS-8040 and LCMS-8050.

Shimadzu:

First, could you outline your research and achievements?

Prof. van Breemen:

My research group investigates natural cancer chemoprevention agents and the safety and efficacy of botanical dietary supplements. We specialize in mass spectrometric studies of natural products that span the range from discovery to clinical investigation. An early proponent of MS-based screening, we use our ultrafiltration-mass spectrometry approach to find pharmacologically active compounds in complex natural product extracts. Then, we use high resolution LC-MS/MS to investigate the metabolism of lead compounds. Finally in support of clinical trials, we use quantitative UHPLC-MS-MS to measure levels of the active natural products and their metabolites as well as biomarkers of efficacy in clinical specimens.

Some of our most successful projects have included studies of carotenoid bioavailability and chemoprevention that are exemplified by our clinical trials of prostate cancer chemoprevention by the tomato carotenoid lycopene. As I am director of the oldest academic center in the United States focused on the safety and efficacy of botanical dietary supplements (UIC/NIH Center for Botanical Dietary Supplements Research), we help to safeguard women's health by investigating mechanisms of action and safety of botanicals used by menopausal women as alternatives to conventional estrogen replacement therapy. Qualitative and quantitative applications of mass spectrometry are essential at every stage of these botanical dietary supplement studies.

Shimadzu:

Why are you interested in those fields?

Prof. van Breemen:

Our interest in natural products as sources of new drugs is based on the fact that at least half of all existing pharmaceuticals are natural products or are derived from them, which should be no surprise as Nature has already preselected these substances for bioactivity. Now that most major pharmaceutical companies have abandoned their natural products drug discovery programs, our search for new natural pharmaceutical agents has become even more imperative. Since the Women's Health Initiative indicated that conventional estrogen replacement therapy might increase risks of certain forms of cancer, cardiovascular disease and even dementia, many women have sought relief from menopausal symptoms by using botanical dietary supplements with the expectation that they may be safer as well as effective. In response, our Botanical Center is working to ensure the safety of these botanical products as well to determine their mechanisms of action and potential efficacy.

Shimadzu:

Then, your article in this journal addresses the quantitative analysis of bisphenol A (BPA) in water using the LCMS-8050. Why are biomedical researchers concerned about human exposure to BPA?

Prof. van Breemen:

BPA is a high volume industrial product used in a wide range of consumer products from polycarbonate bottles to epoxy resins lining the inside of canned foods. The release of BPA into the environment might result in its accumulation in lakes and rivers where it might affect aquatic wildlife due to its endocrine disruption activity. There is particular concern that exposure of children to endocrine disrupting chemicals like BPA might be especially significant.

Shimadzu:

How are our instruments helping you?

Prof. van Breemen:

My research group has witnessed many innovations in mass spectrometry and chromatography over the last 25+ years that include the introduction of novel of ionization techniques such as MALDI and electrospray, the invention of new tandem mass spectrometers such as MALDI TOF/TOF and IT TOF instruments, and the introduction of UHPLC. All of these innovations contribute to our research by enabling faster MS-based screening of botanical and marine extracts, faster structural characterization of natural product lead compounds and faster and more sensitive quantitative analysis in support of preclinical and clinical studies.

Shimadzu:

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

Prof. van Breemen:

For many years, Shimadzu has been a leader in HPLC and UHPLC by providing highly reliable as well as high performance chromatography instrumentation. Shimadzu is also highly respected for providing high quality service support. In the field of MALDI TOF/TOF, Shimadzu offers the highest performance and features not available from most vendors due to innovations such implementation of the late Prof. Robert Cotter's curved-field reflectron and high-energy CID features, which facilitate structural studies using charge-remote fragmentation. Shimadzu's high resolution IT-TOF mass spectrometer offers MS^n capabilities not obtainable with QqTOF instruments, and Shimadzu continues to lead the world in fast triple quadrupole mass spectrometer technology with the LCMS-8030, LCMS-8040 and now the fastest scanning and most sensitive of all, the LCMS-8050.

Shimadzu:

Finally, can you please share any requests that you have with respect to analytical instrument vendors?

Prof. van Breemen:

Like most academic biomedical mass spectrometry laboratories, we are always pushing the limits of technology in terms of speed and sensitivity in the analysis of clinical and research specimens. Fortunately, the performance specifications of mass spectrometers continue to increase and thereby enable us to be more productive than ever. For example, we are looking forward to seeing how much more research we can accomplish each day using Shimadzu's new LCMS-8050 triple quadrupole mass spectrometer.

Shimadzu:

Thank you very much.

Here are selected 2013 publications citing Shimadzu mass spectrometers (7 out of 15 for 2013):

And here are the latest publications by Prof. van Breemen :

(1)Nikolic D, van Breemen RB. Analytical methods for quantitation of prenylated flavonoids from hops. *Curr. Anal. Chem. 9,* 71-85 (2013).

(2)Yuan Y, Yu L-F, Qiu X, Kozikowski AP, van Breemen RB. Pharmacokinetics and brain penetration of LF-3-88, (2-[5-[5-(2(S)-azetidinylmethoxyl)-3-pyridyl]-3-isoxazolyl]-ethanol, a selective $\alpha 4\beta 2$-nAChR partial agonist and promising antidepressant. *J. Chromatogr. B* 912, 38-42 (2013).

(3)Gaba RC, Yap FY, Martinez EM, Li Y, Guzman G, Parvinian A, van Breemen RB, Kumar N. Transarterial sorafenib chemoembolization: preliminary study of technical feasibility in a rabbit model. *J. Vasc. Interv. Radiol.* 24, 744-750 (2013).

(4)Conda-Sheridan M, Park EJ, Beck DE, Reddy PV, Nguyen TX, Hu B, Chen L, White JJ, van Breemen RB, Pezzuto JM, Cushman M. Design, synthesis, and biological evaluation of indenoisoquinoline rexinoids with chemopreventive potential. *J. Med. Chem.* 56, 2581-2605 (2013).

(5)Yu R, Zhao G, Christman JW, Xiao L, van Breemen RB. Method development and validation for ultra-high pressure liquid chromatography/tandem mass spectrometry determination of multiple prostanoids in biological samples. *J. AOAC Intl.* 96, 67-76 (2013).

(6)Hajirahimkhan A, Simmler C, Yuan Y, Anderson JR, Chen SN, Nikolić D, Dietz BM, Pauli GF, van Breemen RB, Bolton JL. Evaluation of estrogenic activity of licorice species in comparison with hops used in botanicals for menopausal symptoms. *PLoS One* 8(7):e67947 Jul 12 (2013). doi: 10.1371/journal.pone.0067947

(7)Andreani A, Leoni A, Locatelli A, Morigi R, Rambaldi M, Cervellati R, Greco E, Kondratyuk TP, Park E-J, Huang K, van Breemen RB, Pezzuto JM. Chemopreventive and antioxidant activity of 6-substituted imidazo[2,1-b]thiazoles, *Eur. J. Med. Chem.* 68C, 412-421 (2013).

Quantitative analysis of bisphenol A in water and serum using UHPLC-MS-MS

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[1]Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 South Wood Street, Chicago, IL 60612 USA

ABSTRACT

Endocrine disruptors represent a major toxicological and public health issue, and the xenoestrogen bisphenol A (BPA) has received much attention due to its high volume of production for plastics and widespread human exposure. The measurement of BPA in biological and environmental samples is essential for risk assessment. However, such analyses are challenging due to the trace levels of BPA in these samples and the possibility of false positive determinations due to background contamination. To overcome these limitations, we developed a highly sensitive and selective method using ultrahigh pressure liquid chromatography (Shimadzu Nexera) and a new generation triple quadruple mass spectrometer (Shimadzu LCMS-8050).

Key Words

Bisphenol A, bisphenol A-glucuronide, serum, UHPLC-MS-MS, LCMS-8050, water quality

1. Introduction

Used as a plasticizer in epoxy resins such as those lining canned foods, as a constituent of thermal papers used in cash register receipts and as a monomer for polycarbonate plastic used in production of water bottles, bisphenol A (BPA) is a high volume industrial chemical with considerable human and environmental exposure.[1,2] BPA (Fig. 1) has estrogenic effects and has been shown to function as an endocrine disruptor.[3] There is concern that environmental BPA exposure can adversely impact aquatic life and human health, especially in children who might be more sensitive to endocrine disruptors.

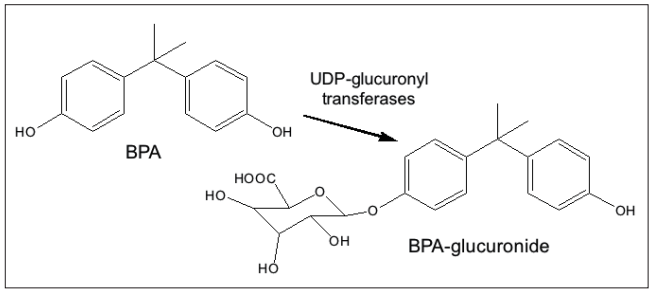

Fig. 1. Chemical structures of BPA and its primary human metabolite, BPA-glucuronide.

Human exposure to BPA can result from such diverse sources as consumption of canned foods, drinking water, dental composites, and cash register receipts. The large quantity of BPA produced world-wide each year has resulted in BPA contamination of rivers and lakes that can affect wildlife as well as humans using these resources for drinking water. Therefore, methods are needed for the quantitative analysis of BPA in water.

After absorption, BPA is metabolized in the liver of adults to form primarily BPA-monoglucuronide (BPA-G; Fig. 1) and to a lesser extent, BPA-sulfate, before being excreted in urine.[4] Since the activities of glucuronyltransferases that conjugate BPA with glucuronic acid are low at birth and increase for the next several weeks, neonates might be exposed to higher concentrations of unconjugated BPA than adults. While levels of unconjugated BPA in adult human serum are typically low (≤0.5 ng/mL), higher levels have been reported in neonates.[4] To enable risk assessment of BPA exposure, BPA and BPA-G need to be determined in serum not only of adults but also of neonates and infants, from whom serum sample sizes are typically quite small.[5]

BPA has been measured in surface water samples using gas chromatography-tandem mass spectrometry (GC-MS),[6] high performance liquid chromatography-tandem mass spectrometry (HPLC-MS-MS)[7] and ultrahigh pressure liquid chromatography-MS-MS (UHPLC-MS-MS).[8] Most methods for the measurement of BPA and BPA-G involve chemical or enzymatic deconjugation to convert BPA-G to BPA prior to quantitative analysis. For example, Kosarac, *et al*.[9] enzymatically deconjugated BPA-G prior to derivatization and measurement of total BPA in serum using GC-MS-MS. Among the few methods that measure both BPA and BPA-G directly in serum, most rely on HPLC-MS-MS[10] or UHPLC-MS-MS[11] and utilize negative ion electrospray without need for derivatization.

We developed methods based on UHPLC-MS-MS for the analysis of BPA in water and analysis of both BPA and intact BPA-G in serum. Unlike GC-MS-MS assays, our approach requires no derivatization of BPA, and unlike GC-MS-MS and some HPLC-MS-MS based assays, our method avoids hydrolysis of BPA-G. UHPLC was utilized instead of HPLC to shorten chromatographic separation time (less than 2 min per analysis), and when volumes of serum samples are small, our method is unique in that only 25 µL of serum are required.

2. Experimental

2-1 Materials

HPLC-grade solvents (acetonitrile, methanol and water) were purchased from Burdick & Jackson (Honeywell, Muskegon, MI). BPA and [*rings*-$^{13}C_{12}$]-BPA-G were purchased from Sigma-Aldrich (St. Louis, MO). Bisphenol A mono-β-D-glucuronide (BPA-G) was obtained from the Midwest Research Institute (Kansas City, MO), and [d_6]-BPA was purchased from Cambridge Isotope Laboratories (Andover, MA).

2-2 Sample Preparation

Standard solutions. Stock solutions of BPA and BPA-G were prepared in methanol at final concentrations of 1 mg/mL each. Working standards were made by serial dilution from stock solutions. Calibration standards were prepared by mixing 1 µL of each working standard with 24 µL blank mouse serum or water and vortex mixing.

Serum. Each unknown serum sample (25 µL) or calibration standard (25 µL) was mixed with 100 µL acetonitrile containing the surrogate standards 5 ng/mL [d_6]-BPA and 5 ng/mL [$^{13}C_{12}$]-BPA-G. The mixture was vortexed for 1 min, centrifuged for 15 min at 13000 x *g* at 4˚C, and then the supernatant was removed and evaporated to dryness. The residue was reconstituted in 25 µL of 50% aqueous methanol, and a 5 µL aliquot was injected onto the UHPLC-MS-MS system for analysis.

Water. Each water sample (50 mL) was spiked with [d_6]-BPA, acidified with 50 µL 33% HCl (aq) and loaded onto a Waters (Milford, MA) Oasis HLB 5cc 200 mg LP glass solid phase extraction cartridge that had been conditioned using 4 mL portions of methyl-*t*-butyl ether, methanol and water. The cartridge was washed with 4 mL water, and then BPA was eluted using 4 mL methanol and then 4 mL of 10% methanol in methyl-t-butyl ether. The combined methanol and methyl-*t*-butyl ether eluates were evaporated to dryness under a stream of nitrogen, and the residue was reconstituted in 100 µL of 50% aqueous methanol for analysis using UHPLC-MS-MS.

UHPLC-MS-MS. Chromatographic separations were carried out using a Shimadzu (Kyoto, Japan) LCMS-8050 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera UHPLC system. BPA and BPA-G were separated on a Waters (Milford, MA) Acquity UPLC BEH (2.1 x 50 mm, 1.7 µm) C18 column. A 1.5 min linear gradient was used from 10-100% acetonitrile in water followed by a hold at 100% for 0.4 min at a flow rate of 0.4 mL/min. The total run time including equilibration was 3.5 min. The column oven temperature was 45 °C, and the injection volume was 5 µL.

Negative ion electrospray mass spectrometry with selected reaction monitoring (SRM) and a dwell time of 50 ms per transition was used for the measurement of each analyte. Deprotonated molecules were used as the precursor ions, and the most abundant two product ions (Fig. 2) were used as quantifiers and qualifiers, respectively, for SRM. For BPA, the SRM transitions were *m/z* 227 to 212 (quantifier) and *m/z* 227 to 133 (qualifier); and for the surrogate standard [d_6]-BPA, the SRM transitions were *m/z* 233 to 215 (quantifier) and *m/z* 233 to 113 (qualifier). For BPA-G and its surrogate standard [13C12]-BPA-G, the SRM transitions were *m/z* 403 to 227 (quantifier) and *m/z* 403 to 113 (qualifier); and *m/z* 415 to 239 (quantifier) and *m/z* 415 to 113 (qualifier), respectively.

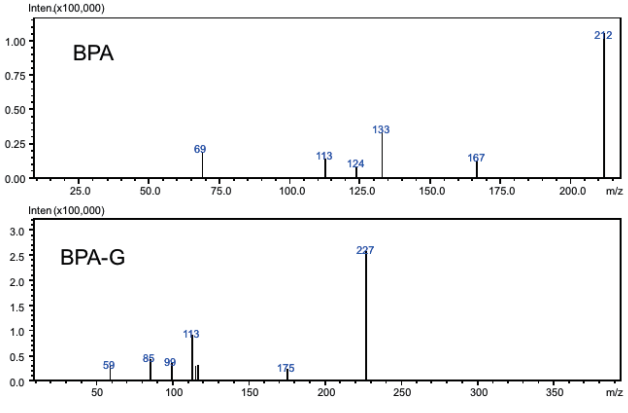

Fig. 2. Negative ion electrospray product ion tandem mass spectra of the deprotonated molecules of BPA (*m/z* 227) and BPA-G (*m/z* 403). The two most abundant product ions of each were selected for use during SRM.

