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NEWS 02 2020

Polymers and fluorescence

Fluorescence spectroscopy of industrial base polymers

Balance of power

LCMS-8060NX: performance and robustness without compromising sensitivity and speed

360° drinking water analysis trilogy

Automatic, simultaneous and rapid analysis of pesticides

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Chemical, Petrochemical, Biofuel and Energy

Clinical

Environment

Food, Beverages, Agriculture

Pharmaceutical

Plastics and Rubber

Automotive

Enrico Davoli with the PESI-MS system (research-use only [RUO] instrument)

Global solution through global collaboration

Cancer diagnosis: Shimadzu innovation centers as a partner of scientific research

Beginning with one man in Kyoto, Japan, in 1875, the Shimadzu Corporation now has more than 10,000 employees worldwide working under the company's philosophy, "contributing to society through science and technology." For its next generation of scientific excellence, Shimadzu has established Shimadzu Innovation Centers (SICs) across the globe, with the European Innovation Center (EUIC) opening in 2017 in Duisburg, Germany.

Silvia Giordano and Enrico Davoli in front of the Mario Negri Institute, Milan

Based on the growing network Shimadzu has nurtured since its arrival in Europe in 1968, the EUIC has worked on over a dozen ongoing projects that aim to change how societies preserve food, practice medicine, and protect the environment. Here we introduce a project between EUIC and an academic institution that will advance cancer treatment.

Research to improve cancer detection

There is no doubt who proposed to collaborate on the cancer project between Shimadzu and Dr. Enrico Davoli at the Mario Negri Institute for Pharmacological Research in Milan, Italy, and the first president of the Italian Mass Spectrometry Society. After its successful collaboration with Professor Sen Takeda of Yamanashi University, Japan, to study a new system that combines artificial intelligence (AI) with probe electrospray ionization mass spectrometry (PESI-MS) to enhance research-based detection of cancer cells of the liver (Yoshimura et al., 2012), Shimadzu proposed to do the same with Davoli in Europe.

Hepatocellular carcinomas are a leading cause of cancer death. Common treatment involves resecting the cancer cells, but there is argument in the field on just how much healthy tissue taken in the resection is best for patient survival, with some researchers arguing a small buffer zone that removes healthy tissue is acceptable and others arguing nothing but cancerous tissue should be removed. During the operation, the surgeon will pass a specimen to a pathologist who will perform histology to determine whether more resection is needed. Each specimen takes about 30 minutes to prepare and analyze, which not only cause higher costs, but also the likelihood of infection.

New science for our partners

Japan ranks near the top of patient outcomes for liver cancer, in large part because of Japan's innovative diagnostic technologies (Kudo, 2018). In the project between Davoli and Shimadzu, AI is trained to analyze a specimen from PESI-MS data without any histology or other laborious preparation. AI will output a binary decision – normal or cancer – with a statistical probability. The whole process takes no more than two minutes from the time the surgeon acquires the specimen to the AI decision.

After Takeda and Shimadzu had shown the effectiveness of this approach in Japan, the question lingering was whether it could extrapolate to other countries, since the AI was originally trained with data from Japanese specimens. That was when Shimadzu contacted Davoli, asking if he could acquire tissue samples from Italian hospitals. Davoli was very excited to take part.

"For a researcher, having a prototype and the first to try something is a desire your whole life. The instrument is brand new. The approach is brand new. It was a very good opportunity," he said.

The early results are promising, and Davoli is extending his collection to samples for other solid tumors with funding from Shimadzu. "Our plan is to work with other European nations to test PESI-MS on other populations," he says, adding that EUIC has been instrumental at building the continental research network.

Working like an academic partner

In this way, SICs can be viewed as a hybrid of industry and academia. All SICs aim to create new commercial markets, but explains Dr. Ann-Christin Niehoff, EUIC Product Manager of Imaging, markets do not only grow from economic need, but also training expertise.

"More than 90 % of Ph.D. chemists do not stay in academia. We help build their career," she says, which is why Shimadzu funds Ph.D. students and will even be co-investigators on grants. Examples include collaborative projects at Limoges Hospital, France, and the University College London, England, where EUIC is supporting Ph.D. grants for three years.

Shimadzu believes that no matter the excellence of its instruments, the primary goal of its SICs is to benefit society. SICs therefore view their research collaborations as academic partnerships that never compromise academic independence, encouraging research publications and supporting academic education.

Prof. Sen Takeda of University of Yamanashi at Shimadzu Global Innovation Summit 2017

The success of SICs is built on new ideas, which is why our teams are always in consultation with university researchers to learn about scientific problems that Shimadzu innovation can help solve. It is also why SICs build research networks, having introduced Davoli to Takeda. Takeda was invited to speak at our hosting event, Global Innovation Summit 2017 in Kyoto, where he got to meet our other partners in academia as well and share their latest findings in analytical research.

By "contributing to society through science and technology", Shimadzu and its SICs want to translate academic research into scientific solutions for some of the world's greatest challenges.

The information including affiliates and titles of the persons in this article are current as of the time of interviewing (December 2018).

All the instruments in this article are research-use only (RUO) instruments and not for use in diagnostic procedures. Shimadzu is an analytical equipment provider and in certain regions, including the USA, Shimadzu products may be regulated as for research purposes only. If you have questions on approved use of a Shimadzu device, please contact your regional office.

References

Kudo, M. (2018). Management of Hepatocellular Carcinoma in Japan as a World-Leading Model. Liver Cancer 7, 134-147.

Yoshimura, K., Chen, L.C., Mandal, M.K., Nakazawa, T., Yu, Z., Uchiyama, T., Hori, H., Tanabe, K., Kubota, T., Fujii, H., et al. (2012). Analysis of renal cell carcinoma as a first step for developing mass spectrometry-based diagnostics. J Am Soc Mass Spectrom 23, 1741-1749.

»Plug und Play« disease screening solution?

The MALDI-8020 in screening for Sickle Cell Disease

3D illustration of sickle cell

In 2006, the World Health Assembly passed a resolution recognizing the Sickle Cell Disease (SCD) as a public health priority and called on countries to tackle the illness. This resolution was also adopted by the United Nations in 2009. In this context, numerous screening programs have been set up in many laboratories around the world.

SCD is a group of inherited red blood cell disorders. It is caused

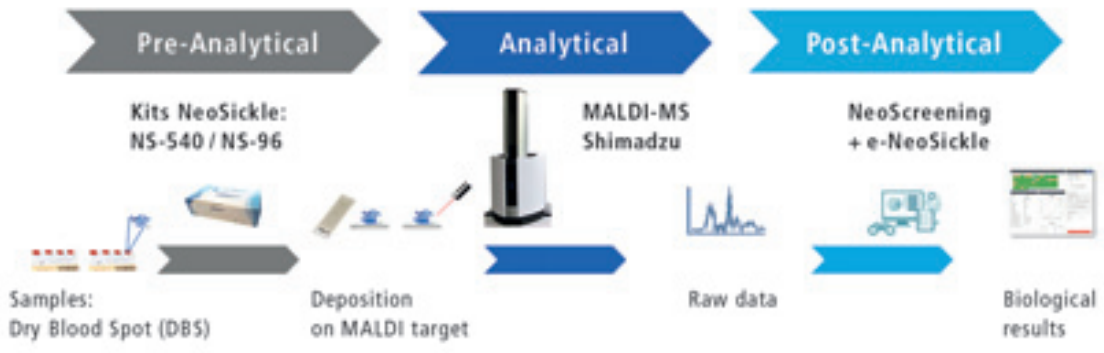

Figure 1: Workflow of automated screening

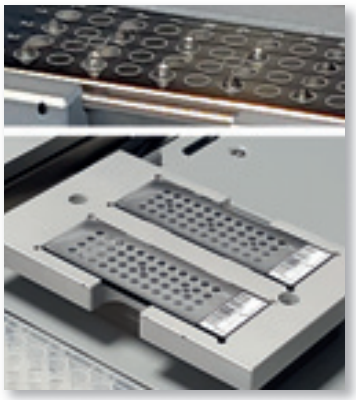

Figure 2: Specific support for Fleximass target

by abnormal hemoglobin (HbS) resulting from mutations in the hemoglobin beta. The condition was first described as early as 1910. Today, approximately 80 % of sickle cell disease cases are found in tropical regions, particularly sub-Saharan Africa, but have also increased in Europe in recent decades due to migration.

How to meet the needs of public health measures

Disease screening programs are front-line public health measures, and as such must be based on robust analytical methods and data-processing software. Cost effectiveness is a further requirement and has prompted the implementation of high-throughput screening units that reduce item costs. Lastly, the greatest possible use of automation enables the medical team to focus exclusively on abnormal samples. The Shimadzu MALDI-MS (Mass Spectrometer) together with technology packages from Biomaneo company in Dijon, France including sample processing kit, data processing and management software were designed to address these challenges.

Two-tier procedure for more accurate results

Screening programs are typically organized in two-tier procedures: The first tier is normally a routine method which must be able to detect the pathological samples with a single mutation and classify unambiguously into three groups: heterozygotes without HbS variants, heterozygotes with HbS variants and HbS homozygotes. The second level is based on a second standard reference method and is used to confirm the first results.