3. Results and Discussion

Since BPA is a constituent of many plastics including some used in laboratory plastic ware and cap liners of vials and bottles, the solvents and materials used for BPA and BPA-G extraction and analysis had to be tested for BPA. Several brands of HPLC-grade methanol, methyl-*tert*-butyl ether and acetonitrile tested positive for BPA. Eventually, all laboratory ware and solvents utilized for sample preparation and analysis were confirmed to be free of BPA contamination.

The recoveries of BPA and BPA-G from serum using solvent/solvent extraction were ~90% and ~87%, respectively. The standard curves for BPA and BPA-G in water and serum were linear. For example, the standard curves for BPA and BPA-G in mouse serum shown in Fig. 3 were linear over the range of 0.2 to 25 ng/mL (r^2 = 0.998) and 0.1 to 25 ng/mL (r^2 = 0.996), respectively. The mouse serum used to prepare these curves contained traces of BPA and BPA-G despite attempting to raise the animals in a BPA-free laboratory environment. This problem illustrates the ubiquitous nature of BPA in the laboratory and in the environment.

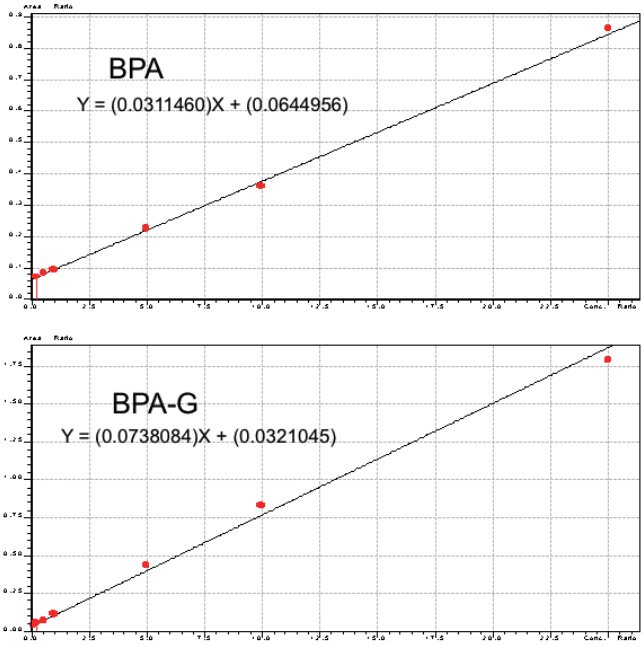

Fig. 3. Standard curves obtained for BPA and BPA-G in mouse serum using UHPLC-MS-MS with negative ion electrospray and SRM.

The lower limit of quantitation for BPA in water was 0.01 ng/mL, and the lower limits of quantitation for BPA and BPA-G in mouse serum were 0.5 ng/mL and 0.2 ng/mL, respectively. Although these LLOQ values for BPA and BPA-G in serum are not the lowest in the literature, the serum sample size used in our experiments (25 µL) was at least 10-fold smaller than previous methods. For example, using UHPLC-MS-MS Gerano *et al*[13]. reported an LLOQ of 0.1 for both BPA and BPA-G in serum using a sample size of 250 µL serum. Therefore, on a sample size basis, our method is as sensitive as any in the literature.

The method for measuring BPA in water was applied to the analysis of drinking water and surface water samples from the Chicago area, Jamaica and Ghana. BPA in water in these samples ranged from none detected to up to 0.186 ng/mL. These data are

being reported in a study of human exposure to BPA through drinking water.[15] An example of the analysis of BPA in type I purified laboratory water is shown in Fig. 4. It should be noted that even many laboratory water purification systems produce water contaminated with BPA, since they often contain polysulfone filters that leach BPA monomer.[12]

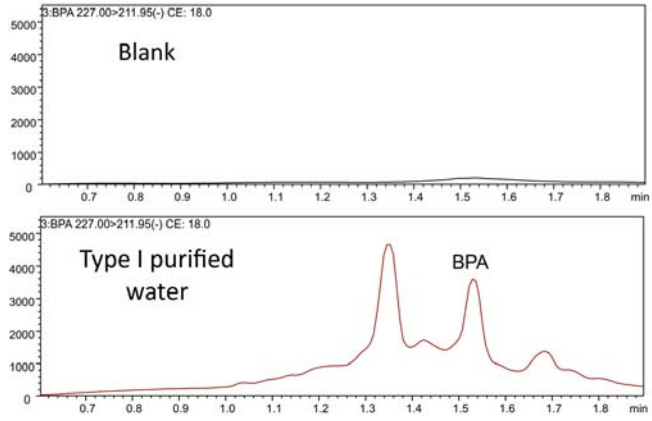

Fig. 4. Negative ion electrospray UHPLC-MS-MS analysis of a system blank and BPA in type I high purity laboratory water.

Our method for measuring BPA and BPA-G in serum was applied to an on-going study of in utero and neonatal exposures of mice and rats to BPA. The need for a method that utilizes small volumes of serum was prompted by studies such as Prins *et al*.[5] that had to pool sera from 8 to 10 rat pups to obtain 500 µL serum for a single analysis of unconjugated BPA and then another 500 µL serum for analysis of total BPA (after hydrolysis of BPA-G). In our study, 25 µL serum from a single mouse was analyzed for both BPA and BPA-G as shown in Fig. 5.

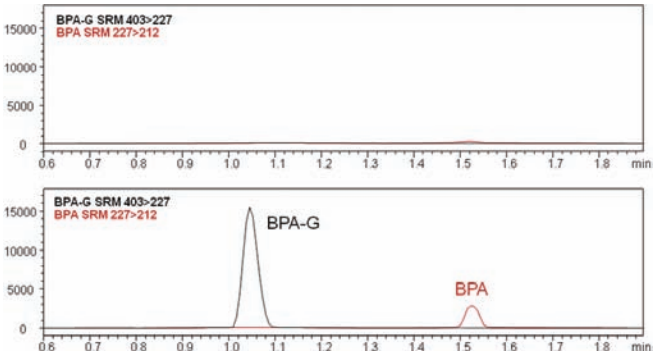

Fig. 5. UHPLC-MS-MS with negative ion electrospray and SRM analysis of BPA (retention time 1.52 min) and BPA-G (retention time 1.04 min) in mouse serum after receiving dose of vegetable oil vehicle as a control (top) or in mouse serum after administration of oil containing 10 µg/kg BPA (bottom).

4. Conclusion

A fast and sensitive UHPLC-MS-MS method was developed for the quantitative analysis of BPA in water and BPA and its major metabolite BPA-G in serum. Unlike GC-MS-MS assays, BPA may be measured using UHPLC-MS-MS without derivatization, and BPA-G may be measured directly without hydrolysis to BPA and then derivatization. These advantages simplify analysis and shorten sample preparation times. Compared with previous LC-MS-MS assays for BPA and BPA-G, the new UHPLC-MS-MS assay takes only 3.5 min (approximately 4-fold faster than HPLC-MS-MS) and requires only 25 µL serum instead of 500 µL or more. Simpler sample preparation, faster analysis and smaller sample sizes will enable biological and environmental studies of the effects of BPA to be carried out more efficiently than was possible previously.

Acknowledgements

We would like to thank Dr. Gail Prins of the University of Illinois College of Medicine for providing the serum samples and Dr. Amy Luke of Loyola University Chicago for providing water samples. We thank Mr. Zane Hauck for assistance with sample preparation and Shimadzu for making the Nexera and LCMS-8050 instruments available for these studies.

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Unique & Innovative Functions for Next Generation Separation Technologies

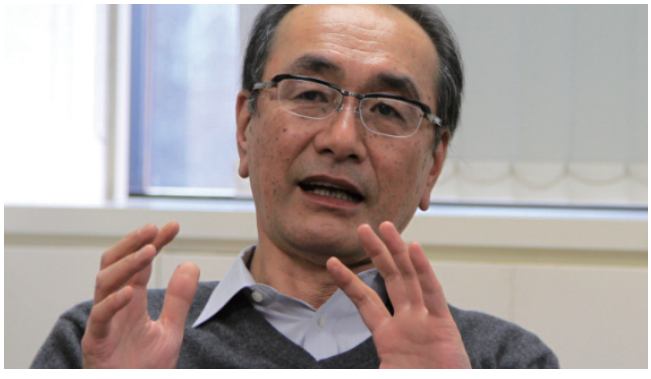
We interviewed two customers with whom we have built good relations for over 10 years. They have advised us during the development of instruments and methods for pharmaceutical analyses. Recently, we released the Nexera X2 UHPLC that features two innovative functions: ***i***-PDeA (Intelligent Peak Deconvolution Analysis) and ***i***-DReC (Intelligent Dynamic Range Extension Calculator). We asked them about each technology. For further information about ***i***-PDeA and ***i***-DReC, please see each technical report that follows the interviews.

i-PDeA - Intelligent Peak Deconvolution Analysis - (See pp. 39-42)

What is the background to how the benefits of this technology were brought to light?

Pharmaceutical development normally entails a developmental period of several decades and research and development expenditures of around 100 billion yen. Therefore, the development of efficient research practices is of increasing importance. Meanwhile, the efficacy and safety of pharmaceutical products are invariable requirements, and regulatory authorities are demanding ever-stricter conditions for their approval. With regards to regulations on quality assurance practices for pharmaceutical CMC (the ICH "Q" series of guidelines), subsequent to guidelines that pertain to pharmaceutical products, ICH Q11, which references active pharmaceutical ingredients (APIs), has recently been endorsed.

These ICH guidelines perceive the optimization of methods for the manufacture of APIs and pharmaceutical products that occurs between the developmental phase and the commercial production phase of pharmaceutical manufacture in terms of what is called a Design Space. This Design Space approach requires the scientific validity for process conditions defined at each process operation be included in an application for approval. In other words, this approach requires that quality be built in to the product development lifecycle. This change has brought about a major reform in research practices pertaining to chemistry, manufacturing and control (CMC). The evidence that becomes the scientific basis for process optimization is analytical data, where an enormous amount of analytical data is used to form the basis of the optimization of each process operation. Consequently, a major focus of optimization is the speed and power of the analyses performed to obtain this data. Analytical methods necessary for the optimization of process operations at the commercial production scale need to incorporate three important elements: speed, simplicity, and accuracy. An outcome of this is the importance of working towards analytical methods that work in real time, or what is also called process analytical technology (PAT).



Dr. Naoki Asakawa, Eisai Co., Ltd.

The development of ***i***-PDeA occurred against this backdrop, and originated from eluate testing that then took 24 hours to perform.

We changed from using conventional HPLC to a UV-based analytical method, and to analysis of the derivative spectrum of UV spectra to ensure specificity. I also proposed the development of ***i***-PDeA analytical software, with which spectra can be obtained instantaneously.

Could you tell us what analytical applications and particular tests you see this feature being useful for?

Although ***i***-PDeA has only just been developed as a commercial product and it will find many applications in the future, the current primary focus is its use as a PAT.

In particular, the 85-mm long path length cell for the SPD-M30A detector and dynamic range extension using ***i***-DReC dramatically increase the concentration range of analytes, expanding the range of applications to high-sensitivity and high-concentration sample analysis. I believe this will further increase the potential range of applications for ***i***-PDeA.

In addition to applications such as (1) real-time eluate testing and (2) quantitative monitoring of target products from synthesis reactions, I think ***i***-PDeA can be used for (3) verifying specificity (peak purity) during HPLC analytical method validation and (4) HPLC used for impurity testing.

(1) For eluate testing, ***i***-PDeA is extremely attractive as drug specificity can be identified easily using instantaneously obtained UV spectra. Using ***i***-PDeA is both quick and simple, and provides quantitative analysis in real time. In the future, I think ***i***-PDeA will see fruitful application as an analytical technique suited to manufacturing process control that is geared towards optimizing the method of manufacturing pharmaceutical products, or in other words as a PAT.

(2) For synthesis reactions, as long as there is no interference from the reaction solvent in terms of UV absorption, ***i***-PDeA can be used for real-time monitoring of reaction progress. Also, because reaction liquids become extremely concentrated, for cases in which dynamic range extension (***i***-DReC) can be applied, ***i***-PDeA is likely to become a groundbreaking development for API manufacture. Also, the 85-mm long path cell can be used for analysis of trace components, and can probably be used for monitoring the presence of degradation products and byproducts during a reaction over time.

(3) HPLC analytical method validation encompasses specificity testing, where a required degree of peak purity in the chromatogram is employed as a means of verification. Because ***i***-PDeA easily determines

whether non-target substances are present in a particular sample, its use enables the elucidation of HPLC conditions that provide a high degree of reliability.

(4) Controlling the presence of impurities is an extremely important and complex part of pharmaceutical development. Pharmaceutical impurities include the impurities present in starting materials (raw ingredients) as well as byproducts found in intermediates and APIs. Many of these impurities are similar structurally to useful components, and their identification can be difficult when the resolving power of HPLC is insufficient for their quantitation. For example, uncertainty can arise over whether to apply tangent skim or peak splitting to determine the area of an impurity peak. ***i***-PDeA extracts the impurity peak with specificity to always provide objective peak integration that produces scientifically accurate results not affected by human factors.

While there is the conventional method of improving resolution by increasing throughput time to increase separation of impurities, if ***i***-PDeA recognizes differences between UV spectra, impurities can be quantitated within a short period of time. In addition, when chromatographic separation is insufficient for impurity analysis, it may be assumed that ***i***-PDeA can be used for quantitation as long as there is a difference present in UV spectra.

Also, because an 85-mm optical path length cell allows ***i***-PDeA to carry out highly sensitive analysis, ***i***-PDeA is expected to be used to analyze GTIs (genotoxic impurities) at ppm levels. GTI analysis is an essential part of process control testing and release testing that is performed during the phases of pharmaceutical development through to commercial production. At present, highly sensitive and highly selective LC/MS analysis methods are generally employed for this analysis. However, considering its stable detection, speed, simplicity, and routine performance, including the ease of equipment control, I think a synergistic application of chromatographic technology with the Nexera X2 and the analytical specificity of ***i***-PDeA would be adequate for this purpose. I believe there is an extremely wide range of applications for ***i***-PDeA, and I would like to see further data be accumulated in support of this.

Could you talk about any functionality you think should be included in *i*-PDeA, or other matters that could be improved?

I believe we need to see improvements in hardware performance and for new devices to be developed to better support the use of ***i***-PDeA in a variety of sampling methods, including the eluate testing and reaction monitoring I mentioned earlier.

Please give us your opinion of Shimadzu, and what you would like to see from them.

The idea for ***i***-PDeA was given to Shimadzu around five years ago. I wonder whether Shimadzu could not have developed ***i***-PDeA a little earlier, though I suppose conventional methods were deemed good enough at the time, or no one raised any criticism against them. However, it must be recognized that to stick to conventional methods is a complacent approach that halts progress. We must look to the future in order to advance, despite the strong tendency to view the future through the rear-view mirror of conventional wisdom. I think it is in people's nature to be conservative. While maintaining a focus on the past can result in work getting done, you cannot expect to see any progress while doing so. The promise of progress goes hand in hand with a certain amount of effort and hardship.

The technical capabilities exhibited by Shimadzu in their commercialization of ***i***-PDeA within six months of deciding to proceed with the project are both admirable and astonishing. And I feel that the timing of this commercialization of ***i***-PDeA is apposite for those who work in pharmaceutical development. I believe applications for ***i***-PDeA will continue to appear, and think it certain that ***i***-PDeA will, as a PAT, become a groundbreaking technology with respect to the manufacturing process control required by regulatory authorities.

Thanks go to all those involved who listened to users and answered their queries.

Thank you for giving us your valued opinions.

i-DReC - Intelligent Dynamic Range Extension Calculator - (See pp. 43-46)

Mr. Takeuchi, based on your evaluation of the *i*-DReC function, could you share some background on why you were interested in the wide dynamic range of the *i*-DReC, what types of samples were giving you problems, and so on?

One of the things we do is use LC for quantitative analysis in the early-stage of drug discovery work. Unlike many physics, chemistry, and drug-manufacturing departments, where analysis is performed using validated instruments or measurements are made for application processes, we focus not only on data quality and accuracy, but also on analytical efficiency to process as many compounds as quickly as possible.

The samples Shimadzu tested for us contained high concentrations of compounds that would normally be diluted before quantitation.

Mr. Takahiro Takeuchi, Teijin Pharma Ltd.

Pretreatment processes such as dilution can affect accuracy. In addition, dilution and some other pretreatment processes are required when considering the solvent use (which takes additional time). Highly viscous solvents, moreover, require separate consideration. For example, the sample quantity available for administration tests is often very limited, so we were already looking for ways to eliminate such negative factors by measuring concentrated samples directly without pretreatment. However, typical PDA detectors have a limited dynamic range.

Eventually, we considered using MS or another detection method with a wide dynamic range, but MS requires dealing with matrix effects and has to consider internal standard corrections. It was at that point that we were introduced to the ***i***-DReC and provided samples. I thought the technology was theoretically possible, but after seeing the good range and linearity of calibration curve results, I realized that this was truly a useful function.

Did you have any apprehensions about the *i*-DReC technique?

I knew it was important to specify optimal wavelength settings, but I didn't know what would result from extremely low molecular weight causing a lack of multiple maximum absorption wavelengths, from barely detectable peaks on the low wavelength end, or from very low absorptivity. As it turned out, when we calculated the linearity for even rather low-wavelength and sloped regions of the spectrum, linearity was quite high, which convinced us that it was a useful technique.

What about in terms of improving analytical efficiency?

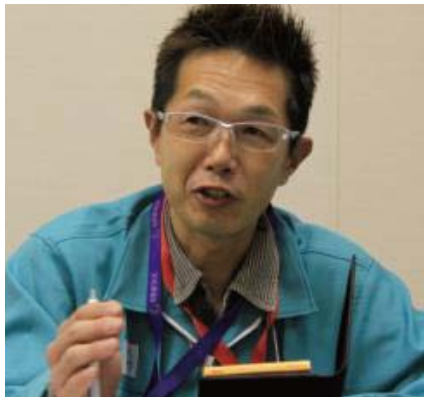
It definitely allowed us to improve analytical efficiency. With conventional techniques, dilution requires particular care, because not only the type of solvent, but also the solubility is important. If viscosity is too high, it can also affect results from LC analysis. It is also important to determine a dilution rate that allows the concentration of a target component to fall within the quantitative range of the calibration curve. Furthermore, since calibration curves are often prepared using standard samples, subtracting the background noise from the diluting solvent must be considered. Background noise effects can be quite large in MS, which is why an internal standard needs to be added. Using MS provides a wide dynamic range, but it requires considering a lot of factors. Therefore, I think that in reality, PDA results provide higher accuracy for our measurement purposes.

i-DReC is only one of the PDA functions, so have you thought of connecting the PDA detector in series with an MS system after it in some cases?

Yes, it would probably be used more often as an LC/MS system, because we also want to obtain MS data.

Aside from quantitation of high-concentration samples, would you use it for simultaneous quantitation of components with large differences in concentration, such as impurities and principal components?

When we study the synthesis process for manufacturing, we must consider the synthesis route. Therefore, it is important to establish analytical methods (simultaneous multi-component analysis) that can quantitatively determine, based on how long the reaction is performed, the amount of principal components produced and the loss of impurities and raw materials, for example. In such cases, the



principal components are often detected at their optimal wavelengths, which are not necessarily the same as the optimal UV wavelengths for impurities or raw material components. Consequently, detection can require considerable effort. Also, if only MS is used for analysis, the raw materials or other components can be made up of low-molecular-weight molecules, which can prevent adequate detection and make it difficult to determine appropriate analytical conditions for simultaneous quantitative analysis. Similarly, when considering processes, reactions must be checked with simultaneous quantitation that is conducted in as short a time as possible. In contrast, if calibration curves are prepared in advance, quantitative analysis can be accomplished very quickly by loading PDA data and using the ***i***-DReC function.

Did you see the *i*-DReC settings screen in LabSolutions?

Yes, I did. Only two windows were added and there were few settings needed to be specified. I think it only involved setting wavelength and threshold values. I was shown on the spot how to generate results, which did not seem very difficult. Without having to specify all sorts of parameters, it seemed rather user-friendly.

What do you think about the reliability of results obtained using *i*-PDeA?

When it comes to data reliability, I am concerned about how the data may be interpreted. After all, the information in the final results is not extracted directly from raw data. On the other hand, it is not that data taken from different instruments are combined into one, but rather that information from simultaneously acquired data is used. Therefore, it may be safe to conclude that no analytical problems arise as long as data consistency is maintained. I think it would be important to confirm consistency by using actual samples to compare results from previous methods with results obtained using ***i***-PDeA. In that respect, the tests conducted by Shimadzu probably serve to demonstrate satisfactory data consistency for the given samples.

Thank you so much for providing your samples and helping us with your valuable comments.

Great Potential for Further Applications

High-Sensitivity Cell and New Dynamic Range Extension Function Extend Measurement Concentration Range of Nexera X2 Series UHPLC by a Factor of 50

LC analysis often requires performing multiple analyses to analyze samples with different concentration levels. For instance, when using the same detection method to quantify compounds with significantly different concentration or sensitivity levels, or when measuring impurities, other trace components, and primary components.

Nexera X2 systems, when equipped with an SPD-M30A high-sensitivity cell (85 mm optical path length) and with the ***i***-DReC* function enabled, minimize this issue. With such a configuration, Nexera X2 systems can obtain information about both extremely small and large peaks from a single analysis. Consequently, Nexera X2 systems can reduce the number of analyses required and increase productivity. In particular, it provides an effective way of shortening the time (and improving efficiency) of analyses when each measurement takes a long time, due to various constraints on analytical conditions, for example.

Features:

• The ***i***-DReC function can be used with either SR-Cells (10 mm optical path length) or high-sensitivity SR-Cells (85 mm optical path length) and measurements can be performed using a single PDA detector.

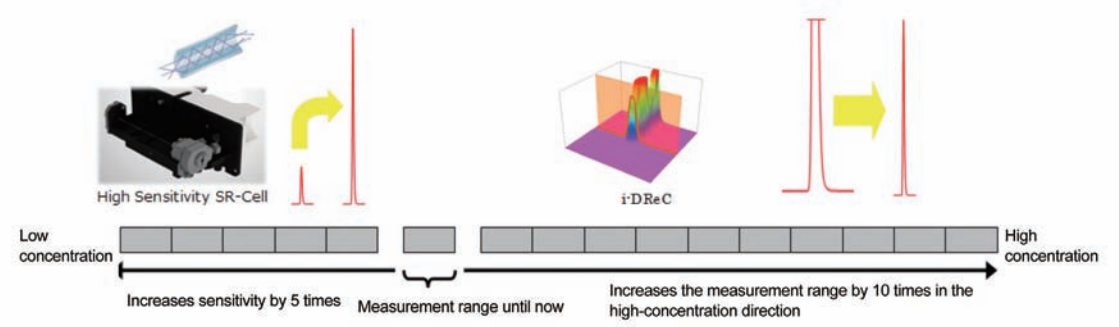
• Using a high-sensitivity SR-Cell boosts the sensitivity of SPD-M30A detectors by about 5 times and allows detecting extremely small peaks.

• The ***i***-DReC function extends the measurement range by about 10 times in the high-concentration direction and automatically corrects the peak area and height values of saturated peaks.

• Using the ***i***-DReC function with the high-sensitivity SR-Cell extends the Nexera X2 measurable concentration range by up to 50 times.

• Obtain results from a single dataset acquired using a single PDA detector.

• ***i***-DReC is included as a standard feature of LabSolutions software.



This ability to measure a wider range of concentrations means the system can now be used for an even wider range of applications. These include analyzing ultra-trace impurities, trace contaminants or hazardous substances in genotoxicity testing in the pharmaceutical industry, and confirming the purity, monitoring the processing, or testing the stability or dissolution of synthetic compounds.

Furthermore, results can be obtained from a single dataset acquired using a single PDA detector. This makes it easier to obtain more consistent results than when changing the concentration level and performing multiple analyses. The following is an example of results obtained from analyzing a pharmaceutical mixture sample using a Nexera SR system (with a high-sensitivity SR-Cell).

Table 1: Comparison of Area% Corrected by ***i***-DReC and Obtained from Analyzing Two Sample Concentrations

Peak No.	Retention Time (min)	Area Ratio (%) Corrected by ***i***-DReC	Area Ratio (%) Obtained from Two Samples (50 ppm and 1000 ppm)	Error
(1) Main	4.634	96.6789	96.7171	-0.0382
(2)	5.448	2.8749	2.8419	0.0330
(3)	3.900	0.1993	0.1970	0.0023
(4)	4.910	0.1019	0.1007	0.0012
(5)	5.091	0.0469	0.0464	0.0005
(6)	4.487	0.0295	0.0291	0.0004
(7)	4.226	0.0248	0.0245	0.0003
(8)	4.975	0.0248	0.0245	0.0003
Imp1	4.056	0.0062	0.0061	0.0001
Imp2	4.331	0.0076	0.0075	0.0001
Imp3	4.376	0.0052	0.0051	0.0001

Using the ***i***-DReC function in combination with a high-sensitivity SR-Cell requires only a single analysis of the sample to detect peaks (1) through (8). Then, based on those impurities, area value results can be obtained for primary components by using pre-specified threshold values to automatically correct area values as though the peaks had not been saturated. In this example, results with an extremely small error factor were obtained from analyzing two sample concentrations.

In contrast, using previous methods, peak information for primary components was first obtained by analyzing a low-concentration sample (50 ppm). Then the peak information for trace components was obtained by analyzing a high-concentration sample (1000 ppm). This required calculating the total area ratio percent value.

* ***i***-DReC is a function that determines peak area and peak height in PDA detector data with peak intensity saturation (intensity has exceeded a pre-specified threshold value) by obtaining a chromatogram that is automatically shifted to a position where peaks are at a wavelength with low absorption. Then, the sensitivity ratio between the wavelengths in the spectral data is corrected to calculate the peak area and height values at the target wavelength.

Fractional Determination of Co-eluted Compounds Using a New Data Processing Method for Photodiode Array Detector

Principle and Summary of i-PDeA (Intelligent Peak Deconvolution Analysis)

Toshinobu Yanagisawa[1]

Abstract

The i-PDeA derivative spectrum chromatogram method was developed as a new data processing technique for photodiode array detectors for HPLC. A derivative spectrum is created by performing differential processing on the UV-Vis absorption spectrum at each measurement time. Plotting the derivative spectrum values at the specified wavelength against retention time creates a derivative spectrum chromatogram that is able to separate co-eluted peaks. The high selectivity of the derivative spectrum chromatogram can detect unexpected impurities and quantitate the target component only, without effects from interfering components that elute simultaneously. This paper formulates the theory of the derivative spectrum chromatogram method into mathematical expressions and reports details of verification of the basic performance using standard samples.

Keywords:PDA data processing, peak deconvolution, derivative spectrum chromatogram, Nexera X2, UHPLC

1. Basic Theory of the Derivative Spectrum Chromatogram Method

1-1. Separation of Two Component Co-eluted Peaks

Fig. 1 shows the absorption spectra of two components (target component x and y), and Fig. 2 shows the derivative spectra differentiated along the wavelength axis. In Fig. 2, the derivative is zero for component x in the derivative spectrum at wavelength λ_x and zero for component y at wavelength λ_y.

Denoting the spectrum for target component x as $s_x(\lambda)$ and the peak profile as $p_x(t)$, and similarly the spectrum for target component y as $s_y(\lambda)$ and the peak profile as $p_y(t)$, the 3D chromatogram $S(t,\lambda)$ for the two-component system in which component x and y both elute can be expressed as:

$$S(t,\lambda) = p_x(t)s_x(\lambda) + p_y(t)s_y(\lambda)$$

Partial differentiation at wavelength λ gives the derivative spectrum chromatogram at wavelength λ_d as:

$$\left.\frac{\partial S}{\partial \lambda}\right|_{\lambda=\lambda_d}(t) = p_x(t)s_x'(\lambda_d) + p_y(t)s_y'(\lambda_d)$$

As the derivative spectrum chromatogram at wavelength λ_x where the component x derivative becomes zero is

$$s_x'(\lambda_x) = 0$$

we get,

$$\left.\frac{\partial S}{\partial \lambda}\right|_{\lambda=\lambda_x}(t) = p_y(t)s_y'(\lambda_x) \quad \text{------ (1)}$$

Similarly, as the derivative spectrum chromatogram at wavelength λ_y where the component y derivative becomes zero is

$$s_y'(\lambda_y) = 0$$

we get,

$$\left.\frac{\partial S}{\partial \lambda}\right|_{\lambda=\lambda_y}(t) = p_x(t)s_x'(\lambda_y) \quad \text{------ (2)}$$

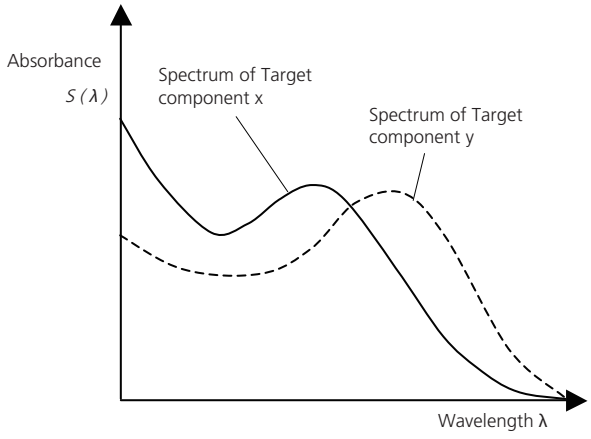


Fig. 1 Spectra of two components

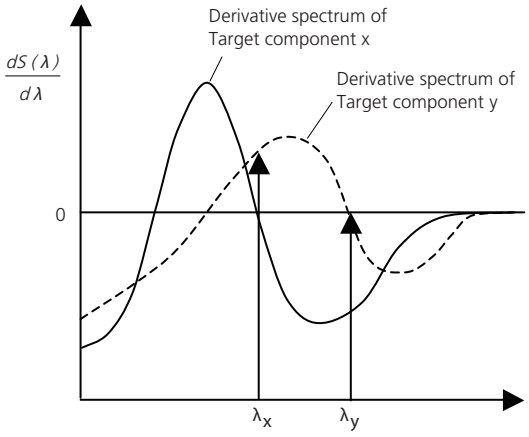


Fig. 2 Derivative spectra of two components

1. Analytical & Measuring Instruments Division

As $s_x'(\lambda_y)$ and $s_y'(\lambda_x)$ are non-zero constants in equations (1) and (2), it can be seen that the derivative spectrum chromatogram at wavelength λ_x shows the elution profile for component y only, and the derivative spectrum chromatogram at wavelength λ_y shows the elution profile for component x only.

That is, the derivative spectrum chromatogram at wavelength λ_x can separate component y only, and the derivative spectrum chromatogram at wavelength λ_y can separate component x only.