Screening for hemoglobinopathies can be performed with newborns, in order to ensure early care for children. This care at an early age enables a considerable increase in life expectancy. The second option of screening can be carried out in young adults or adults in the case of primary prevention, enabling people to know their status of homozygous AA or heterozygous XS. People who inherit one sickle cell gene and one normal gene have sickle cell trait (SCT) and are heterozygote XS. People with SCT do not usually have any SCD symptoms but can pass the trait on to their children.

Efficient analytical method

Neonatal screening is usually performed centrally in specialized laboratories. The biological sample used for this screening is a dry blood spot on blotting paper.

The use of MALDI-MS in hospital laboratories has already proved its value, in particular through the identification of micro-organisms (use of VITEK®MS for example). MALDI-MS techniques meet the requirements of screening. Specifically, the techniques used must meet the criteria of automation, sensitivity, resolution, robustness, speed of analysis, resistance to high analytical rates and ease of use.

Through the implementation of screening for sickle cell disease, Biomaneo company has shown that the MALDI-8020 benchtop mass-spectrometer meets the quality criteria mentioned above and makes the use of MALDI-MS in laboratories easier, convenient and fast.

New method, high-throughput, easy to use

Biomaneo has developed a 'plug and play' analytical solution that combines a sample treatment kit, the MALDI-MS technology and a software to interpret results automatically by combining them with clinical data (figure 1).

The first NeoSickle® kit was developed in two versions: the first is dedicated to high-throughput analysis using a robot; for this a support to use the Fleximass target of MALDI-8020 was specifically created (figure 2), and the robot script adapted.

The second version is a kit for labs which analyses nearly 200 samples per day manually. The Fleximass target is adapted for both of these options.

Resolution is an extremely important criterion in the analyses of hemoglobinopathies

Due to abnormal hemoglobin, SCD arises from mutations in the hemoglobin beta gene (HbS) in which the glutamic acid is substituted by valine at position 6, resulting in 30 Da lowering of the mass. The first NeoSickle solution is based on the whole protein analysis which needs a sufficient resolution at the mass of approximately 15 kDa. Figure 3 shows the resolution capability of the MALDI-8020 in line with expectations to detect the different hemoglobin chains HbS, HbA and HbF.

With this first approach, it is not possible to separate the other chains of hemoglobin variants such as HbC and HbE which have a mass difference of 1 Da compared to the normal HbA chain. For this reason, the second approach used by Biomaneo consists of analyzing hemoglobin chains peptides, and the resolution and dynamic range of the MALDI-8020 benchtop allows for separation of the peptides of interest. Figure 4 (page 6) illustrates the possibility to separate the sample groups homozygote AA or heterozygotes AE.

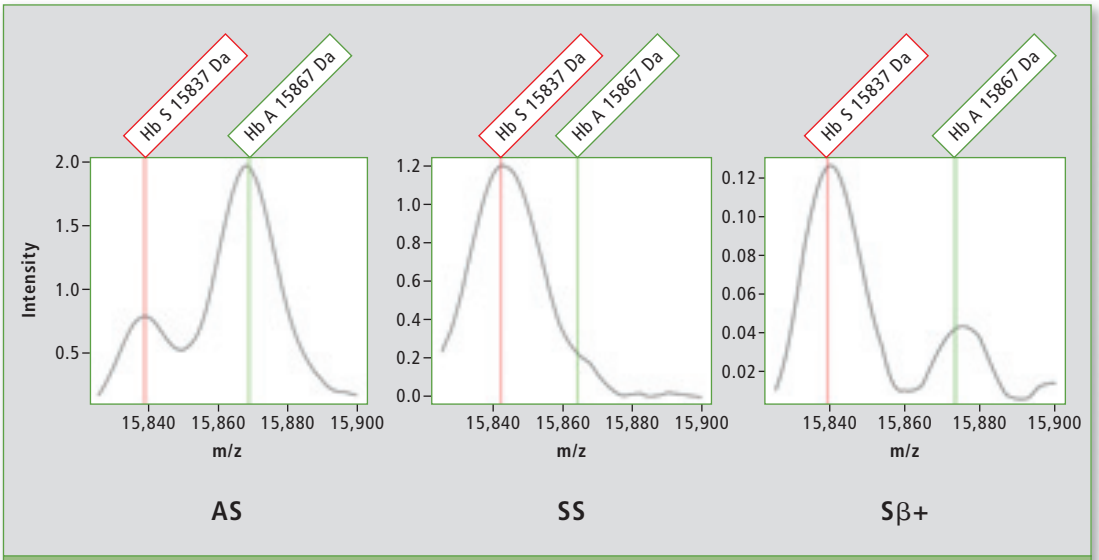

Figure 3: Spectrum obtained from Heterozygote AS, Sbeta+ and Homozygote SS samples with the detection of hemoglobin HbA and HbS chains

Sensitivity and dynamic range of detection

The other crucial criteria in SCD screening are the sensitivity and dynamic range of detection. In fact, the sample from very premature newborns has a very low percentage, less than 3 % of the adult hemoglobin chain compared to other compounds in the sample (foetal beta hemoglobin and alpha hemoglobin chain). Detection and differentiation of the HbS beta hemoglobin chain in these samples is very important in order to avoid re-blood sampling and/or misclassify the newborns. The study demonstrates the high quality of the MALDI-8020 instrument with a percentage of classification of very premature babies above 90 %.

Screening for SCD is performed in centralized laboratories analyzing a large number of samples per day. The technology used for screening must therefore be automated and/or convenient to use for the technician. Usability is an indispensable criterion for the establishment and use of mass spectrometry in analytical laboratories. The usability of the MALDI-8020 benchtop is clearly an advantage for the laboratory where the number and turnover of technicians, not specialists of MALDI-MS, is very high.

MALDI-8020 is the system of choice

The MALDI-8020 meets these criteria with well-calibrated and standardized methods. Use of the MALDI-8020 is intuitive, allowing its rapid integration into laboratory studies and facilitating the work of technicians. In addition, the system is very compact and can be easily placed on any laboratory bench.

Throughput in screening laboratories is a strong requirement to be considered in the introduction of new analytical methods. The spectra acquisition method implemented in the NeoSickle solution combined with the performance of the MALDI-8020 benchtop allows the analysis of a MALDI target containing 48 samples in less than three minutes. This throughput, including the change of target in the instrumentation, will allow 576 analyses per hour.

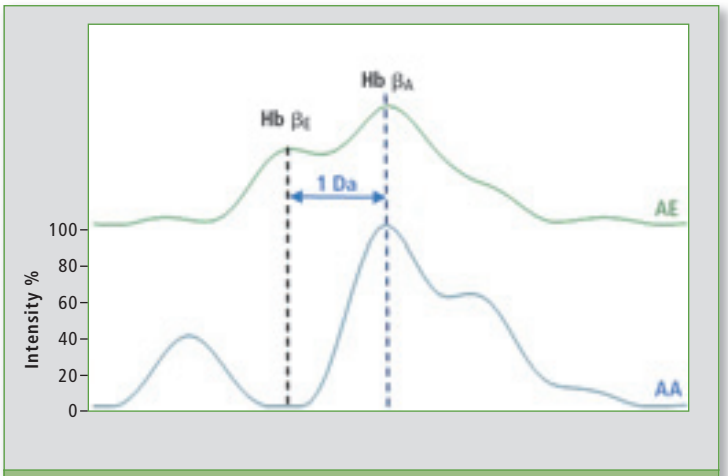

Figure 4: Analysis of specific peptides from hemoglobin chains HbE and HbA

For SCD, the main French screening laboratory analyses about 600 samples per day, meaning over 150,000 samples per year or about 20 % of births. Less than six hours of analysis per day with the MALDI-8020 would be sufficient to screen all newborns in a single laboratory.

Fully serves the needs of screening laboratories

Beyond the daily throughput, the last criterion concerns the robustness of the instrumentation over time and the low maintenance requirements. To maintain instrument performance with an objective of 500,000 tests per year, Shimadzu's MALDI-8020 provides TrueClean automated source cleaning. It can be used to clean the extraction electrode in-situ without breaking instrument vacuum; it is an automated and rapid (< 10 min) UV laser-based procedure. Moreover, the wide-bore ion optics minimize the risk of source contamination over time, providing a robust platform.

These characteristics and results demonstrate the suitability of the MALDI-8020 bench-top mass spectrometer for the detection of hemoglobinopathies and more generally for the high-throughput analysis of complex blood samples. It very clearly satisfies the needs of screening laboratories.

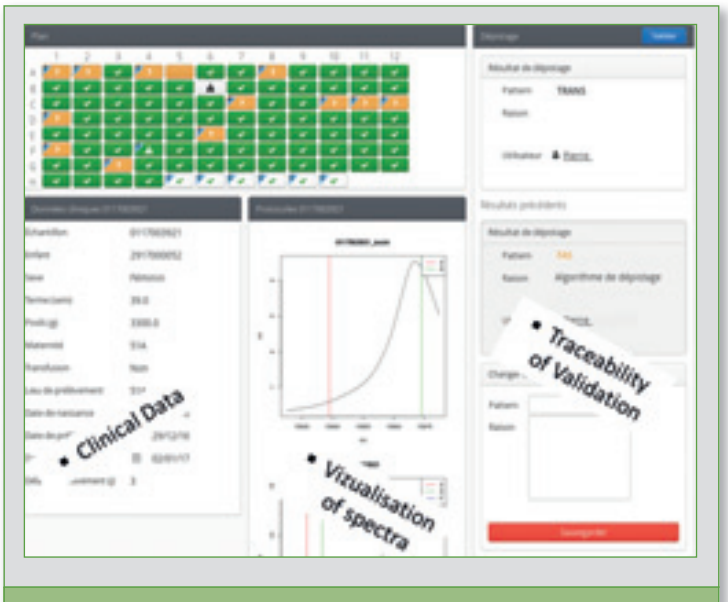

Figure 5: Screenshot of the NeoScreening LIMS after analysis of one plate

The power and speed of MALDI instruments means that they will very rarely be used full time for just a single application. For hospitals or laboratories, it is effective to optimize the investments made in these technologies. System versatility is an important financial criterion to be taken into account. For laboratories with a small number of screening analyses, the advantage of the MALDI-8020 is the management of acquisition parameters. Indeed, the acquisition methods integrate all the parameters necessary to control the instrumentation, making the MALDI-8020 applicable for multiple applications without the risk of interfering with individual analyses.

To enhance the power of automated high-throughput data production by the MALDI-8020, Biomaneo has developed a software package (LIMS: Laboratory Information Management System) to integrate the results and assist in the interpretation of mass spectrometry data. The LIMS NeoScreening® allows production of the files necessary to control the MALDI-8020 and launch an analysis run, while also retrieving data automatically and then processing and classifying each sample result.

Conclusion

The speed of acquisition and the simplicity of changing methods makes the MALDI-8020 benchtop ideal for many applications and an indispensable measuring instrument for biochemistry laboratories.

In the context of SCD screening, the MALDI-8020 meets all requirements. This compact, simple to use and high-performance instrument allows screening in most cases.

The balance of power

LCMS-8060NX balances enhanced performance and robustness without compromising sensitivity and speed

The new LCMS-8060NX coupled to the Nexera LC-40 series

Since 2010, Shimadzu is shaking the market in triple quadrupole LCMS by introducing game changing innovations. The groundbreaking process started with the LCMS-8030 featuring fast polarity switching of 15 ms, combined with ultra-fast electronics for short Dwell Time (DW) and Pause Time (PT), fast scanning speed and fast collision cell. These technologies are now well-established on the market under Ultra-Fast Mass Spectrometry (UFMS). In 2015, the LCMS-8050 introduced 5 ms polarity switching and scan speed at 30,000 amu/s. It improved the market by presenting new ways of using a tandem LC-MS.

Due to the speed of all these new technologies, multiple MRMs/compound methods (more than the standard 2 MRM Multiple Reaction Monitoring technique) started to be developed, enabling better identification with the MRM Spectrum Mode and simplified multicomponent/contaminants analysis review by switching acquired MRMs in post-run analysis in case of interferences.

Subsequent systems, the LCMS-8060 (2016) and LCMS-8045 (2017) are based on that successful LCMS-8050 platform. They offered the market a range of sensitivity depending on individual needs without sacrificing user flexibility.

User-friendly, robust and highly sensitive

A general market demand is the robustness of high-sensitivity instruments. The LCMS-8060 is already a highly robust system used in clinical and food safety applications. The need for more robustness as well as user-friendly method development and analysis is a key driving force for Shimadzu, innovating existing lab solutions and creating new ones to steadily support the market.

A new addition to the LC-MS portfolio is the LCMS-8060NX Triple Quadrupole solution. With enhanced ease-of-use, robustness and a wide range of default parameters, the new system achieves the balance between high sensitivity and robustness.

With the new IonFocus ion source, the LCMS-8060NX efficiently separates ions from neutral particles, allowing only the ions into the system driven by the Focus voltage. It releases the neutral particles through the drain.

A newly extended range for default parameters in the ion source expands the possibilities for a high-sensitivity analysis. The improvement of the integrated ion pathway establishes the LCMS-8060NX as the class leading triple quadrupole LC-MS on the market.

Analytical Intelligence for simplified lab management

In combination with the brand-new LC-40 series and its Analytical Intelligence concept, it offers an outstanding solution for routine testing.

Under the umbrella of Analytical Intelligence, Shimadzu's analytical systems will be successively equipped with automated functions, enabling users to easily review instrument status, optimize resource allocation and achieve higher throughput.

The concept simplifies lab management and enables higher productivity, maximum reliability and better connectivity, applying IoT, Artificial Intelligence and M2M.

Customized software solutions for any measurement

Macro programming for Shimadzu UV-Vis and FTIR

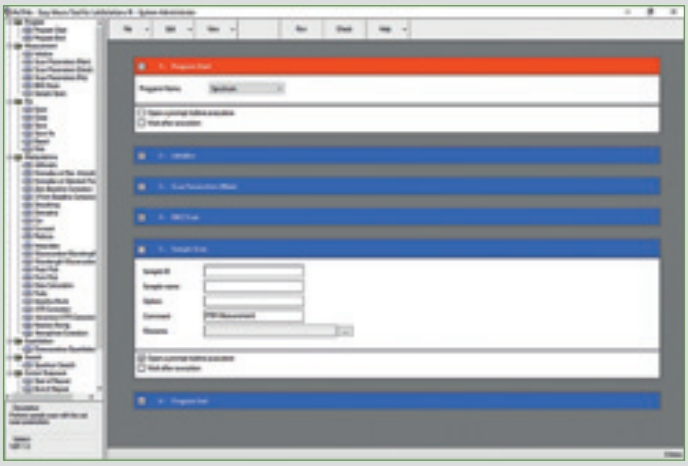

Figure 1: Example of an Easy Macro for a simple measurement with pre-defined measurement parameters

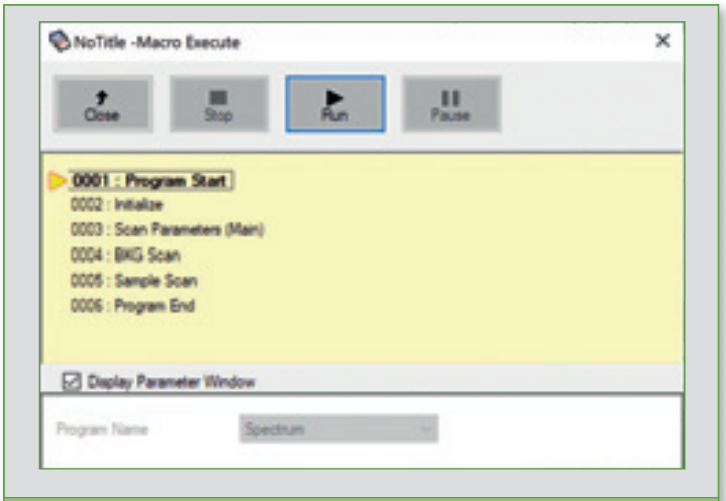

Figure 2: Execute macro window. The LabSolutions IR program used for the easy macro is run in the background. The macro execution can be stopped at any step.

The data output of spectrophotometers has come a long way since the time of mechanical writers. Today, the user gets a full-blown software suite with lots of different measurement options and integrated data processing. While the specifications – such as scan speed, signal-to-noise ratio or resolution – are still improving gradually with each new instrument, the software included has become an equally important selling point.

Even though LabSolutions IR and UV already offer a wide variety of tools, there are cases which demand a custom solution to a very specific problem. Whereas one customer might require a simplified user interface and automated analyses to support the company's technicians in their daily routine, a different client might be bound to a very specialized data processing which is not common enough to be implemented into the LabSolutions software.

Such requests are served by customized software or macros. Shimadzu offers different tools depending on the complexity of the task. This article gives a brief overview of the possibilities of controlling Shimadzu FTIR and UV-Vis instruments by a customized software.

FTIR – Easy Macro

The starting point for most FTIR macros is the Easy Macro tool included in LabSolutions IR. It allows users to automate usual analysis by 'drag and drop' of easy to understand building blocks, such as "Sample Scan", "Smoothing" or "Spectrum Search". Customized messages can be shown between the steps, e.g. telling the user to set the sample or clean the ATR. Figure 1 gives an example of an Easy Macro.

In this example, the spectrum software is used. It is started in step 1, then the instrument is initialized in step 2 and the scan parameters are set in step 3. When the macro is run, the spectrum software opens in the background; without the command "Scan Parameters", it will just use the last set parameters. Steps 4 and 5 first measure the background, and then the sample. The sample and file name can be defined by the macro, but in this example a prompt will be shown to allow the user to enter sample and file name. Each Easy Macro must start with the command "Program Start" and end with the command "Program End".

Easy Macro provides the benefit that even users without any knowledge of programming can develop such a macro. Because of

Tool	Easy Macro	VB Macro
Instrument	IRSpirit, IRAffinity-1s, IRTracer-100	IRSpirit, IRAffinity-1s, IRTracer-100
Used by	Customer or Shimadzu	Shimadzu
Application	Simple automation, starting VB macros	Complex automation, IRPilot, EP validation
Features	Simple drag & drop of pre-defined code blocks	Visual Basic source code in LabSolutions IR
Advantages	Easy to understand Prevents syntax errors Can load more complex macros	Full integration in LabSolutions IR Includes classes from VB, such as user forms Very complex applications possible
Disadvantages	Only spectrum or postrun applications	Not accessible for the customer

Table 1: Comparison of macro tools for Shimadzu FTIR

Figure 3: User interface of the IRPilot

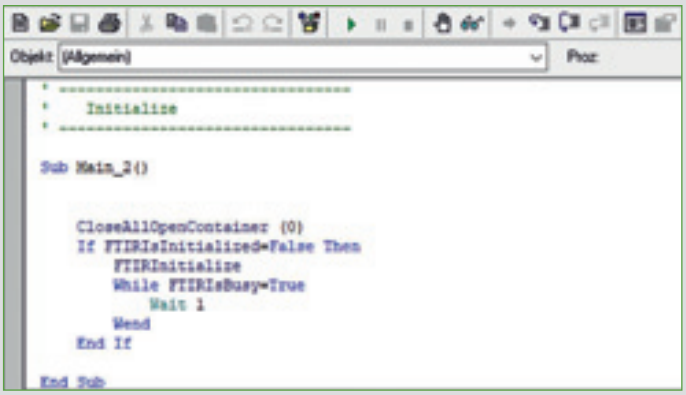

Figure 4: The initialize command from Easy Macro as visual basic source code

the pre-defined building blocks, syntax errors and malicious code are prevented. The user can see in the execute window if an Easy Macro tries loading a potentially malicious basic file, and prevent the execution.