1-2. Impurity Detection

i-PDeA can detect whether impurity components exist in addition to the major component. This method can be applied when one major component is mixed with other impurity components. The 3D chromatogram S (t, λ) can be expressed as follows if the major component elution profile is denoted as $p_x(t)$, the impurity component elution profiles as $p_y(t)$, $p_z(t)$…, the major component spectrum as $s_x(\lambda)$, and the impurity spectra as $s_y(\lambda)$, $s_z(\lambda)$…

$$S(t,\lambda) = p_x(t)s_x(\lambda) + p_y(t)s_y(\lambda) + p_z(t)s_z(\lambda)...$$

Then, the derivative spectrum chromatogram at wavelength λ_x at which the major component derivative spectrum chromatogram $s_x'(\lambda)$ value becomes zero is given by

$$\left.\frac{\partial S}{\partial \lambda}\right|_{\lambda=\lambda_x}(t) = p_y(t)s_y'(\lambda_x) + p_z(t)s_z'(\lambda_x) + ...$$

Therefore, the derivative spectrum chromatogram at wavelength λ_x eliminates the major component elution profile and expresses the elution profiles of the impurities besides the major component.

2. Examples of Analysis Using the i-PDeA Functions

2-1. Impurity Detection in Standard Samples

This section demonstrates that an impurity in a methylnaphthalene (MN) standard was detected using the derivative spectrum chromatogram method.

Analytical Conditions

Pump	: Shimadzu LC-30AD×2
Detector	: Shimadzu SPD-M30A
Column oven	: Shimadzu CTO-20AC
Controller	: Shimadzu CBM-20A
Autosampler	: Shimadzu SIL-30AC$_{MP}$
Column	: Shimadzu Shim-pack XR-ODS (30 mmL. × 3.0 mmI.D., 2.2 µm)
Flow rate	: 1 mL/min
Column temp.	: 40 °C
Sampling	: 80 msec
Slit width	: 1 nm
Time constant	: 240 msec
Wavelength range	: 190 nm to 700 nm
Injection volume	: 1 µL

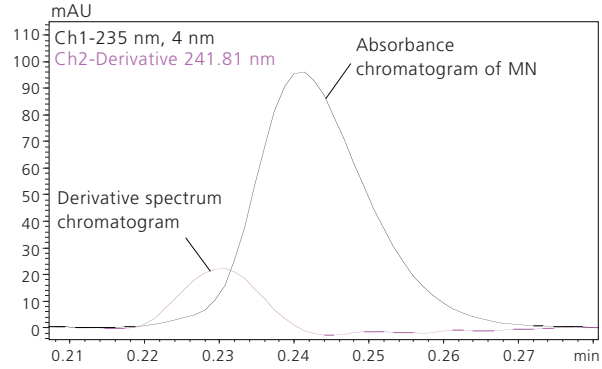
Fig. 3 shows the absorbance chromatogram at 235 nm and derived spectrum chromatogram at 1st derivative zero wavelength under several types of analytical conditions.

In (a), the impurity is detected using i-PDeA, even though MN and its impurity overlap chromatographically.

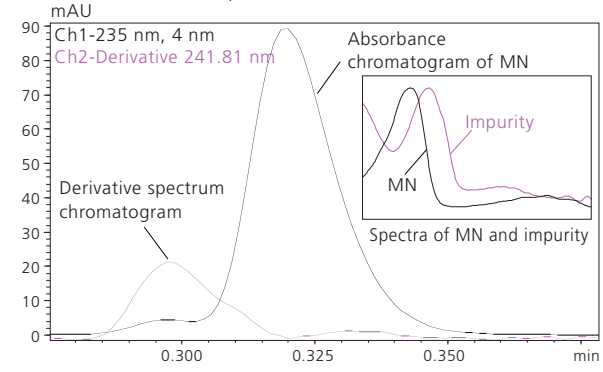
In (b), MN and the impurity begin to separate chromatographically. By comparing the spectra at the apex of each peak, MN and the impurity can be confirmed to be different compounds.

In (c), MN and the impurity are completely separated chromatographically. The derived spectrum chromatogram shows no significant signal at the retention time of the MN peak, which shows the signal of the MN peak does not include a contribution from the impurity.

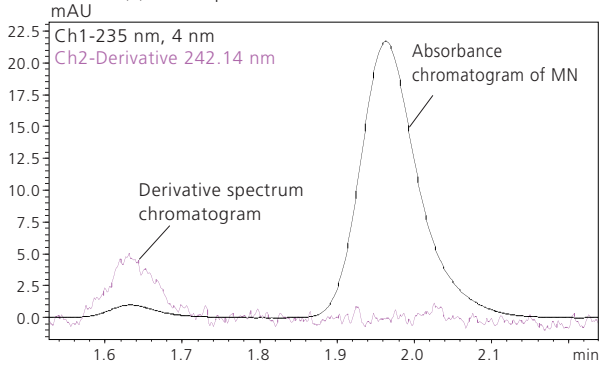
As described in this example, whether impurity peaks are chromatographically resolved or co-eluted with the main component, i-PDeA easily detects their existence.



(a) Mobile phase : Acetnirile95% / Water5%



(b) Mobile phase : Acetonitrile85% / Water15%



(c) Mobile phase : Acetnitrile50% / Water50%

Fig. 3 Absorbance chromatogram and derivative spectrum chromatogram

2-2. Quantitation of a Mixture of Two Components

This section demonstrates that the derivative spectrum chromatogram method can separate and quantitate two chromatographically co-eluted peaks in a data set acquired for a mixture of two components.

Difluorobenzophenone (DFBP) and Valerophenone (VP) standards, in 5 different relative concentrations of 100/1,100/10,100/50, 100/100,100/200, were used to acquire the derivative spectrum chromatograms of DFBP and VP. A calibration curve was created (Table 1) and quantitative analysis of each sample mixture was performed (Tables 2 and 3).

Analytical Conditions

Pump	: Shimadzu LC-30AD×2
Detector	: Shimadzu SPD-M30A
Column oven	: Shimadzu CTO-20AC
Controller	: Shimadzu CBM-20A
Autosampler	: SIL-30AC$_{MP}$
Mobile phase	: Acetonitrile45% / Water55%
Column	: Shimadzu Shim-pack XR-C8 (50 mmL. × 3.0 mmI.D., 2.2 µm)
Flow rate	: 2 mL/min
Column temp.	: 40 °C
Sampling	: 80 msec
Slit width	: 1 nm
Time constant	: 240 msec
Wavelength range	: 190 nm to 700 nm
Injection volume	: 1 µL

Fig. 4 shows the spectrum comparison of DFBP and VP. Fig. 5 shows the absorbance chromatogram of the mixed sample (DFBP/VP= 100/200) at 210 nm and the derived spectrum chromatogram at 255.93 nm (1st derived zero wavelength of DFBP) & 216.93 nm (1st derived zero wavelength of VP).

Fig. 6 shows the absorbance chromatogram of the mixed sample (DFBP/VP=100/1) at 210 nm. Due to the low concentration in the sample, the VP peak is hidden in the DFBP peak. The ellipse in Fig. 6 shows the derivative spectrum chromatograms, which are used by i-PDeA to find and integrate the peak.

The VP calibration curve, created by using the integrated peak area for VP in the derivative spectrum chromatogram, was used to calculate the quantitative amount of VP in each sample. The results are shown in Table 2. In the case of the lowest VP concentration sample (DFBP/VP = 100/1), the concentration was calculated to be 1.023 (2.30% error).

In the same way, the DFBP calibration curve was created and used to calculate the quantitative amount of DFBP in each sample. The results are shown in Table 3. Of note in these results is the reproducibility of peak area for a 1 µL sample injection (<1% RSD) as well as <3% error in the quantitative calculation.

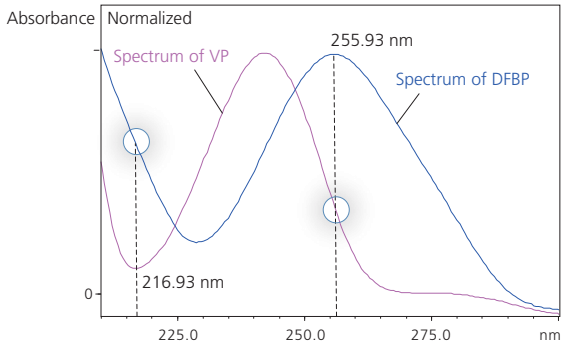


Fig. 4 Spectrum comparison; DFBP and VP

Table 1 Calibration data points created by derivative spectrum chromatogram of VP standard sample (R^2=0.9999309)

Sample relative concentration (VP)	Retention time (min)	Area	Concentration	Error (%)
1	1.804	2,984	0.964	-3.62
10	1.801	30,368	9.876	-1.24
50	1.804	151,922	49.439	-1.12
100	1.802	310,801	101.149	1.15
200	1.802	613,207	199.572	-0.21

Table 2 Quantitation result of VP in DFBP/VP mixed sample

Sample relative concentration (DFBP/VP)	Retention time (min)	Area	Concentration	Error (%)
100/1	1.808	3,167	1.023	2.30
100/10	1.807	30,372	9.878	-1.22
100/50	1.802	153,206	49.856	-0.29
100/100	1.806	309,596	100.757	0.76
100/200	1.815	620,556	201.964	0.98

Table 3 Quantitation result of DFBP in DFBP/VP mixed sample

Sample relative concentration (DFBP/VP)	Retention time (min)	Area	Concentration	Error (%)
100/1	1.746	359,670	102.225	2.23
100/10	1.742	357,969	101.741	1.74
100/50	1.737	357,497	101.607	1.61
100/100	1.742	357,891	101.719	1.72
100/200	1.751	351,528	99.911	-0.09

Area %RSD=0.87 (injection volume:1 µL)

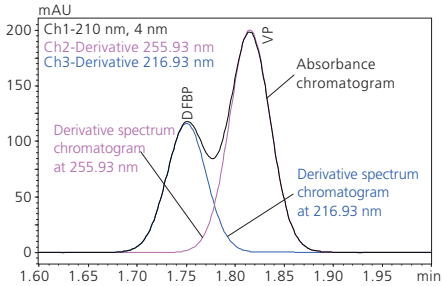


Fig. 5 Peak separation in the mixed sample (DFBP/VP=100/200)

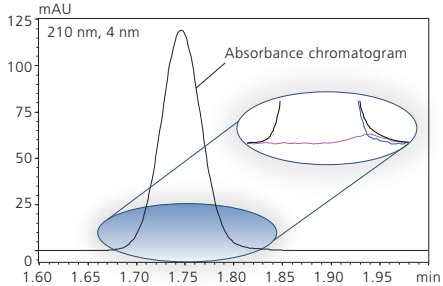
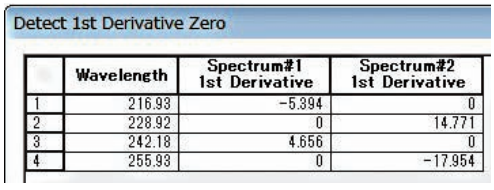


Fig. 6 Absorbance chromatogram of the mixed sample (DFBP/VP=100/1)

3. Summary of i-PDeA Settings

The parameters for the i-PDeA function are set as part of the data processing method of the photodiode array detector. Once the analytical protocol is defined, it can be applied for routine analysis. The following is a brief summary of the i-PDeA parameters.

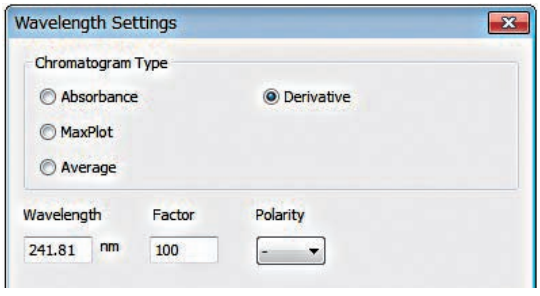
1. The Savitzky-Golay method is used to determine the first derivative spectrum, from which a list of wavelengths where the 1st derivative value is zero is generated. Spline interpolation is applied to calculate the wavelength closest to the 1st derivative zero wavelengths, and the results are displayed in a table.



	Wavelength	Spectrum#1 1st Derivative	Spectrum#2 1st Derivative
1	216.93	-5.394	0
2	228.92	0	14.771
3	242.18	4.656	0
4	255.93	0	-17.954

2. i-PDeA most effectively resolves co-eluted peaks when the peak height in the derivative spectrum chromatogram for one component, taken at a 1st derivative zero wavelength of another component, is sufficiently large. If the shape of the spectra of two components is very similar, i-PDeA cannot be applied.

   To extract the derivative spectrum chromatogram, plotting the derivative spectrum values at the specified wavelength against retention time, select "Derivative" for Chromatogram Type and using the wavelength obtained by the Detect 1st Derivative Zero function, set the value (with 2 decimal places) of the Wavelength in the Wavelength Settings window of the Multi-Chromatogram table.



3. The derivative spectrum chromatogram has positive value when the slope of the spectrum is up and negative value when the slope of the spectrum is down. Peak direction in the derivative spectrum chromatogram can be adjusted by setting the polarity, and peak size can be adjusted by setting the factor.

   Set integration and quantitation parameters for the derivative spectrum chromatogram.

   The derivative spectrum chromatogram can be handled the same as other multi-chromatograms for quantitative purposes.

4. Conclusion

The excellent performance and reproducibility of the SPD-M30A photodiode array detector and the Nexera X2 system make this new separation methodology possible. The i-PDeA function helps increase the speed of analysis and enhances laboratory productivity. To summarize the key benefits:

- Co-eluted peaks can be separated mathematically, using derivative spectrum chromatograms
- Poorly resolved peaks are processed and visualized as pure peaks with no contribution from co-eluting components
- Impurity peaks hidden by, or even in, the target peak can be detected
- Fast and accurate quantitative analysis is possible, even without complete chromatographic separation
- Simple post-run analysis procedure

The i-PDeA function provides a new solution, which is useful for identification and quantitation of impurities. The use of this feature is expected to increase laboratory efficiency and produce more reliable analytical data.

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New Data Processing Method for Photodiode Array Detector

Principle and Summary of i-DReC (Intelligent Dynamic Range Extension Calculator)

Toshinobu Yanagisawa[1]

Abstract

A new data processing method for a photo diode array (PDA) detector, Intelligent Dynamic Range Extension Calculator (i-DReC) enables the automatic calculation of peak area and height, utilizing spectrum similarity in the high concentration range where UV signal is saturated. When the integrated chromatographic peak area exceeds a user-defined threshold value, i-DReC automatically shifts the chromatographic profile to a wavelength with less UV absorption to prevent signal saturation. The absorption ratio between the original target wavelength and the wavelength used by the i-DReC function is applied as a correction factor to the peak area of the acquired chromatogram, thereby calculating the peak area and height at the original target wavelength. The i-DReC dramatically extends the linear dynamic range of calibration curves, enabling reliable quantitation of high concentration samples without need for sample dilution and reinjection, which would otherwise be required.

Keywords:PDA data processing, dynamic range extension, Nexera X2, UHPLC

1. Basic Principle of i-DReC

High concentration samples can produce saturated UV spectral absorbance, which significantly affects peak area calculation and causes a loss of linearity in the relationship of peak area to concentration. The i-DReC calculates an absorbance ratio between the original target wavelength and another wavelength that provides less absorbance in a spectrum on the down-slope of the chromatographic peak where neither wavelength's absorbance is saturated. The corrected peak area and height are then calculated by multiplying the measured peak area and height by the absorbance ratio.