Most operators meet Easy Macro by the validation functions included with their Shimadzu FTIR, which are started by such macros. Each building block of the Easy Macro generates visual basic code which is interpreted by LabSolutions IR.

FTIR – Visual Basic (VB) macro

The visual basic code behind such an Easy Macro can be extracted and used as the basis for more complex Lab Solutions VB macros, like the IR Pilot included with the Shimadzu IRSpirit, shown in figure 3.

The visual basic macro editor included in LabSolutions IR offers a full development environment for the trained specialist. Finalized macros are either added to the toolbar of LabSolutions IR Postrun or Spectrum, or they are started by the "Load Basic File" command of Easy Macro. Figure 4 shows a screenshot from the LabSolutions IR VB macro editor with the code for the "Initialize" building block from Easy Macro.

UV – Automatic Control

In the world of LabSolutions UV-Vis, there is no counterpart for Easy Macro, since LabSolutions UV-Vis already offers a high degree of automation – e.g. for text export, postrun calculations or spectrum evaluation. But it is possible to further automate LabSolutions UV-Vis and connect it to customized software by the Automatic Control option. A typical application for this option is the communication between LabSolutions UV-Vis and an autosampler software.

The scripts use commands similar to the building blocks of LabSolutions FTIR Easy Macro. Text files with these commands are placed in a special folder which is scanned continuously by LabSolutions UV-Vis. LabSolutions reads the commands from "command" text files and writes the result into "response" text files.

The syntax of these commands is easy to understand, and the only prerequisite of the programming environment is to be able to read and write text files. As the user rights and audit trail settings of LabSolutions DB are not circumvented by such scripts, LabSolutions UV-Vis Automatic Control can be used even in a validated environment. A bi-directional communication between LabSolutions UV-Vis and a third-party lab management system is possible by writing command files for the LabSolutions UV-Vis Automatic Control Option and then reading the text or pdf report generated after each measurement.

UV – string commands

The most accessible way to read out data from UV-Vis instruments by customized software is with the external commands documented in the instruction manuals of UV-1280 and UV-1900i. They are also known as string commands, because the communication is established by simple ASCII strings sent over a virtual COM port.

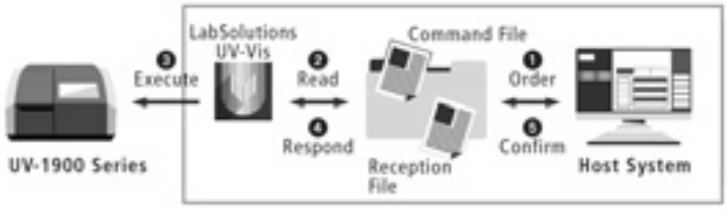

Figure 5: Schematic of the communication between LabSolutions UV-Vis and a host system by the Automatic Control option

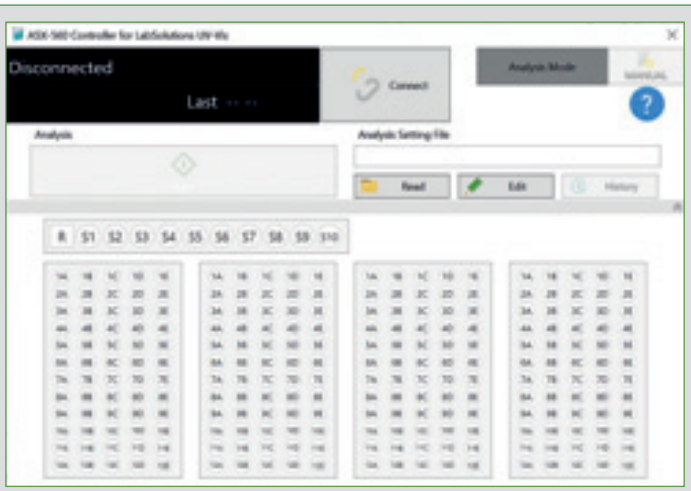

Figure 6: Autosampler control as example for a LabSolutions UV Automatic Control application

Tool	LabSolutions UV-Vis Automatic Control	String Commands	UV-OCX
Instrument	UV i-Selection (UV-1900i, UV-2600i, UV-2700i, UV-3600i plus, SolidSpec-3700i)	UV-1280, UV-1900i	UV-1280, UV-1900i, UV-2600i, UV-2700i
Used by	Customer or Shimadzu	Customer or Shimadzu	Shimadzu
Application	Simple automation, Autosampler control, LIMS integration	PC control of UV-1280, excel-macros, third-party software	Custom software with full instrument control
Features	Scripts for LabSolutions UV-Vis by simple text files	ASCII-commands for simple instrument control	Indirect instrument control by Active X
Advantages	Code independent from instrument Easy to implement LabSolutions DB/CS user rights still valid	Commands are published Small, efficient code Free choice of programming language	Nearly full control of instrument functions Communication errors prevented by design Developer is supported by Microsoft IntelliSense
Disadvantages	Only specific functions of LabSolutions UV accessible	Solid programming skills required	No LabSolutions integration

Table 2: Comparison of macro tools for Shimadzu UV-Vis

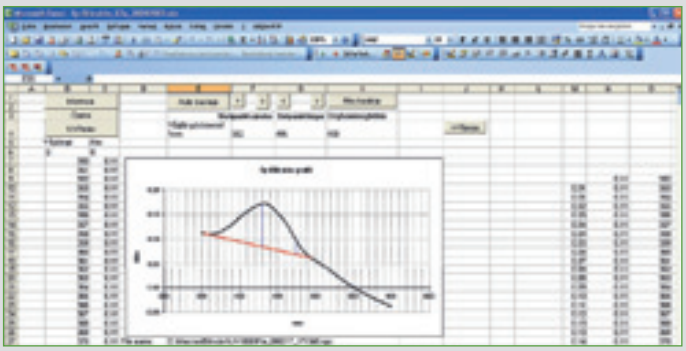

Figure 7: Excel-Macro for Bilirubin in Spinal Fluid as typical customized application using string-commands

These commands are typically used in customized Excel-macros with instrument control or to program drivers for software with control of instruments from multiple vendors. Example code for the implementation in Microsoft Visual Basic 2015 is given in the manual along with the required settings of the virtual com port, but a much deeper understanding of interface programming is required compared to developing software with the LabSolutions Automatic Control.

UV – OCX commands

Another possibility for direct instrument control is given by the option UV-OCX. Here, an Active X control is used to translate commands written in a VBA or Microsoft.net Framework source code into machine code for the spectrophotometer. It is possible to control UV-1280, UV-1900i, UV-2600i and UV-2700i.

Compared to string commands, programming with OCX commands requires less understanding of interfaces, as the communication with the instrument is done by the OCX control and communication errors (such as setting impossible wavelength values) are prevented by this interface. The UV-OCX is implemented as a class with pre-defined methods and parameters. This spares one step compared to the string commands, where the programmers must write their own class to translate between e.g. VBA code and ASCII-strings.

When a programming environment such as Microsoft Visual Studio or the Microsoft Excel VBA editor is used, the UV-OCX even implements helpful functions, e.g. intelligent code completion, to prevent syntax errors.

Conclusion

Macro programming is a powerful tool to support customers. Some macros enable applications which are not covered by the standard software, while other macros serve to automate tedious tasks and prevent user errors. The best approach depends not only on the complexity of the application, but also on the experience of the programmer.

Table 1 compares the different tools to program custom software for Shimadzu FTIR, and table 2 shows the tools for Shimadzu UV-Vis spectrophotometers.

Figure 10: The optional VisEase software which was developed with UV-OCX

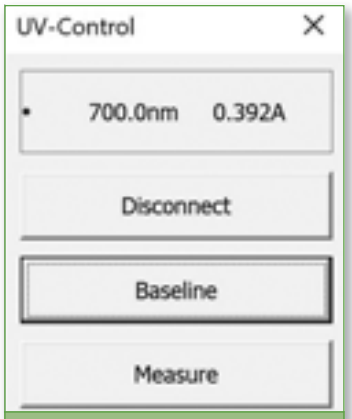

Figure 8: Simple instrument control window in a VBA form made with UV-OCX

Figure 9: UV-OCX instrument control embedded directly in an Excel sheet

Ensuring steroid-free food supplements

Identification of steroids in pharmaceuticals and food supplements with LCMS-8045

Anabolic steroids are often encountered in samples of nutritional supplements and pharmaceutical preparations, either due to doping-related activities [1] or cross-contamination issues during production [2]. Developing methods to identify steroids in such samples can be challenging, since the method should be able to monitor numerous steroids with large differences in terms of polarity. Compounds to be monitored comprise relatively polar steroids such as anastrozole as well as low polarity steroid esters which have been occurring with increasing frequency in food supplement samples [3].

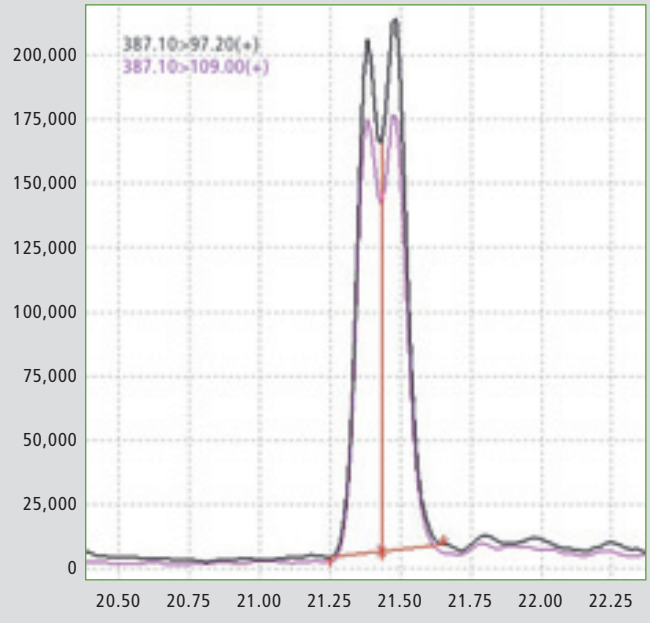

Figure 1: Chromatogram of standard solution, exhibiting partial separation of testosterone caproate and testosterone isocaproate

The method developed includes testing for testosterone caproate and testosterone isocaproate, a challenging set of isomers which were partially separated (Resolution 0.6 as per Eur.Ph.) enabling the analytical method to identify which of the two isomers is contained in the sample. With respect to tibolone, three MRM transitions are reported with no need to resort to derivatization, which is usually the case with tibolone and its metabolites. Finally, a comprehensive rationale is provided for choosing MRM transitions for steroid esters, based on fragments of the free steroid.

Instrumentation, method parameters and sample preparation

The experiments were conducted with Nexera X2 UHPLC system coupled to LCMS-8045 triple quadrupole mass spectrometer. The system consisted of two LC-30AD pumps, a SIL-30AC autosampler and a CTO-20AC column oven with a flow selection valve. The valve was used to divert mobile phase flow to waste during the first 1.5 minutes and avoid contamination of the mass spectrometer with early-eluting matrix components.

Chromatographic separation was achieved with Zorbax SB-C18 Rapid Resolution HT, 50 x 4.6 mm, 3.5 µm (part number 835975-902) maintained at 40 °C. The mobile phase was a mixture of 0.1 % aqueous formic acid and methanol, with gradient elution and a flowrate of 0.4 mL/min. This set of parameters resulted in retention times ranging from 2.4 - 24.0 minutes and a total runtime of 31 minutes including the equilibration step.

Liquid chromatography

Method development strategy aimed at including a broad spectrum of compounds with

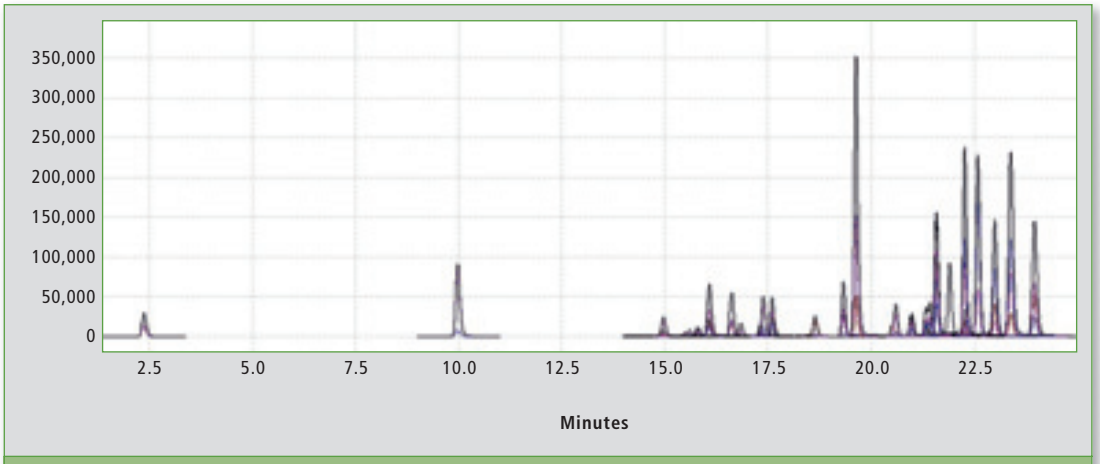

Figure 2: Chromatogram of the standard solution

Time (min)	% B
0.0 - 5.0	35
5.0 - 20.5	35 → 100
20.5 - 25.0	100
25.01 - 31.0	35

Table 1: Gradient elution program

respect to polarity while ensuring a reasonable runtime, with a total of 16 steroid esters being included in the analytical method. Regarding the mobile phase flowrate, 0.5 mL/min and 0.4 mL/min were tested, with the latter being chosen due to increased sensitivity.

Two mobile phase compositions were tested with gradient elution, the first being a mixture of 1 mM aqueous ammonium acetate/1 mM methanolic ammonium acetate and the second being a mixture of 0.1 % aqueous formic acid/ methanol. The second mobile phase was chosen to ensure simpler preparation procedure. The change in mobile phase acidity using formic acid appeared to have minor effects on retention time of the compounds since early eluting compounds exhibited approximately 20 % higher retention, whereas late-eluting compounds exhibited approximately 10 % higher retention.

Two analytical columns were tested, namely one Zorbax SB-C18 Rapid Resolution HT, 50 x 2.1 mm, 1.8 µm (part number 822700-902) and one Zorbax SB-C18 Rapid Resolution HT, 50 x 4.6 mm, 3.5 µm (part number 835975-902). Given that the first column contained sub-2 µm particles, considerably shorter sample runtime was expected, however comparable retention times were obtained with both columns. Since the UHPLC column was prone to overpressure and clogging after a few analyses, the 3.5 µm column was preferred for the application, with an injection volume of 2 µL.

Autosampler temperature was initially set to 4 °C to ensure stability of the samples. Because heavy precipitation of matrix components was observed when the vials were exposed to 4 °C, the autosampler was kept at room temperature and 24-hour stability was established for the analytes.

Sample preparation

In order to keep sample pretreatment simple, effortless steps were employed. The solid dosage form samples were homogenized and 100 mg was weighed in a 10 mL volumetric flask. Due to the high solubility of the compounds in methanol, the homogenized sample was dissolved in methanol to ensure high recovery for all compounds (Test Solution 1). Four Test Solutions (TS) were then prepared (TS 2-5) for injection into the instrument.

Because matrix components contained in TS1 were not always soluble in mobile phase, 1.0 mL of this solution was filtered through a 0.22 µm nylon filter and diluted to volume with mobile phase to avoid precipitation inside the column (Test Solution 2, dilution 1 : 10). 1.0 mL each of TS2, TS3, and TS4 was transferred to 10 mL volumetric flasks and diluted to volume with mobile phase (TS3, dilution 1 : 102; TS4, dilution 1 : 103; TS5, dilution 1 : 104). 2 µL of TS3 and TS4 were injected into the instrument. TS2 would be injected if low intensity peaks were obtained with TS3 and TS4. TS5 would be injected if high intensity peaks were obtained with TS3 and TS4.

Group	Precursor ion	Product ion 1	Product ion 2
Testosterone			
Testosterone	289.4	109.1	97.0
Methyltestosterone	303.4	109.1	97.1
Testosterone propionate	345.1	109.1	97.0
Testosterone phenylpropionate	421.6	105.1	97.1
Testosterone isocaproate	387.1	109.0	97.2
Testosterone caproate	387.1	109.0	97.2
Testosterone enanthate	401.6	271.3	183.0
Testosterone decanoate	443.7	109.2	97.0
Testosterone undecanoate	457.7	109.0	97.1
Trenbolone			
Methyltrenbolone	285.0	227.1	198.0
Trenbolone acetate	313.0	253.1	107.5
Trenbolone enanthate	383.1	253.1	107.5
Trenbolone hexahydrobenzylcarbonate	411.1	253.0	107.5
Nandrolone			
Nandrolone	275.4	257.2	239.0
Nandrolone phenylpropionate	407.1	257.1	105.2
Nandrolone decanoate	429.7	257.3	239.0
Methenolone			
Methenolone (For reference only)	303.0	83.0	187.0
Methenolone acetate	345.0	83.1	97.0
Methenolone enanthate	415.1	82.9	187.1
Boldenone			
Boldenone (For reference only)	287.5	134.6	120.5
Boldenone cypionate	411.6	135.4	125.1
Boldenone undecylenate	453.2	135.2	67.2
Drostanolone			
Drostanolone (For reference only)	305.0	161.0	215.0
Drostanolone propionate	361.1	269.2	215.2

Table 2: Rationale for the choice of MRM transitions

Mass spectrometry

The compounds to be determined were divided into six groups of similar structure: testosterone and testosterone esters, trenbolone esters, nandrolone and nandrolone esters, methenolone esters, boldenone esters and drostanolone esters. The fragments of steroid esters of the same group were expected to be, to a certain extent, common, which was confirmed during the initial mass spectrometry experiments as can be seen in table 2. Therefore, common fragments mentioned in table 2 may be used to choose MRM transitions for other steroid esters, based on fragments of the free steroid.