■Calculation Algorithm

1. i-DRec is automatically applied when the intensity of a target peak exceeds the user-defined threshold value. If the threshold value is not exceeded, i-DReC is not applied.
2. The wavelength used for correction by i-DReC (λb) can be set either manually or automatically. When set manually, λb is a user-defined parameter. When set automatically, λb is determined as follows:
   - A UV spectrum is acquired at the retention time of the target peak.
   - The spectrum is analyzed to determine an appropriate wavelength for which the absorbance is not saturated, which is then set as λb.
3. The chromatogram at λb is extracted from the 3D data and integrated to determine peak area and height.
4. A UV spectrum is extracted from the chromatogram (at the original target wavelength (λa)) at a point on the down-slope of the peak (between the peak apex and peak end) where the absorbance of neither λa nor λb are saturated.
5. An absorption ratio (k) is calculated from the spectrum in (4). The intensity (Ia) of the spectrum at λa is divided by the intensity (Ib) of the spectrum at λb, as follows:

   k=Ia/Ib
6. Peak area and height of the measured peak in the chromatogram at λb are corrected by the absorption ratio to determine the effective area and height at λa, as follows:
   Peak area at λa = (peak area at λb) × k
   Peak height at λa = (peak height at λb) × k

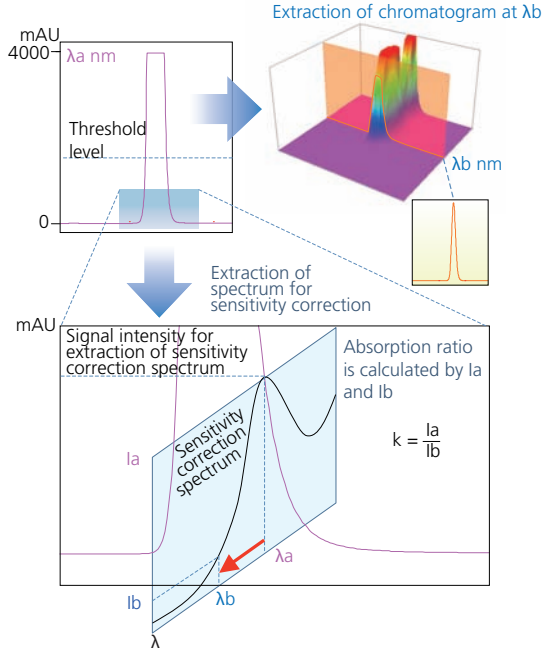


Fig. 1 Basic principle of i-DReC

1. Analytical & Measuring Instruments Division

2. Examples of i-DReC Applications

2-1. Extending the Linear Dynamic Range of Calibration Curves

This section demonstrates the extension of a calibration curve's linearity into a high concentration range, using standard solutions of Rhodamine with concentrations ranging from 0.01 g/L to 10 g/L. The following conditions were used for analysis.

Analytical Conditions

Pump	: Shimadzu LC-30AD×2
Detector	: Shimadzu SPD-M30A
Column oven	: Shimadzu CTO-20AC
Controller	: Shimadzu CBM-20Alite
Autosampler	: Shimadzu SIL-30AC
Mobile phase	: Ammonium formate buffer 45% / ACN 55%
Column	: Shimadzu Shim-pack VP-ODS (4.6 mmL. × 150 mmI.D., 5.0 µm)
Flow rate	: 1 mL/min
Column temp.	: 40 °C
Sampling	: 80 msec
Slit width	: 1 nm
Time constant	: 80 msec
Wavelength range	: 190 nm-700 nm
Cell light path	: 10 mm
Injection volume	: 2 µL

Fig. 2 shows the UV absorbance spectrum of Rhodamine, A Calibration curve was created based on peak area in the extracted chromatogram at 554 nm, the wavelength of maximum absorbance, and is shown in Fig. 3a. At 1 g/L or greater concentration, the calibration curve exhibits the loss of linear relationship between peak area and concentration.

Fig. 3b shows the same calibration curve with i-DReC applied to extend the linearity into the high concentration range. In this example, 347 nm was selected manually as the wavelength for correction, and the spectrum used for sensitivity correction was extracted at an intensity of 700 mAU. The original peak area and the corrected peak area calculated by i-DReC is shown in Table 1. After correction by i-DReC, the calibration curve based on the corrected peak areas exhibited excellent linearity with an unweighted correlation factor of 0.9999078 and 0.9995750 weighted by $1/(\text{concentration})^2$ over the concentration range of 0.01 g/L to 10 g/L.

Fig. 3C shows the error in concentration values obtained by inverse estimation using the i-DReC corrected calibration curve with weighting of $1/(\text{concentration})^2$. Even though the i-DReC corrected calibration curve extended the linear range of the original calibration curve by an order of magnitude, over the full range of concentration, the error in calculated concentration value was within 5%.

Table 1 Calibration points of Rhodamine samples

#	Conc.(g/L)	Peak area average (uAUsec) (n=2)	
		Original	i-DReC
1	0.01	267,847	267,847
2	0.02	544,266	544,266
3	0.08	2,089,341	2,089,341
4	0.1	2,622,781	2,622,781
5	0.2	5,255,999	5,255,999
6	0.5	12,072,748	**12,282,271**
7	0.8	18,539,104	**19,887,814**
8	1	21,823,608	**24,644,792**
9	2	33,708,885	**49,250,552**
10	5	53,883,445	**126,813,723**
11	8	65,182,276	**198,990,013**
12	10	71,500,307	**245,336,353**

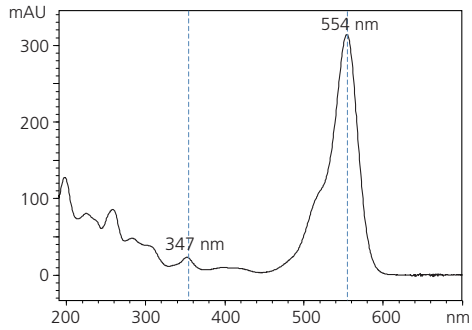
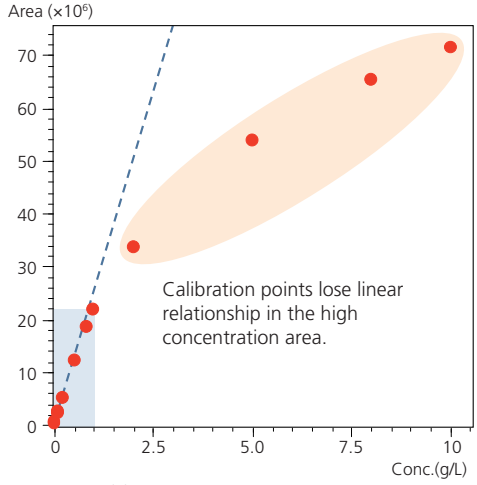
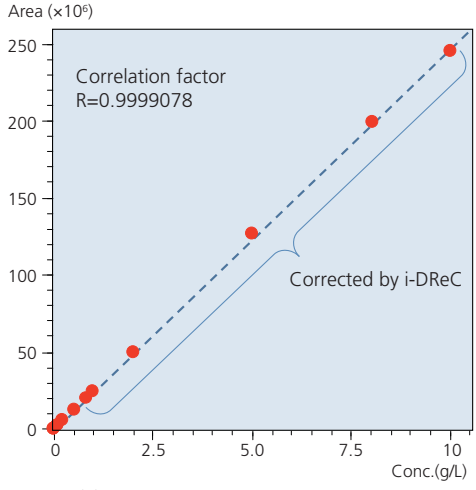


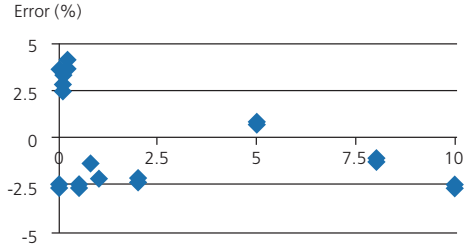
Fig. 2 Spectrum of Rhodamine



(a) Calibration curve at 554 nm



(b) Linearity range extended by i-DReC



(c) Error of corrected calibration points

Fig. 3 Calibration curve of Rhodamine

2-2. Simultaneous Quantitation of Main Component and Minor Impurities

This section demonstrates the use of i-DReC to simultaneously quantify a relatively high concentration major component in a pharmaceutical sample and the relatively low concentration impurities. Samples in which the concentration of the main component ranged from 0.01 g/L to 1 g/L where analyzed using the SPD-M30A photodiode array detector equipped with the high sensitivity cell. Fig. 4 shows the calibration curve for the main component based on peak area in the extracted chromatogram at 250 nm.

Analytical Conditions

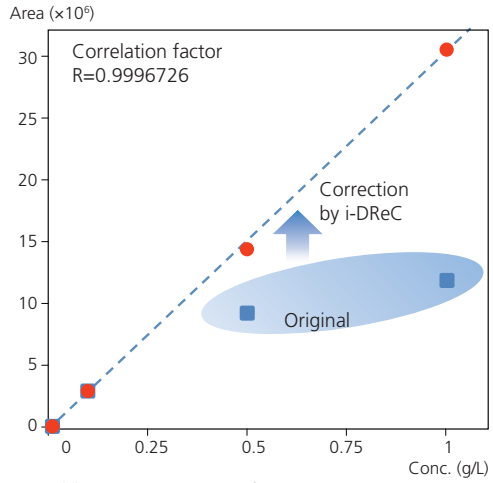
Pump	: Shimadzu LC-30AD×2
Detection	: Shimadzu SPD-M30A
Column oven	: Shimadzu CTO-20AC
Controller	: Shimadzu CBM-20Alite
Autosampler	: Shimadzu SIL-30AC
Column	: Shimadzu Shim-pack XR-ODS (150 mmL. × 3.0 mmI.D., 2.2 µm)
Mobile phase A	: 5% MeCN + 0.05% TFA
Mobile phase B	: 95% MeCN + 0.05% TFA
Time program	: 2% (0–1.2 min) → 2–98% (1.2–8.9 min) → 98% (8.9–10.8 min) → 98–2% (10.8–11.1 min) → STOP (14 min)
Flow rate	: 1 mL/min
Column temp.	: 40 °C
Sampling	: 160 msec
Slit width	: 8 nm
Time constant	: 160 msec
Wavelength range	: 190 nm-700 nm
Cell light path	: 85 mm
Injection volume	: 1 µL

As shown in Fig. 4a, calibration points for concentrations above 0.5 g/L deviate from linear relationship. In this example, 280 nm was selected manually as the wavelength for correction, and the spectrum used for sensitivity correction was extracted at an intensity of 200 mAU. The absorption ratio was calculated and used to correct the peak areas and extend the linear dynamic range of the calibration curve, resulting in a correlation factor R of 0.9996726 weighted by 1/(concentration)^2 over the concentration range of 0.01 g/L to 1 g/L.

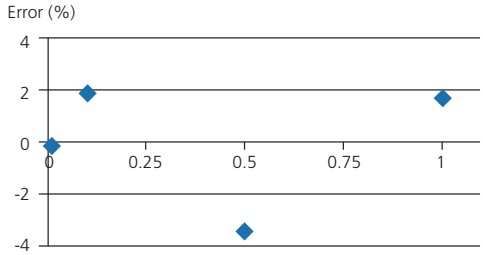
Fig. 4b shows the error in concentration values obtained by inverse estimation using the i-DReC corrected calibration curve with weighting of 1/(concentration)^2 is within 4%. The reproducibility of the peak area (n=6) of the main component and impurities, as well as the peak area ratio between the main component and impurities, is shown in Table 2. An example chromatogram for the sample is shown in Fig. 5. In this example, i-DReC was only applied to correct the peak area of the main component, which provided a saturated signal at 250 nm.

Table 2 Peak area reproducibility of the pharmaceutical sample

Peak	Retention Time(min)	Mean Area (µAUsec)	Area %RSD	Area Ratio(%)
①Main	4.634	31,123,746	0.06	--
②	5.448	925,522	0.12	2.974
③	3.900	64,161	0.08	0.206
④	4.910	32,810	0.15	0.105
⑤	5.091	15,103	0.16	0.049
⑥	4.487	9,487	0.26	0.030
⑦	4.226	7,981	0.28	0.026
⑧	4.975	7,981	0.44	0.026
Imp1	4.056	2,001	0.27	0.006
Imp2	4.331	2,440	0.85	0.008
Imp3	4.376	1,663	0.65	0.005



(a) Calibration curve of the main component



(b) Error of corrected calibration points

Fig. 4 Linearity evaluation

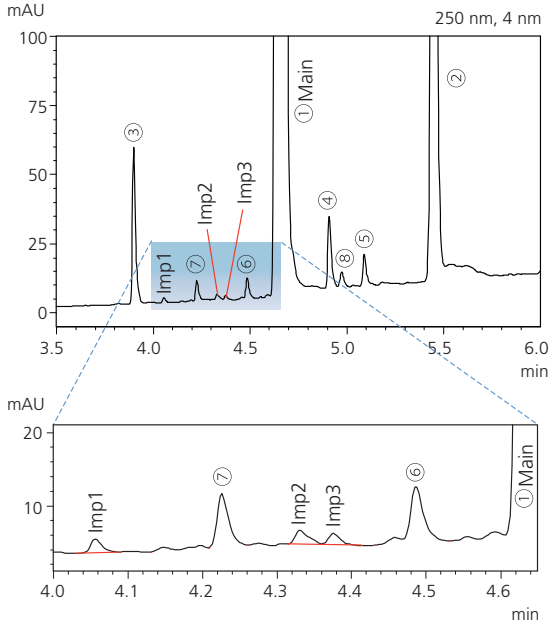


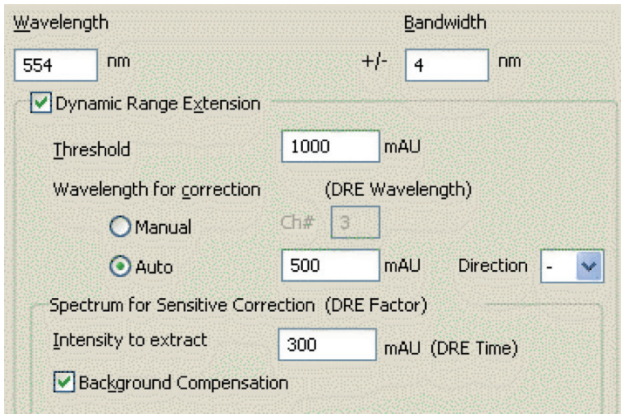
Fig. 5 Chromatogram of the pharmaceutical sample

As shown in Table 2, i-DReC correction provided peak area reproducibility of 0.06% RSD for peak 1, the main component. Peak area reproducibility for impurity peak 3 (Imp3), whose peak area was 0.005% of the main component, was less than 1%.

3. Summary of i-DReC Settings

i-DReC parameters are set as part of the data processing parameters for the photodiode array detector and can be applied in data processing methods for routine analysis without requiring post-run operations. The following is a summary of parameters and their descriptions.

1. i-DReC parameters are set in the multi chromatogram table.



Parameter	Description
Dynamic range extension	Select whether i-DReC is applied or not.
Threshold	When intensity at peak top is over the threshold, i-DReC is applied to the peak for correction.
Wavelength for correction (manual/auto)	Select whether wavelength for correction will be set manually or automatically.
Ch#	When "Manual" is selected, set the channel number of the chromatogram that will be used.
Intensity for correction wavelength	When "Auto" is selected, set the target intensity for determining an appropriate correction wavelength.
Direction (+/-)	When "Auto" is selected, set the direction to search for an appropriate correction wavelength. (+ = longer wavelength, – = shorter wavelength)
Intensity to extract	Set intensity for extraction of sensitivity correction spectrum.
Background compensation	Select whether background compensation is used or not for sensitivity correction spectrum.

The concentration range over which i-DReC effectively extends the linear dynamic range is dependent upon the shape of the spectrum of target peaks. The lower the slope of the spectrum around the correction wavelength, the more reliable the correction.

2. A mark indicating whether i-DReC has been applied, the wavelength used for correction, the retention time of the spectrum used for sensitivity correction, and the calculated sensitivity correction factor can be shown in the peak table and compound table.

Item	Description
Mark	C = peaks to which i-DReC was applied E1-E4 = error in i-DReC calculation
DRE wavelength	For peaks to which i-DReC was applied, this is the wavelength used for correction.
DRE factor	For peaks to which i-DReC was applied, this is the absorption ratio used for sensitivity correction.
DRE time	For peaks to which i-DReC was applied, this is the retention time of the spectrum used for sensitivity correction.

i-DReC requires that spectrum similarity is maintained across the peak. When peak separation is insufficient, i-DReC may not be able to be applied.