With respect to testosterone group, m/z 109 was used for quantitation and m/z 97 was used for confirmation. Both ions are characteristic fragments of testosterone and their use is proven in the literature [1-3]. Moreover, because the two ions correspond to fragments of the steroid core of the molecule, their applicability to all testosterone esters was investigated and confirmed. A similar rationale was followed for the group of nandrolone and nandrolone esters, m/z

Compound	Retention time (min)	MRM Quantitation	Reference ion 1	Reference ion 2	C std (ug mL^{-1})	S/N ratio
Clenbuterol	2.4	277.3 → 203.3	167.5	—	0.05	1.9 x 10^3
Anastrozole	10.0	294.0 → 225.1	210.1	—	0.05	1.2 x 10^4
Fluoxymesterone	14.9	337.0 → 281.2	317.2	299.2	1.0	204
Oxandrolone	15.0	307.0 → 289.2	93.4	—	1.0	213
Nandrolone	15.2	275.1 → 239.1	257.2	—	5.0	87
Methyltrenbolone	15.6	285.0 → 227.1	198.0	—	0.05	61
Methandienone	16.1	301.0 → 283.1	283.1	149.1	0.5	694
Testosterone	16.1	289.1 → 109.2	97.0	—	0.05	44
Methyltestosterone	16.9	303.1 → 108.8	96.8	—	0.05	207
Mesterolone	17.4	304.5 → 269.2	229.2	—	5.0	103
Trenbolone acetate	17.7	313.0 → 253.1	107.5	—	0.05	388
Danazol	17.6	338.4 → 148.0	121.2	—	0.5	891
Stanozolol	17.4	328.6 → 81.0	121.1	—	1.0	471
Tibolone	16.6	313.3 → 295.1	91.1	105.1	5.0	252
Methenolone acetate	19.3	344.6 → 82.8	187.1	297.2	1.0	557
Oxymetholone	18.6	333.0 → 159.1	279.1	—	1.0	110
Testosterone propionate	19.7	345.1 → 109.1	97.0	271.0	0.05	2.2 x 10^3
Nandrolone phenylpropionate	20.7	407.1 → 105.2	257.1	—	0.05	616
Drostanolone propionate	21.0	361.1 → 269.2	215.2	—	5.0	552
Testosterone phenylpropionate	21.0	420.6 → 105.0	97.1	—	1.0	646
Testosterone isocaproate	21.4	387.1 → 97.2	109.0	—	0.05	148
Trenbolone enanthate	21.5	383.1 → 253.1	107.5	—	0.05	867
Testosterone caproate	21.5	387.1 → 97.0	109.1	—	0.05	188
Trenbolone hexahydrobenzylcarbonate	21.6	411.1 → 253.0	107.5	—	0.05	2.1 x 10^3
Boldenone cypionate	21.6	411.1 → 135.1	125.1	—	0.5	3,216
Testosterone enanthate	21.9	401.1 → 96.8	109.0	—	0.5	194
Methenolone enanthate	22.3	415.1 → 187.1	144.9	—	0.5	2.0 x 10^3
Boldenone undecylenate	22.6	453.2 → 135.2	67.2	—	1.0	3.0 x 10^3
Nadrolone decanoate	23.0	429.4 → 257.3	274.6	239.0	0.2	1.5 x 10^3
Testosterone decanoate	23.4	443.2 → 109.2	97.0	43.1	0.2	1.6 x 10^3
Testosterone undecanoate	24.0	457.2 → 109.0	169.1	—	0.2	1.1 x 10^3

Table 3: MRM transitions for each compound

257 and m/z 239. For the group of trenbolone esters, m/z 253 and m/z 107 were used. For the group of boldenone esters, m/z 135 was applied, and for drostanolone propionate, m/z 215 was used.

The optimized mass spectrometry parameters were:

Positive ionization: (ESI capillary voltage set to -4 kV)
Heat block temperature: 400 °C
Desolvation line temperature: 250 °C
Heating gas temperature: 350 °C
Drying gas: 10.0 L/ min
Nebulizing gas: 3.0 L/min
Heating gas: 10.0 L/min

Dwell time was set to 20 ms, except for:
Nandrolone: (100 ms)
Fluoxymesterone: (50 ms)
Methyltrenbolone: (150 ms)
Methenolone acetate: (50 ms)
Drostanolone propionate: (150 ms).

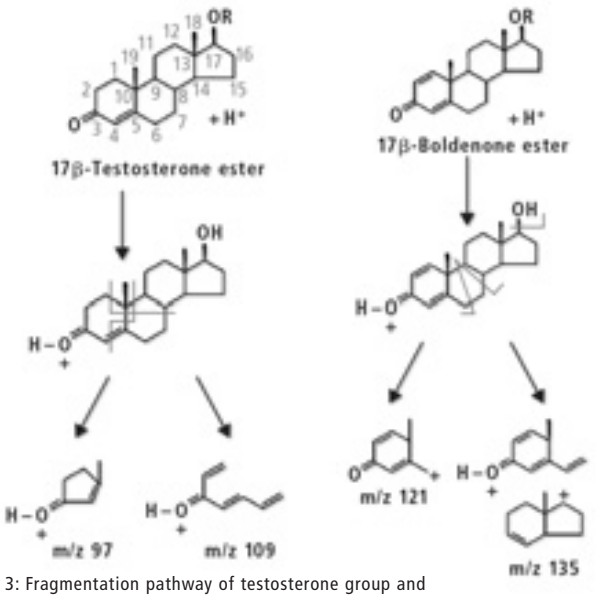

Figure 3: Fragmentation pathway of testosterone group and boldenone group of compounds [3]

Conclusions

An analytical method was developed to identify 31 steroids in pharmaceutical preparations and food supplements. This is the first method to identify testosterone caproate in the presence of its isomer, testosterone isocaproate, and vice versa. Key parameters that affect the analysis of all 31 steroids have been identified and taken into account.

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Literature

[1] C. Borges, N. Miller, M. Shelby, M. Hansen, C. White, M.H. Slawson, K. Monti, D.J. Crouch, Analysis of a challenging subset of World Anti Doping Agency-Banned steroids and anti-estrogens by LC-MS-MS, Journal of Analytical Toxicology, 31 (2007) 125-131.

[2] S. Fekete, J. Fekete, K. Ganzler, Validated UPLC method for the fast and sensitive determination of steroid residues in support of cleaning validation in formulation area, Journal of Pharmaceutical and Biomedical analysis, 49 (2009) 833-838.

[3] M. Thevis, Y. Schrader, A. Thomas, G. Sigmund, H. Geyer, W. Schanzer, Analysis of confiscated black market drugs using chromatographic and mass spectrometric approaches, Journal of Analytical Toxicology 2008;32:232-240.

360° drinking water analysis: Episode 2

Automatic, simultaneous and rapid analysis of pesticides in drinking water by online SPE and UHPLC-MS/MS

EU citizens use up to 156 liters of water per day. Groundwater and surface water such as rivers, lakes, reservoirs and seawater are sources for drinking water. Its safety and high quality are essential for public and individual health as well as for the economy.

Hazards in source waters include pathogenic microorganisms and chemicals from domestic, agricultural, commercial or natural sources. Defects in quality and quantity generate massive problems and cause high social and economic costs. Strict and steady controls are therefore essential.

As seen in Episode 1 in the Shimadzu NEWS 1/2020, Shimadzu's ICPMS-2030 and TOC-L meet analytical requirements for effective control of drinking water, are user-friendly and provide an economical solution for modern laboratories. This Episode 2 deals with a sensitive online SPE-UHPLC-MS/MS method.

Background

In 1998, the European Union (EU) adopted the Drinking Water Directive 98/83/EC1 (DWD) [2]. For more than 20 years, the DWD has regulated the quality of water intended for human consumption. Evaluation of this directive was included in the Commission Work Programme 20152 as part of the Commission's Regulatory Fitness and Performance programme (REFIT), to assess whether this instrument remains fit for purpose. It is the first full evaluation of the DWD and has been published as a Commission Staff Working Document in December 2016 (SWD, 2016, 428 final).

Cooperation between the DWD and agriculture is a crucial factor for providing safe and high-quality drinking water. Agricultural practices like fertilization and plant protection with pesticides have a significant impact on drinking water quality. To prevent agricultural contamination, parameters like nitrate and pesticides have been included in the DWD. These standards contributed to reducing the release of fertilizers and crop protection products to preserve the environment. Decreasing releases of nitrate or pesticides into the envi-

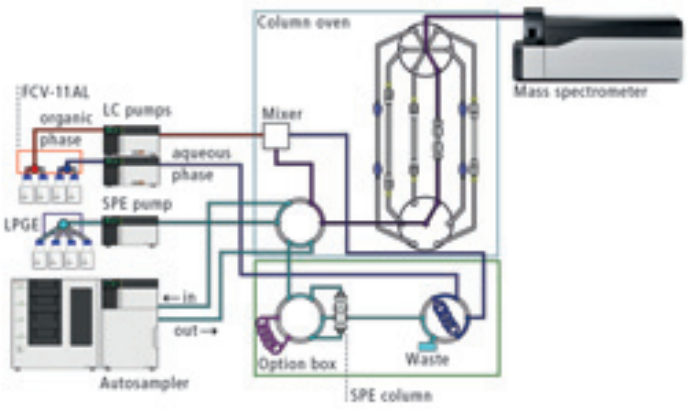


Figure 1: Scheme of online SPE and UHPLC-MS/MS
(Number of patent: WO 2016/098169 A1)

The total abstraction of fresh water across Europe is around 182 billion m^3/year, drawn in roughly equal amounts from groundwater and surface water sources. Drinking water in the EU stems from around 11,000 large suppliers and 85,000 small suppliers, serving around 80 % and 20 % of the population respectively.

More than 60 % of the EU water infrastructure consists of water services provided by publicly-owned companies – the rest are regulated entities with different levels of private ownership. Regulatory options range from largely decentralized management of private companies (subject to antitrust and price regulation) to public ownership, ministerial guidance and budget control. The European water sector is a major economic player (1 % of GDP), with an annual turnover in the EU of about 80 billion Euro. It provides around 500,000 full-time jobs and a yearly investment of 7 billion Euro [1].

ronment is the driving force for ensuring safe and high-quality drinking water.

Pesticides in drinking water

Pesticides are often applied for crop protection. Their intensive use and slow natural degradation make them serious contaminants of surface and underground water – the most important sources for drinking water. This exposure can be dangerous for animals, humans and ecosystems, with an immediate or long-term effect. In relation to these compounds, some studies have revealed health impacts such as alterations of the nervous system, immune system diseases, fertility and development problems as well as cancer. This lead to the DWD setting a concentration limit of 0.1 µg/L for individual pesticides and 0.5 µg/L for the total sum of pesticides.

Determination of 272 pesticides in water

Reference separation techniques require a tedious pretreatment protocol to reach the thresholds set by environmental standards. Shimadzu proposes a fast and sensitive online SPE-LC-MS/MS method for simultaneous highly sensitive quantification of 272 pesticides in surface and groundwater. This method uses high quality solvents and reagents for mobile phases (LC-MS grade Biosolve[1]). Two surface waters and one groundwater sample were spiked with standard pesticides purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Six calibration levels at 1, 10, 20, 50, 100 and 500 ng/L were prepared three times in each matrix.

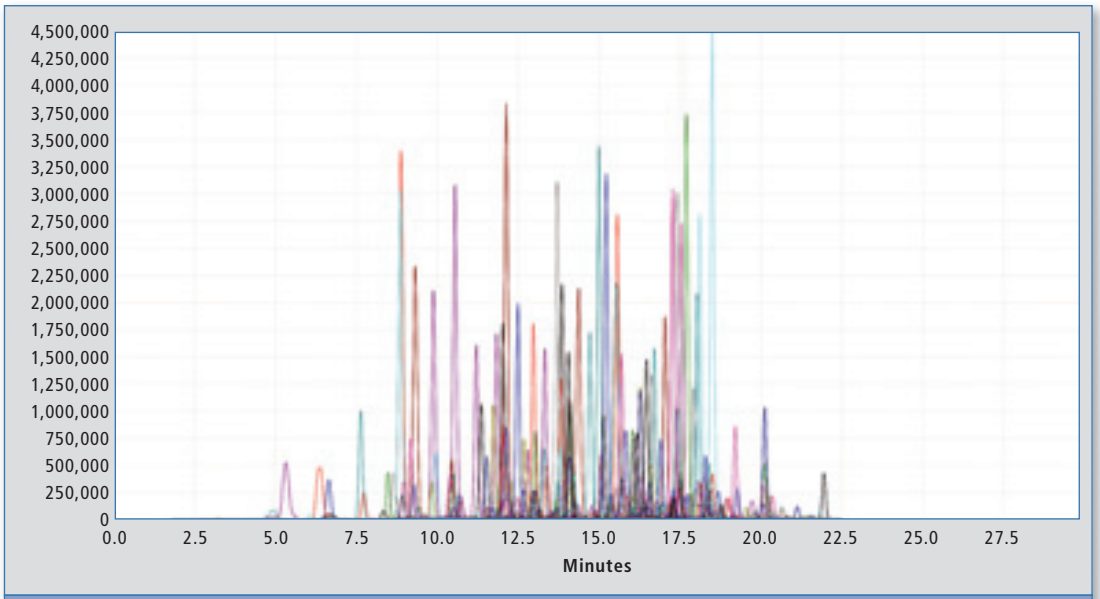


Figure 2: Chromatogram of ground water spiked at 100 ng/L

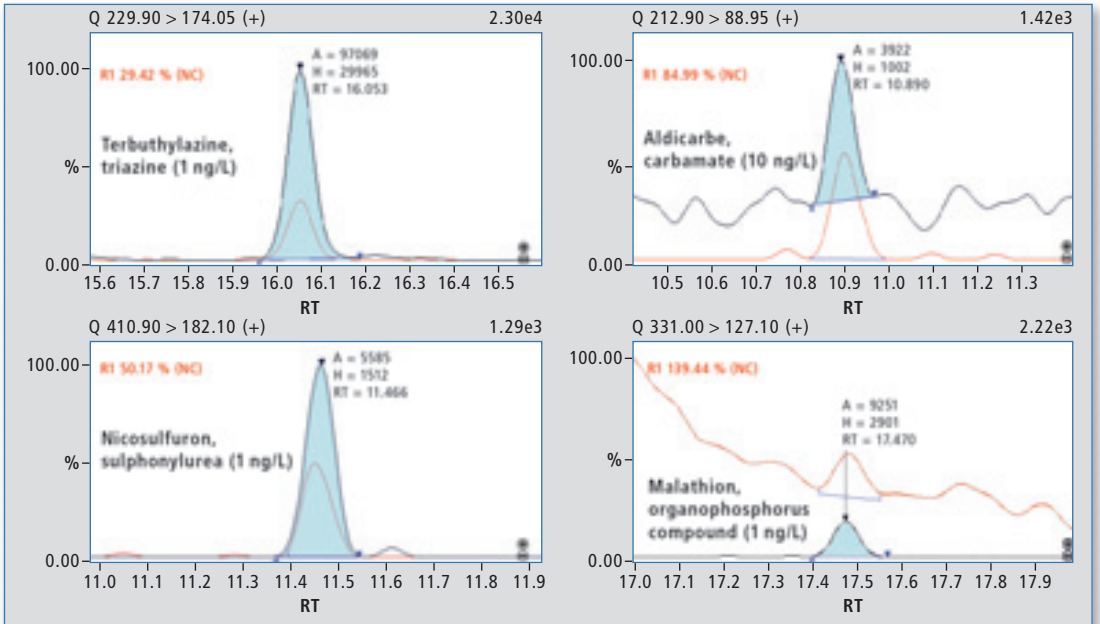


Figure 3: Example of chromatogram at the LLOQ

SPE-UHPLC conditions: Nexera X2

System: Shimadzu Nexera X2
Column SPE: Mayi-ODS C18
Column LC: C18AQ CS Interchim 2,6 µm 150*3 mm
A/B (SPE): water/acetonitrile + 0.002 % formic acid + 2 mM ammonium formate
A: water + 0.002 % formic acid + 2 mM ammonium formate
B: 50/50 acetonitrile/methanol + 0.002 % formic acid + 2 mM ammonium formate
LC flow: 0.7 mL/min
SPE elution flow: 0.2 mL/min
Oven temperature: 40 °C
Injection volume: 1,000 µl

MS conditions: LCMS-8050

System: Shimadzu LCMS-8050
ESI ionization mode: positive and negative
Dwell time: 4 to 199 msec to obtain at least 15 points per peak
Nebulizing gas flow: 2.8 L/min
Heating gas flow: 10 L/min
Drying gas flow: 10 L/min
Desolvation line: 150 °C
Heat block temperature: 300 °C
Interface temperature: 350 °C

An analysis demonstrated the different concentration levels prepared on three water samples (figure 2).

One of the previous matrices, spiked at different levels, was selected to establish the calibration curve. The other doped water samples worked as controls. The 23 different isotopic standards were added to perform an internal calibration. To overcome the matrix effect, ISTD were selected to cover the main families of pesticides such as phenylureas, triazines, carbamates, sulphonylureas and organophosphorus compounds.

The lower limits of quantification (LLOQ's) were established for each compound by a signal to noise ratio (S/N) greater than 10. Table 1 classifies the compounds according to their limits of quantification: 1, 10 and 25 ng/L. The LLOQs obtained fit the requirements of the European directives and the guidelines of the World Health Organization (WHO).