3. The peak area and height corrected by i-DReC can be used in normal quantitation processes. The simple implementation allows the seamless use of i-DReC for routine analysis.

4. Conclusion

i-DReC's ability to calculate corrected peak area and height for high concentration samples is made possible by the improved performance of the SPD-M30A photodiode array detector and the excellent reproducibility of the Nexera X2 system. The key features of i-DReC can be summarized as follows.

- Extension of the linear dynamic range using spectral similarity.
- Simultaneous quantitation of both low and high concentration compounds in a single injection.
- Requires the use of only one PDA detector.
- Standard samples are not necessary for correction.
- Simple method settings allow the use of i-DReC in routine analysis.

i-DReC can be applied to samples containing a wide range of compound concentrations. The use of i-DReC improves the efficiency of sample pretreatment processes and laboratory productivity.

Posters from Recent Conferences

These posters were presented at ASMS 2013 (June 9-13, 2013, Minneapolis) and HPLC 2013 (June 16-20, Amsterdam). Click the title URLs to download the posters of interest.

Poster 1 ASMS

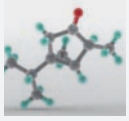
Development and Validation of LC/MS/MS Method with Ultra Small-Volume Injection for Quantitative Determination of Alprazolam in Human Plasma

An LC/MS/MS method with an ultra small-volume injection for quantitative determination of alprazolam in human plasma has been developed and validated using the Shimadzu LCMS-8080. The results indicate that the extra small injection volume LC/MS/MS method did not change matrix effect behavior but greatly reduced contamination of the dirty plasma matrix to the interface and ion optics. As such, the method is more robust and suitable for bioanalysis in heavily loaded research and service laboratories. The method also makes it possible to handle a very small amount of available sample due to limited sample sources.

Poster 2 ASMS

Development of high speed CYP cocktail inhibition assay using UHPLC-MS/MS

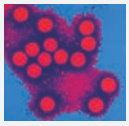
A high-speed CYP cocktail inhibition assay using Nexera UHPLC combined with the LCMS-8080 has been developed. With the conventional method using Shim-pack XR-ODS II columns (flow rate at 0.2 mL/min), all compounds eluted within 4.5 min (the cycle time was 7 min). In contrast, the UHPLC method successfully reduced the cycle time to within 60 seconds without sacrificing the data quality as the next sample was injected by overlapping with the last part of the previous analysis. UHPLC-MS/MS improves the throughput 7 times compared with a conventional system.



Poster 3 ASMS

Characterization of products formed by forced degradation of Amlodipine Besylate using LC/MS/MS

Amlodipine was degraded under three different conditions and degradation products were studied. Structures were predicted using fragmentation patterns. Initially, a chromatographic method was optimized using the gradient mode for Amlodipine degradation products. The ESI MS/MS parameters were optimized, both for ionization source and collision energies, for the observed degradation products. Depending on the degradation type, possible chemical reactions were predicted and supported by fragmentation information from the LCMS-8080.



Poster 4 ASMS

Detecting Nucleoside Post Enzymatic Cleavage Modifications in RNA Using Fast Product Ion Scanning Triple Quadrupole Mass Spectrometry

Scientists rely on LC-MS-MS for detection and quantitation of nucleosides. LC-MS-MS methods use MRM analysis for the highest sensitivity and selectivity; however, these methods only detect analytes whose MRM transitions are known in advance. Modified nucleosides are not easily identified by traditional MRM-based methods as many species share common precursor and product ions. We developed LC-MS methods that utilize extremely fast product ion scanning for detection of nucleosides obtained from commercial suppliers. Product ion spectra for each known nucleoside were acquired and inspected for common product ions. A mixture of modified nucleoside standards was analyzed as well.

Poster 5 ASMS

Identification of antibacterial component from extract of *Garcinia indica* fruit rinds using LC/MS/MS

Ionization of the sample for mass spectrometric analysis was carried out using the DUIS mode available in the LCMS-8040. Here, both Electro-Spray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) techniques are used simultaneously; hence, polar to slightly mid-polar molecules can be analyzed in the same run. The LCMS-8040 has an 'optimization of method' feature in which the mass spectrometer selects the best product ion(s) and optimizes voltages and collision energies for the precursor to product transition.

Poster 6 ASMS

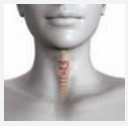
Study of antibacterial activity of Essential Oil components obtained from pericarp of *Zanthoxylum rhetsa* (Indian origin) using HS-GCMS

Initially, GCMS parameters were optimized by using direct liquid injection of EO. The individual components found in EO were separated on Rtx-5Sil MS column. The n-alkane standard (C-7 to C-35) was injected for determination of LRIs of the components. The FFNSC library with LRIs was used for qualitative confirmation. The headspace parameters for the EO in presence of complex matrix like diluent, broth and culture were optimized.

Poster 7 ASMS

Structural Elucidation of N-glycans Originating From Ovarian Cancer Cells Using High-Vacuum MALDI Mass Spectrometry

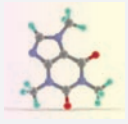
MS2/MS3 was able to characterize with high detail the N-glycans of ovarian cancer cells and identify the different isobaric N-glycans occupying a single MS1 peak. Furthermore, with controlled high-energy fragmentation, MS3/MS4 was able to confirm and characterize antenna modifications such as sialylation and fucosylation based on diagnostic ions.



Poster 8 ASMS

Sensitive assay of free thyroid hormones by on line SPE-UHPLC-MS/MS in human plasma

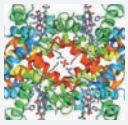
Because of the low limit of quantification to attain, particular attention was given to extraction recovery and ion suppression. To improve assay ruggedness and accuracy, deuterated analogues were used as internal standards. Mobile phase composition was optimized to generate the highest ionization efficiency. Both positive and negative ionization were investigated.



Poster 9 ASMS

Fragmentation Outcome Modelling: Prototype software for prediction of CID fragment ions for small molecule structures

The fragmentation of each molecule was unique with each fragment ion being a unique mass. For comparative purposes the total number of fragment ions successfully predicted for each software package is expressed as a percentage of the total number of experimental fragment ions. Results are presented as per the above order. Fragmentation Outcome Modeling was the only application to successfully predict every fragment ion for each of the three examples given here.



Poster 10 ASMS

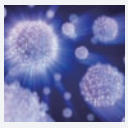
Development and evaluation of Nano-ESI coupled to a triple quadrupole mass spectrometer for quantitative proteomics research

Digested BSA (Bovine Serum Albumin) was analyzed to optimize the conditions of the LC and the triple Q MS parameters. In summary, we improved the sensitivity of the triple Q MS by reducing the LC flow rate to nL/min. A few femtomoles of injected peptides could be analyzed by nano-flow LC-MS. In this study, we developed and evaluated a novel quantitative system for proteomics with the results suggesting that this system could be applied to shotgun proteomics.

Poster 11 **ASMS**

Strategies for structure elucidation using Ultrafast Mass Spectrometry (UFMS): Using nMS^2 as an alternative to MS^3

We have applied UFMS to generate multiple product ion spectra (PIS) across a UHPLC peak with CID at varying energies. The nMS2 approach yields an array of MSMS spectra derived from the same parent ion but exhibiting mechanistically distinct fragment ions. UFMS has enabled on-the-fly detection and identification of novel compounds (e.g. metabolites) with arrayed MS2 data. Unlike MSn, the nMS2 approach is not subject to the transfer loss limitations on sensitivity because the same parent ion is selected from the first mass filter rather than from collisional debris.



Poster 12 **ASMS**

Novel bacterial classification method by MALDI-TOF MS based on ribosomal protein coding in *S10-spc-alpha* Operon at Strain level

We developed a novel bacterial classification method by MALDI-TOF MS using ribosomal subunit proteins coded in S10-spc-alpha operon as biomarkers, because the operon encodes half of the ribosomal subunit and can be easily sequenced using conventional DNA sequencer. Now we are trying to differentiate other bacterial species that are difficult to discriminate using the mass fingerprinting method. These bacterial species under our evaluation are important in the area of food safety so we expect that our trial will contribute to this area.

Poster 13 **HPLC**

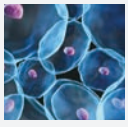
Novel Methods for Detection and Quantitation of Impurities Using a New High Sensitivity Photodiode Array Detector

The presence of impurities in commercial products may seriously affect their quality, resulting in more stringent requirements for impurity monitoring. For this reason, researchers require a more sensitive UHPLC detector. We have developed a new high-sensitivity photodiode array detector with novel data processing methods to detect and quantitate very trace amounts of impurities present in complex samples.

Poster 14 **HPLC**

Screening of 25-OH Vitamin D2,D3 using Shimadzu LCMS-8040

Hypovitaminosis D is a relatively common problem traditionally manifesting in the elderly population and in people with severe liver or kidney disease. Inadequate levels of vitamin D can lead to increased risk of cancer, diabetes mellitus, chronic pain, and hypertension. We evaluated Shimadzu's Triple Quadrupole LCMS-8040 using different commercial kits available for detection of 25-OH Vitamin D and a home-brew method using a considerable number of biological samples.

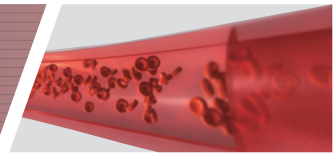


Poster 15 **HPLC**

Glycerophospholipids Analysis by Two-Dimensional Liquid Chromatography Coupled with a Triple Quadrupole Mass Spectrometer

Glycerophospholipids (GPLs) are the major component of biological membranes. They can not only act as a barrier from the external environment, but can also play a key role in a variety of biological processes including membrane trafficking and signal transduction. Thus, analysis of GPLs is one of the most important studies in the metabolomics field. We established a new strategy using two-dimensional liquid chromatography (2D-LC) coupled with ESI-MS/MS (2D-LC-ESIMS).

High-Throughput Bioanalysis with On-Line Sample Pretreatment Using Restricted Access Media The Shimadzu Co-Sense for BA System Solution



1. Introduction

Because of its high sensitivity and selectivity, analysis via LC/MSMS is now widely used in the context of pharmacokinetic research, drug metabolism research, bioequivalence tests, and other measurements of drug concentrations in biological samples (bioanalysis). However, in more than a few situations, there are problems with impurities such as proteins originating from organisms; ionization suppression caused by matrix effects; ionization enhancement; degradation of precision, accuracy, and reproducibility; and carryover. Accordingly, sample pretreatment methods have become a very important topic of investigation. Conventionally, the following have been utilized as serum and plasma pretreatment methods, primarily with the objective of deproteinization.

1) Addition of acids or organic solvents to denature proteins and render them insoluble, for removal by centrifuging
2) Physical removal using ultrafiltration membranes
3) Off-line pretreatment using solid phase extraction cartridges
4) On-line pretreatment using solid phase extraction columns

In method (1), it is important to evaluate whether the drugs are denatured, while in method (2), adsorption by raw materials must be evaluated. When considering treatment time, cost, and data reproducibility, method (4), which can be implemented on-line, seems the most likely to increase overall productivity.

In terms of pretreatment columns in method (4), in addition to reversed phase mode, normal phase mode, and ion exchange mode, columns classified by restricted access media (RAM) are now utilized. Product types have also increased, and have been subject to review [1], [2]. RAM is a highly functional packing material, designed so that the outer surface of the packing particles removes proteins and other biological macromolecules, restricting their internal penetration. Conversely, the internal pores or inner surface captures the target low molecular compounds.

In conjunction with Eisai Co., Ltd., Shimadzu has developed and commercialized unique restricted access type trap columns (Shim-pack MAYI series) classified as RAM, and an LC system with on-line pretreatment functionality via valve switching (Co-Sense for BA). The Co-Sense for BA allows large volumes of plasma, serum and other biological samples to be injected and analyzed. High-concentration proteins and electrolytes, which are unnecessary components, are almost completely removed. Since pretreatment involves direct injection, there is no loss of valuable samples, and stable data can be obtained even with unstable components. Further, the adoption of dilution trap method maintains peak shape and enhances sensitivity by concentrating the intended components, thereby providing stable recovery rates and reproducibility [3], [4], [5], [6], [7]. These features effectively result in the protection of analysis columns and the LC/MS interface, as well as the reduction of matrix effects, enabling the acquisition of efficient, highly reliable results [8].

Note: "Co-Sense for BA", the name of this product, is an abbreviation of "the Collaboration of Shimadzu and Eisai for New Systematic Efficiency." "Co-Sense" refers to "collaboration of sensibilities" the concept behind this product. This refers to the integration of user and manufacturer to create new value. In addition, the MAYI pretreatment column is named using the initials of the four researchers at Eisai responsible for developing it.

2. Methylcellulose-Immobilized Pretreatment Column, Essential to Co-Sense for BA

Operating Principles Behind the Shim-pack MAYI Series of Biological Sample Pretreatment Columns

The outer surface of the silica gel (50 um) is coated with a water-soluble polymer (Methylcellulose). Only the pore interior is chemically modified by octadecyl or other functional groups [5]. Macromolecules such as proteins are blocked by the water-soluble polymer layer on the outer surface and do not enter the pores. As a result, they are not retained by the stationary phase, and are quickly eluted. In contrast, drugs and other typical organic low molecular compounds penetrate into the pores, and are retained by the stationary phase on the inner surface. They are then eluted under appropriate conditions and analyzed with the analysis column. Fig. 1 shows the behavior of drugs (low-molecular compounds) and proteins at the surface of the packing material in the Shim-pack MAYI-ODS.

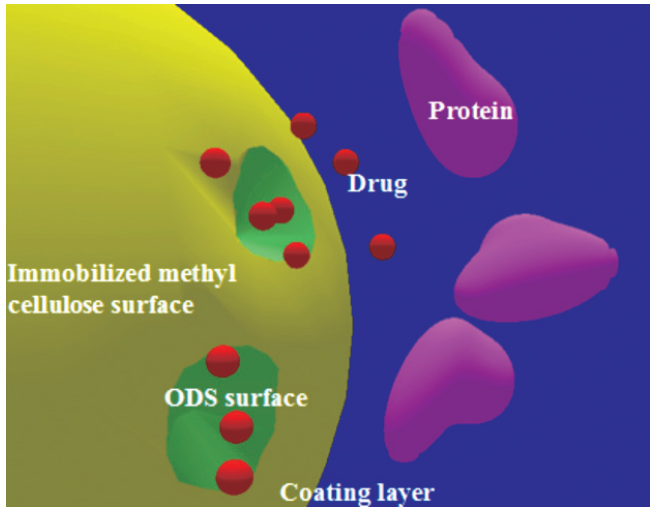


Fig. 1 Behavior of Drugs (Low-Molecular Compounds) and Proteins at the Surface of the Shim-pack MAYI-ODS

In addition to five reversed phase separation modes, including ODS, the Shim-pack MAYI series provides a strong cation exchange mode and a strong anion exchange mode (Table 1).

Table 1 Stationary Phases and Separation Modes in the Shim-pack MAYI Series

Product Name	Stationary Phase	Separation Mode
Shim-pack MAYI-ODS(G)	Octadecyl	Reversed Phase
Shim-pack MAYI-C14(G)	Tetradecyl	Reversed Phase
Shim-pack MAYI-C8(G)	Octyl	Reversed Phase
Shim-pack MAYI-C4(G)	Butyl	Reversed Phase
Shim-pack MAYI-C1(G)	Methyl	Reversed Phase
Shim-pack MAYI-SCX(G)	Sulfonate	Strong Cation Exchange Mode
Shim-pack MAYI-SAX(G)	Trimethylammonium	Strong Anion Exchange Mode

The stationary phase is selected in accordance with the polarity of the target compound. However, the type of extraction solvent from the column and the trap dilution solvent must also be investigated [5], [6], [13]. In investigations utilizing the strong cation exchange mode (SCX), a variety of evaluations have been performed. These include the recovery rate of differing pKa compounds in plasma, evaluation of the decrease in matrix effects in MS detection, and evaluation of metabolites in rat bile through combination with a C4 column [7], [8], [9]. In investigations utilizing the strong anion exchange mode (SAX), results have been evaluated related to the analysis of aspirin and its metabolites (such as acetylsalicylic acid) in rat plasma [10].

With an optimized particle size and a special coating technique, the Shim-pack MAYI column provides a high protein removal efficiency and long-term stability. In addition, by increasing sample permeability, the occurrence of pressure increases (clogging) due to adsorption of sample components has been suppressed, and excellent reproducibility is shown, even in consecutive multi-sample analysis. Fig. 2 shows the changes to the chromatogram when an isopropylantipyrine sample spiked with plasma is injected 300 times. Essentially the same chromatogram is obtained, even after 300 injections. This series consists of cartridge type columns, which are attached to a special column holder.

In addition to these, Eisai has also evaluated weak cation exchange mode columns [11].

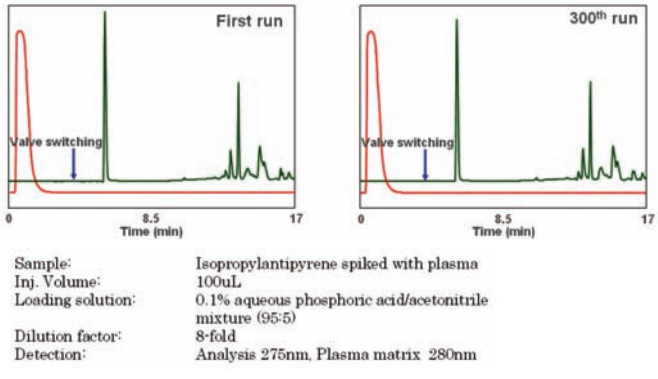


Fig. 2 Evaluation of the Durability of the Shim-pack MAYI-ODS

3. Mechanism behind Co-Sense for BA

Fig. 3 shows the flow schematic for Co-Sense for BA. The standard configuration adopts a sample injection method based on the dilution trap method, to ensure reliable trapping and concentration of intended components.

While the sample injected from the autosampler is introduced to the trap column via the mobile phase from the pump unit for sample injection (a), it is diluted automatically by the dilution solvent delivered from the pump unit for on-line dilution (b), and introduced to the trap column, the Shim-pack MAYI column (c). Thanks to this dilution process, interactions with the matrix and the impact of the sample solvent are suppressed, while the intended components are introduced to the trap column, thus providing reliable trapping and concentration. A liquid containing a buffer solution and a low-concentration organic solvent is used as the dilution solvent.

Intended components concentrated in the trap column are introduced to the analysis line via valve switching. The mobile phase from the pump unit for analysis (d) then delivers them to the analysis column (e) where they are separated and analyzed. In addition to UV and PDA detectors, MS detectors can also be connected.

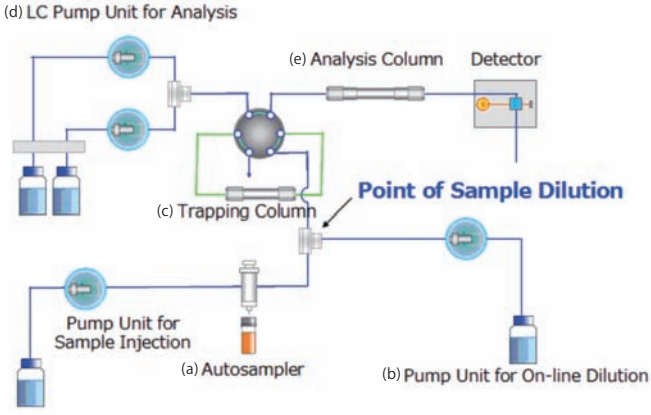


Fig. 3 Flow Schematic for the Standard Configuration of Co-Sense for BA

4. Effective Trapping and Concentration Using a Dilution Solvent

With Co-Sense for BA, the injected sample is diluted at a constant rate. This is performed to suppress interactions between intended components and proteins in the sample, and to release intended components bound to proteins. In this way, even components with a high binding rate are reliably trapped and concentrated. A liquid containing a buffer solution and a low-concentration organic solvent is used as the dilution solvent. As a result, even plasma and serum samples can be injected directly. In addition, high sensitivity is achieved due to excellent recovery rates and peak shapes, even for large-volume injections [6], [7], [9], [10], [12], [13].

Fig. 4 shows a comparison of recovery rates based on the presence or absence of on-line dilution, utilizing indomethacin samples spiked with plasma. With dilution, the recovery rate is almost 100 %. Without dilution, the recovery rate falls to approximately 50 %.

Fig. 5 shows the recovery rates and peak shapes when a dilution trap is used with indomethacin samples likewise spiked with plasma, and large-volume 100 µL, 200 µL, and 500 µL samples are injected. Even with the 500 µL injection, a recovery rate of almost 100 % is achieved, and the peak shape is maintained.

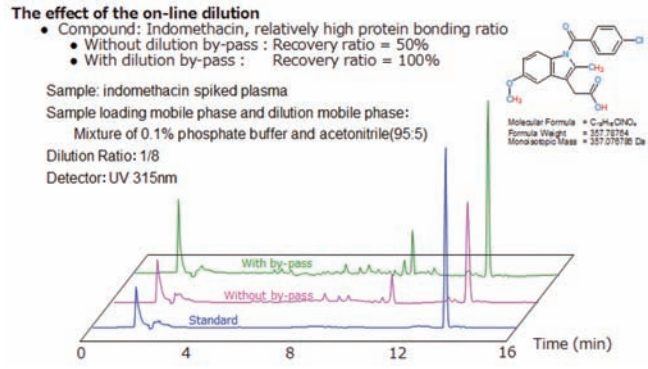


Fig. 4 Effect of On-Line Dilution

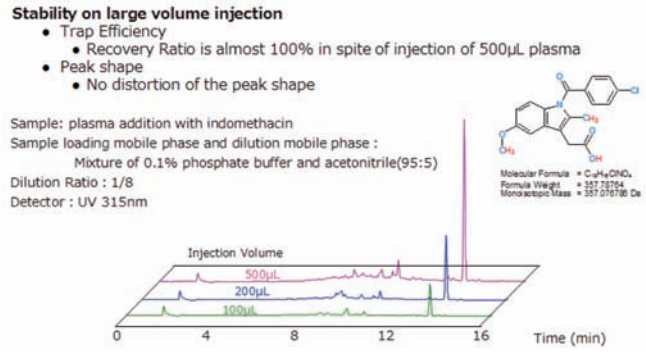


Fig. 5. Stability for Large-Volume Sample Injections

5. Method Validation in Bioanalysis

At the FDA and EMA, guidance and guidelines are enacted with respect to the validation of methods for analyzing the concentration of drugs in biological samples (Bioanalytical Method Evaluation: BMV). In July 2013, the Japanese Ministry of Health, Labour and Welfare also issued BMV guidelines. The thinking behind ISR (Incurred Samples Reanalysis: evaluation of reproducibility for measurement values from actual samples) and other aspects of the European and American guidelines were introduced, and its importance emphasized.

At first glance, Co-Sense for BA appears to be a complicated system, and there might be qualification-related concerns. However, utilization of this system not only concentrates samples but also reduces matrix effects in MS analysis. As a result, in terms of the ongoing issue of method validation, the attainment of more reliable results is anticipated. In addition to generally enforced accuracy, precision, reproducibility, and linearity evaluations, both between-day and within-day variance have also been evaluated, and excellent results have been obtained [7], [8], [10], [12], [13].

6. Use in Other Applications

6.1 Application to Cleaning Validation

Cleaning validations for pharmaceutical production equipment require high-sensitivity analysis. However, the samples used for measurement are often dilute organic solvents. As a result, pretreatment to concentrate samples is required; this can sometimes lower throughput. In cleaning validations, Co-Sense for BA enables direct, large-volume injection without concentrating the samples, which heightens efficiency.

In reversed phase mode, when organic solvents and other solvents with strong elution properties are injected in large volumes as the sample solvent, peak shapes tend to worsen and there may be problems with retention times. In cleaning validations for pharmaceutical production equipment, ethanol and other organic solvents are often used as the sample solvent, which hinders large-volume injection for high-sensitivity analysis. In addition, with ordinary trap injections, recovery rates may decrease due to elution of intended components from the trap column. As a result, in LC analysis for cleaning validation, the sample is often concentrated in advance, leading to decreased throughput.

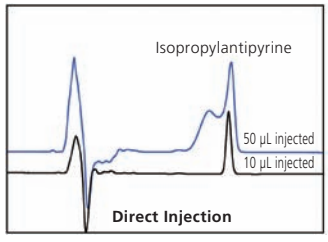


Fig. 6 Chromatograms for Isopropylantipyrine, with Direct Injection of an Ethanol Solution

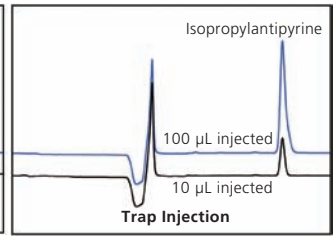


Fig. 7 Chromatograms for Isopropylantipyrine, Trapping Injection without Dilution

Fig. 6 shows the chromatograms when isopropylantipyrine/ethanol solutions are analyzed using direct injection, and when a trapping injection is used without on-line dilution. In large-volume (50 µL) injections via the direct injection method, the isopropylantipyrine peak is leading due to the impact of the sample solvent. In addition, in trap concentration without on-line dilution, elution from the trap column occurs due to the impact of the sample solvent, and the recovery rate decreases. (The peak for a 100 µL injection does not equal 10 times that of a 10 µL injection.)
In sample injections via the Co-Sense for BA dilution trap, even with this sort of large-volume injection of organic solvents, the sample is introduced to the trap column while on-line dilution is performed using water or a buffer solution. As a result, reliable trap concentrations are possible even if guard and other generic columns are used as the trap column. In addition, the intended components are introduced to the analysis column by valve switching after concentration in the trap column, so excellent peak shapes can be obtained. The high-sensitivity analysis of isopropylantipyrine in ethanol is difficult for ordinary systems, but with Co-Sense for BA, an approximately 100 % recovery rate and excellent peaks shapes are obtained even with 100 µL injections. Fig. 8 shows the chromatograms when isopropylantipyrine/ethanol solutions are analyzed with Co-Sense for BA.

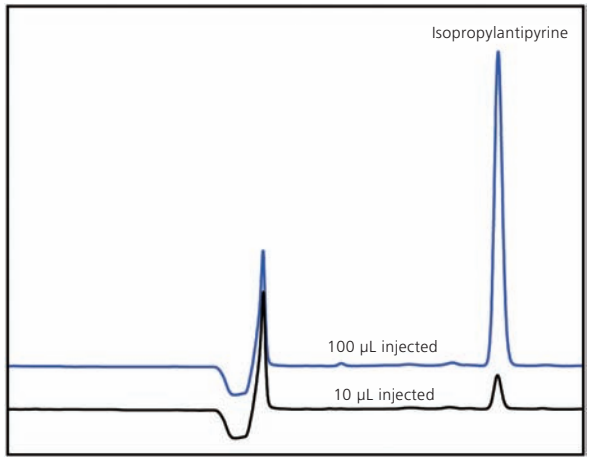


Fig. 8 Chromatograms for Isopropylantipyrine/Ethanol Solutions Using Co-Sense for BA

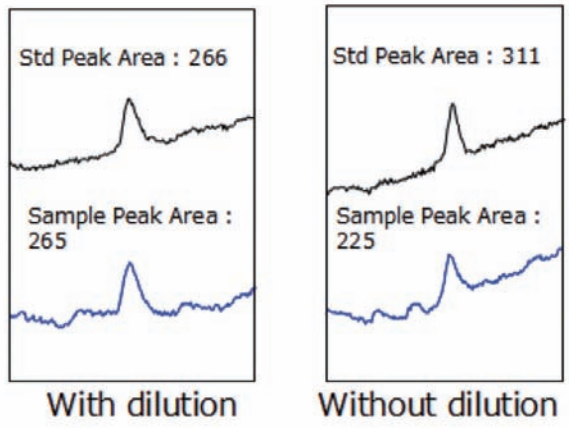


Fig. 9 Chromatograms for Indomethacin in an IgG Solution

6.2 Analysis of Free Bodies and Impurities in Antibody-Drug Conjugates

According to the FDA, investigational new drug (IND) applications for antibody-drug conjugates (ADC) have increased markedly since 2008, and profiles for free bodies and process-derived impurities are also being discussed [14].

In the analysis of impurities and free bodies contained in ADCs, deproteinization is required to eliminate high-concentration antibodies. If deproteinization is performed using organic solvents or acids as a sample pretreatment, the impact of the sample solvent may worsen peak shapes, and enhancing the sensitivity via large-volume injections is often difficult. In addition, for samples with a high organic solvent ratio after deproteinization, the recovery rate may decrease with the ordinary trap injection method due to the impact of the sample solvent. Furthermore, if on-line pretreatment (on-line solid phase extraction) is performed in order to automate the deproteinization, the recovery rate for the intended impurities may decrease due to antibody and drug-derived impurity interactions.

With the Co-Sense for BA dilution trap method, components can be trapped reliably, even with large-volume injections, to enhance sensitivity, thanks to antibody removal (deproteinization) and the suppression of antibody and impurity interactions. Fig. 9 shows the chromatograms from an analysis of indomethacin in IgG using Co-Sense for BA. The sample is an acetonitrile solution of 1 mg/mL IgG and 2.5 ng/mL indomethacin. (The molar ratio of the indomethacin added is approximately 0.1 % that of the antibodies.) The recovery rate when diluted is 99.6 %. However, the recovery rate drops to 72.3 % when not diluted. Utilizing on-line dilution breaks the non-covalent bonds, avoiding a decrease in the recovery rate.

The following shows an example of the analysis of indomethacin-derived impurities. Fig. 10 shows the chromatogram from an analysis of indomethacin utilizing an ordinary LC system. Impurities of 0.07 % are detected. Fig. 11 shows the chromatogram from the analysis of a sample mixture of indomethacin and IgG in a 1:1 molar ratio using Co-Sense for BA. The impact of the antibodies is eliminated, the recovery rate is 100 %, and a CV of 2.8 % is achieved. In addition, using LC/MS for detection makes it possible to reliably identify the presence of drug-derived impurities, even in ADCs.

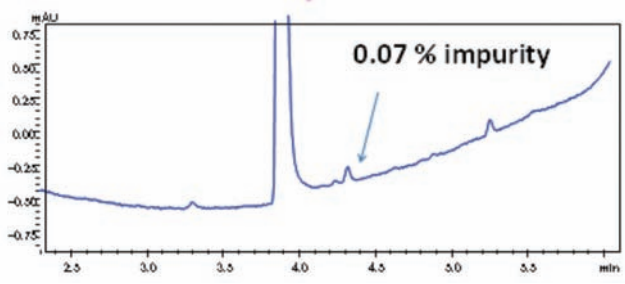
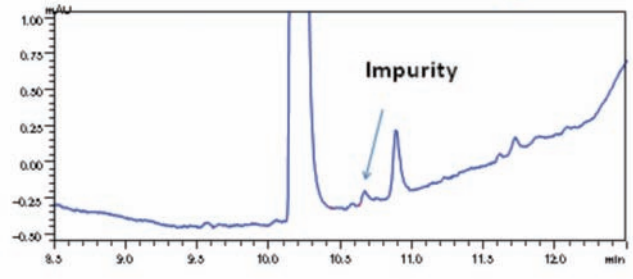


Fig. 10 Chromatogram for Indomethacin Using Normal LC Analysis



On-line pretreatment (deproteination & trapping) Condition
Trapping column : Shim-pack MAYI-ODS(4.6 mmi.d.X10 mmL., 50 µm)
Trapping solvent : water/acetonitrile/formic acid=900/100/1
Trapping flow rate : sample injection:0.3 mL/min dilution:2.7 mL/min (10 times dilution)
Sample injection volume : 100µL
Trapping time : 5 minuites

Separation Condition
Column : ODS (4.6 mmi.d.X50 mmL., 2.7 µm)
Mobile phase A : 0.1% Formic acid/water
Mobile phase B : 0.1% Formic acid/acetonitrile (Gradient)
Flow rate : 0.85 mL/min
Column temp. : 40℃
Detection : PDA 320 nm

Fig. 11 Chromatogram for Indomethacin in IgG Using Co-Sense for BA

At Eisai, Yamamoto et al. analyzed a liposome preparation with an SPE-SPE-HPLC system utilizing MAYI-ODS. In this research, it was possible to simultaneously quantify liposomal doxorubicin and released doxorubicin in plasma, and this has even been applied to a PK study utilizing mice [15].

7. Ideal in Combination with Shimadzu UFMS Series LC/MS

Speed and Sensitivity Beyond Comparison

The LCMS-8050 triple quadrupole mass spectrometer is the top model in the Shimadzu UFMS series. It combines the world's fastest speeds with the highest class of sensitivity. It provides 5 msec ultra-fast positive/negative ionization switching and 30,000 u/sec ultra-fast scanning performance. As a result, quantitative and qualitative measurements can be performed in parallel in a single analysis by combining MRM and scan measurements. Quantitative accuracy is not lost even in such simultaneous MRM/scan measurements.

Accordingly, highly reliable quantitative results and reliable qualitative information can always be obtained. In addition to high quantitative throughput, the system also demonstrates its strengths in the process of screening and optimizing analysis conditions.

Furthermore, a contamination-resistant ionization interface has been adopted, and the design focuses on the robustness of the system as a whole. Fig. 12 is a plot of area values, in which plasma samples spiked with alprazolam were deproteinized, and then analyzed consecutively 1,000 times. From the 1,000 data cycles, an excellent area value reproducibility of %RSD 4.59 % was obtained.

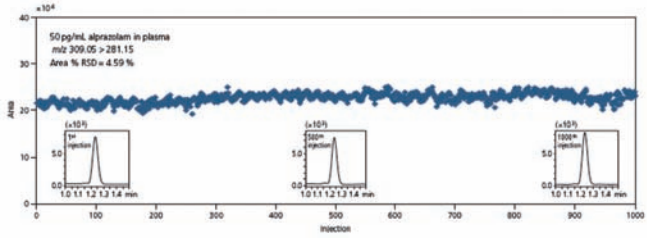


Fig. 12 1,000 Consecutive Analyses of a Deproteinized Blood Plasma Sample Spiked with Alprazolam (LCMS-8050)

In analyses involving plasma and other biological samples, ease of maintenance is an important point. With the LCMS-8050, the ESI capillary is easily replaced, and the desolvation line can also be replaced without compromising vacuum, keeping downtime to a minimum. In addition, Co-Sense for BA does not require special software, so with LabSolutions LCMS, everything from front-end LC to LCMS can be controlled centrally from a single PC.

8. Conclusion

Using a system combining the Shim-pack MAYI biological sample pretreatment column and Co-Sense for BA, plasma, serum and other biological samples can be injected directly in large volumes for analysis. High-concentration proteins and electrolytes, unnecessary components, are almost completely removed, and stable data can be obtained even for unstable components, with no loss of precious samples. In addition, the adoption of Shimadzu's proprietary dilution trap method maintains peak shape and enhances sensitivity by concentrating the intended components, thereby providing stable recovery rates and reproducibility. Furthermore, the system demonstrates its effectiveness in protecting analysis columns and the MS interface, and reducing matrix effects. In terms of items required for method validation, it contributes to the efficient attainment of highly reliable results. In the future, its application will likely extend to other types of analysis, including impurities related to ADCs and other biopharmaceuticals.

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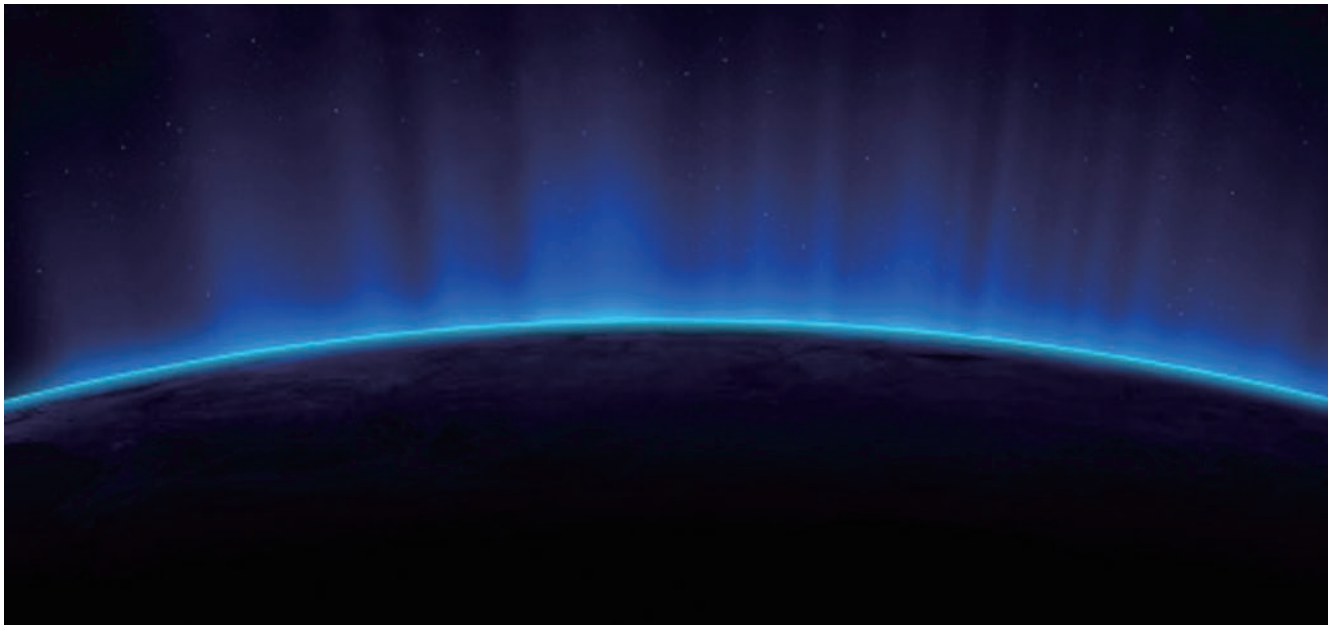
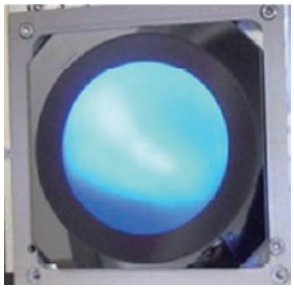


Shimadzu's Diffraction Grating Goes into Space

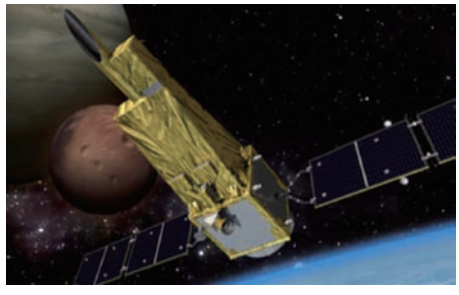
It was launched with the Spectroscopic Planet Observatory for Recognition of Interaction of Atmosphere (SPRINT-A) by the first Epsilon Launch Vehicle .

The Spectroscopic Planet Observatory for Recognition of Interaction of Atmosphere (SPRINT-A), named "HISAKI" later, is the world's first space telescope for remote observation of planets such as Jupiter, Mars, and Venus from the artificial satellite orbit around the earth. It was successfully launched by the Epsilon-1 rocket on September 14 and injected into the planned orbit with the completion of solar array paddle deployment, and sun acquisition.
Our diffraction grading is used in the spectrometer loaded onto "HISAKI". It has started to play an important role in observing extreme ultraviolet (EUV) light from the satellite, and studying the atmospheric outflow and the energy changes in the plasma environment by the interaction of the solar wind.
In November 2007, Shimadzu was asked to design a spectrometer for EUV spectroscopy and to develop and manufacture the diffraction grating for it. In the spring of 2011, we succeeded in achieving the requested diffraction efficiency in the entire range of wavelength and provided a toroidal concave CVD-SiC diffraction grating of the size of 85mm × 85mm × 12mm with the project team.
Visit the JAXA web site at

http://www.jaxa.jp/projects/sat/sprint_a/index_e.html



Shimadzu's diffraction grating (the blue circular portion) attached to a device to evaluate its performance



The spectrometer is equipped in the box on the right side of the cylinder cut off slantingly at the top (the image is from JAXA's HP).

Shimadzu's HMV-G Series Micro Vickers Hardness Testers Receive the 2013 Silver Award for Industrial Design for Analytical Instruments by Instrument Business Outlook.

Instrument Business Outlook (IBO) chose Shimadzu's HMV-G Series of Micro Vickers Hardness Testers as the recipient of its 2013 Silver Award for Industrial Design for Analytical Instruments(*) for their outstanding modern and bold design. With a curved frame softened by a bright color scheme, the HMV-G Series' appearance expresses both technical sophistication and robustness and accessibility.

The concept of the HMV-G Series' initial design was based on feedback from customers who wanted to measure different sizes and types of samples. As a result, the HMV-G Series' frame is not only a striking design feature but also offers a key user function. It has an expanded workspace depth and the opening at the center of the arc-type frame improves operability and visibility. This allows users to easily measure longer and larger-area samples.

Designing a compact instrument with this unique frame, while retaining the main performance features such as stability, was a significant challenge. The biggest difficulty was designing the frame for all kinds of samples to be tested easily and effectively. In order to make the HMV-G Series easy to use, these testers include such functions as automatic indentation-detection and automatic lens switching to avoid human measurement errors. They may also be used with a computer or LCD touch panel.

* IBO's Industrial Design Awards are presented yearly and recognize recently introduced products whose physical appearance and features make a distinct and valuable impression, thus communicating the product's functionality and value. The design elements of each system are integral to the product's purpose and its relationship with the user, particularly in creating an inviting and intuitive user experience.

To be eligible for a Design Award, a product must have begun shipping between August 2012 and July 2013. Award candidates are chosen from the new products that IBO monitors through trade shows, trade publications, press releases and the Internet. Award winners are selected solely based on their industrial design, not on technical capabilities or performance. Criteria include innovation, aesthetics, functionality and features.

IBO is a 12-page, subscription-based newsletter published twice monthly by Strategic Directions International, Inc. (SDi), a management and marketing research firm. SDi is the world's leading source of information on the market for analytical and life science instrumentation and equipment for laboratory and on-site applications. For more information, visit www.strategic-directions.com.

Collaboration with Analysis Center of Tsinghua University in China

Shimadzu has been collaborating with Analysis Center of Tsinghua University (hereinafter referred to as "ACTU") since 2004 to cultivate young talent via an internship program, provide high-end mass spectrometry such as IT-TOF and MALDI-TOF, and offer technical advice and maintenance support in developing new applications. In addition, Shimadzu set up "the Scholarships for ACTU Shimadzu Excellent Graduate" in 2010 and expanded the scope of its scholarship in May 2013 by supporting "The Cross-Straits Tsinghua Chemistry Department PhD Students' Forum/Shimadzu Scholarships Dissertation Assessment and Evaluation Meeting."Nearly130 Ph.D. students from both the mainland and Taiwan Tsinghua University participated. According to Professor Jin-Ming Lin, Director of ACTU, there are many benefits of the collaboration. For example, Shimadzu and ATCU co-issued some publications, one of which achieved the China Association for Instrumental Analysis (CAIA) Award. Secondly, the maintenance and daily use of the instruments provide good examples for the teaching staffs of other schools or faculties in Tsinghua University who are planning to purchase instruments. Thirdly, organizing lectures and training by Shimadzu engineers help students better understand the instruments. Finally, the collaborative research provides information for instrument application and improvement.

The recent achievement of this collaboration is as follows:

1) Liu W, Mao S, Wu J, Lin JM. "Development and Applications of Paper-Based Electrospray Ionization-Mass Spectrometry for Monitoring of Sequentially Generated Droplets"Analyst. 2013 Apr 7;138(7):2163-70

New Products

LCMS-8050

High-Sensitivity, Ultra-Fast Triple Quadrupole LC-MS/MS

The LCMS-8050 is a triple quadrupole LC-MS/MS incorporating proprietary ultrafast technologies as well as a newly developed ion source and collision cell technology. As the high-end model of its UFMS (Ultra-Fast Mass Spectrometry) product line, the LCMS-8050 features high sensitivity, high data quality, and the world's fastest data acquisition rates to meet the growing demand for trace-level quantitation in clinical research and other markets.

Features

- Attogram-level sensitivity and unsurpassed ruggedness with the newly designed Heated ESI source and UFsweeper®III collision cell
- Scan rate of 30,000 u/sec and a 5 msec polarity switching time
- User-friendly operation and easy maintenance

IRTracer-100

Shimadzu's New Fourier Transform Infrared Spectrophotometer Provides High-speed, High-sensitivity Analysis for an Expanded Range of Markets and Applications

The IRTracer-100 Fourier Transform Infrared (FTIR) Spectrophotometer quickly and easily obtains high-quality data for samples in such fields as pharmaceuticals, foods, chemicals and electronics, combining high speed, sensitivity, and resolution with enhanced expandability and easy-to-use software.

Features

- Best-in-class 60,000:1 SN ratio enables quick and easy contaminant analysis
- Rapid scan function obtains up to 20 spectra/second
- High-performance LabSolutions IR Software offers network capabilities, spectra-rich libraries, a powerful search function, and labor-saving easy macro programs

Aggregates Sizer

A New Aggregation Analysis System for Biopharmaceuticals Enables Real-time Quantitative Evaluation of SVP Aggregates

The Aggregates Sizer offers quantitative, high-speed evaluation of 100 nm to 10 µm SVP (sub-visible particle) aggregates that may be present in biopharmaceuticals. Capable of evaluating the concentration (µg/mL) of aggregates that can cause shock symptom or other side effects, it is ideally suited for quality control and for improving the efficiency of biopharmaceutical development.

Features

- Quantitatively evaluates SVP range aggregate concentrations
- Measures aggregates with sensitivity 10 times higher than previous particle size analyzers
- Quantitatively evaluates aggregation processes at intervals as short as one second

EDX-7000/8000

Shimadzu's New EDX-7000 and EDX-8000 High Performance Energy-dispersive X-ray Fluorescence Spectrometers Offer Outstanding Flexibility and Easy Operation

The new energy-dispersive X-ray fluorescence spectrometer series: the EDX-7000 (Measurement range: 11Na to 92U) and EDX-8000 (Measurement range: 6C to 92U), offers excellent sensitivity, resolution, and flexibility. They can be applied to a variety of industries and applications, from environmental assessments such as RoHS or ELV directives for the control of regulated materials to unique general-research analysis needs that require precise, high-sensitivity measurements.

Features

- High sensitivity with the lower limit of detection improved 1.5 to 5 times
- High throughput increased by up to a factor of 10
- Sample chamber as large as 210 mm x 297 mm x approx. 100 mmH
- High resolution by incorporating a state-of-the-art SDD detector
- No liquid nitrogen required with the electronically cooled SDD detector
- Ultra-light element analysis (EDX-8000)

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