Compounds with LOQ at 1 ng/L					
4MBC	Chlorfluazon	Ethidimuron	Ioxynil	Oxamyl	Simazine
Acetamiprid	Chloridazon	Ethiofencarb	IPPMU	Oxasulfuron	Simazine hydroxy
Acetochlor	Chlorsulfuron	Ethiprole	IPPU	Oxazepam	Simetryn
Acide niflumique	Chlortoluron	Enbuconazole	Iprovalicarb	Oxydemeton methyl	Spirotetramat
Alachlore	Clethodim	Fenobucarb	Isoprothiolane	Paclobutrazol	Sulfometuron methyl
Ametryn	Coumafene	Fenothiocarbe	Isoproturon	Penconazole	Sulfosulfuron
Amidosulfuron	Coumatetralyl	Fenpyroximate	Isoxaben	Penoxsulam	Tebuconazole
Aminocarb	Cyanazine	Fensulfothion	Lufenuron	Phosphamidon	Tebufenoside
Atenolol	Cybutrine	Fenuron	Malaoxon	Pinoxaden	Tebutam
Atrazine	Cycloxydim	Fipronil sulfone	Malathion	Pirimicarb	Tebuthiuron
Atrazine desethyl	Cyflufenamid	Fluazinam	Mandipropamid	Pirimicarb desmethyl	Teflubenzuron
Atrazine-OH	Cyproconazole	Flufenacet	Mecarbam	Pirimicarb II	Tepraloxydim
Azaconazole	DCPMU	Fluometuron	Mefluidide	Prochloraz	Terbumeton
Azamethiphos	Desmetryn	Fluopicolide	Mercaptodimethur	Progesterone	Terbumeton desethyl
Azimsulfuron	Dichlorophen	Fluoxastrobin	Metabenzthiazuron	Promecarb	Terbuthylazine
Azinphos ethyl	Dicrotophos	Flupyrsulfuron methyl	Metalaxyl	Prometryn	Terbuthylazine desethyl
Azinphos methyl	Diethofencarb	Fluridone	Metazachlor	Propachlore	Terbuthylazine hydroxy
Azoxystrobin	Difenacoum	Flurtamone	Metconazole	Propazine	Tetraconazole
Bensulfuron methyl	Difenoconazole	Flusilazole	Methomyl	Propiconazole	Thiabendazole
Benthiavalicarb isopropyl	Difethialone	Fluxapyroxad	Metobromuron	Propoxur	Thiacloprid
Bisphenol S	Diisobutylphtalate	Foramsulfuron	Metolachlore	Propoxycarbazone	Thiazafluron
Buturon	Dimetachlore	Formetanate	Metosulam	Propyl paraben	Thiobencarb
Cafeine	Dimethenamid	Fosthiazate	Metoxuron	Proquinazid	Thiophanate methyl
Carbamazepime	Dimethomorph	Fuberidazole	Metribuzin	Prosulfuron	Triadimefon
Carbamazepine epoxyde	Dimetilan	Halosulfuron methyl	Metropolol	Pyrazophos	Triazamate
Carbaryl	Dimoxystrobin	Hexaconazole	Metsulfuron methyl	Pyrifenox	Tribenuron methyl
Carbendazim	Diniconazole	Hexaflumuron	Monolinuron	Pyrimethanil	Trietazine
Carbetamide	Dinoseb	Hexazinone	Monuron	Pyroxsulam	Trietazine-2-OH
Carbofuran	Dinoterb	Hexythiazox	Myclobutanil	Quizalofop ethyl	Triflumizole
Carbofuran 3 hydroxy	Diuron	Hydroxypropazine	Neburon	Rimsulfuron	Trimetoprime
Carboxin	Econazole	Imazapyr	Nicosulfuron	Rotenone	Trinexapac ethyl
Chlorantraniliprole	Epoxiconazole	Imazaquin	Oruface	Sebuthylazine	Triticonazole
Chlorbromuron	Erythromicine	Imidacloprid	Oryzalin	Siduron	Vamidothion
Compounds with LOQ at 10 ng/L					
245T Fenoprop	Cyprosulfamide	Fenarimol	Imazamox	Metazachlor oa	Quizalofop
Aldicarbe	Cyromazine	Fenoxycarb	Imazofulfuron	Methidathion	Roxythromycine
Atrazine desisopropyl	Desethyl terbutylazine 2-0H	Flazasulfuron	Iodosulfuron methyl	Oxadixyl	Tembotrione
Bentazone	Desmedipham	Florasulam	Isoprocarb	Paraoxon	Testosterone
Benzafibrate	Dia2hydroxy	Flufenacet oa	Isoxadifen ethyl	Phenmedipham	Thiencarbazone methyl
Brodifacoum	Dichlorprop-P	Fluroxypyr	Lorazepam	Phoxim	Thiodicarb
Bromadiolone	Diclofenac	Fomesafen	MCPA	Propamocarb	Tolytriazole
Butyl paraben	Dimetachlore oa	Forchlorfenuron	MCPP	Propanolol	Triasulfuron
Chloroxuron	Epitestosterone	Haloxyfop	Medroxyprogesterone	Propyzamide	Triazoxide
Clofentezine	Ethoxysulfuron	Haloxvfop methyl	Mesosulfuron methyl	Prothioconazole	Triclopyr
Clorsulon	Ethyl paraben	Imazalil	Metamitron	Pymetrozine	Trietazine desethyl
Closantel					Tylosine
Compounds with LOQ at 25 ng/L					
2,4-D	Dea 2 hydroxy	Fluquinconazole	Linuron	Titrosulfuron	Triflumuron

Table 1: Limit of quantification in ng/L for each compound

Performance evaluation

Repeatability was determined at the low level of concentration for each water with three injections. Regardless of the water matrix, the repeatability was less than 15 % for all 272 targeted compounds.

The results obtained showed recovery rates from 85 to 115 % for the calibration standards and the control samples.

Conclusion

Shimadzu offers a unique, automated and sensitive method to quantify a large number of pesticides in water. Limits of quantification range from 1 to 25 ng/L with 1 mL sample injection. Recovery rates ranging from 85 - 115 %, while repeatability is less than 15 % for the full list. Finally, this method is appropriate for thresholds of the actual normative environment such as the DWD, and a perfect example of a chromatographic method in combination with mass spectrometry as a powerful, user-friendly and time-saving method meeting the users' needs.

In addition to pesticide contamination, drinking water suppliers and water treatment plants are suffering from elevated concentration levels of nitrates released by extensive agricultural treatment. Episode 3 of our water Trilogy will give an overview on how to analyze anions such as nitrate and more using ion chromatography in the next issue of Shimadzu NEWS.

Referenzes

[1] REFIT EVALUATION of the Drinking Water Directive 98/83/EC, 2016.
[2] European Drinking Water Directive 98/83/EC1.

HMV-G3 series main unit

Versatile testing tool for the automotive industry

New HMV-G3 Series: "Redesigned" with a color camera and data protection functions

The future of mobility, the future use of cars as well as their propulsion, is multi-faceted as never before. Currently, ideas and projects meeting upcoming needs are at the R&D stage in the automotive industry, which is driven by ever-shorter development times. Simultaneously, it is one of the most competitive markets with very high quality standards.

As a global supplier to the automotive supply chain, Shimadzu provides the full range of analytical instrumentation and testing solutions in chromatography, spectroscopy, mass spectrometry, TOC and material testing. This covers engines, motors and power sources, bodies and interiors, environmental conservation, suspension and power transmission systems, electronics, batteries and fuel cells. This diversity of testing methods meets the valid standards, norms, regulations and compliance requirements.

Treatments of drive-related parts are critical parameters

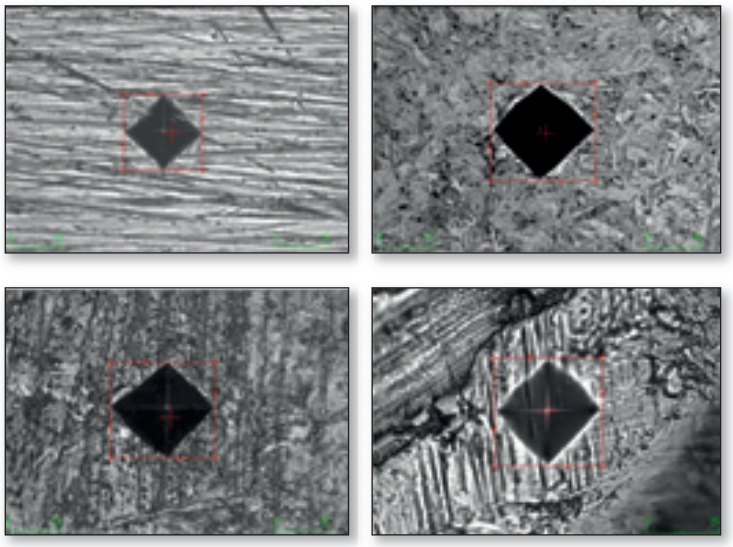
Particularly hardness testing of ferrous and non-ferrous components to international standards is a well-known process in the automotive market. The wear and strength characteristics of rotating and cast parts in the engine or other drive-related parts undergo treatments which are critical parameters. If the treatment is not carried out properly, this can cause additional costs and in a worst case situation, risk passenger safety while traveling by car.

Shimadzu's new HMV-G3 hardness testers target research and production processes. New functions improve data reliability, accommodate ISO standards related to Vickers hardness tests as standard and offer high expandability.

For mechanical parts, metals, layers, ceramics and plastics

The micro-hardness tester is indispensable in metallographic structure research, quality control of manufactured products and for preparing of product certification documents. The ability to measure hardness in limited microscopic areas and to do this with extremely high reliability is required for small precision mechanical parts, metal structures, processed surface layers and plating layers. In addition to metals, it is also useful in the evaluation of fine ceramics and engineering plastics.

The HMV-G3 series instruments measure Vickers hardness at very low test force levels. A regular quadrangular pyramidal diamond, referred to as an indenter, applies a force to a sample. The hardness of the sample can be calculated by measuring the indentation formed on the surface.



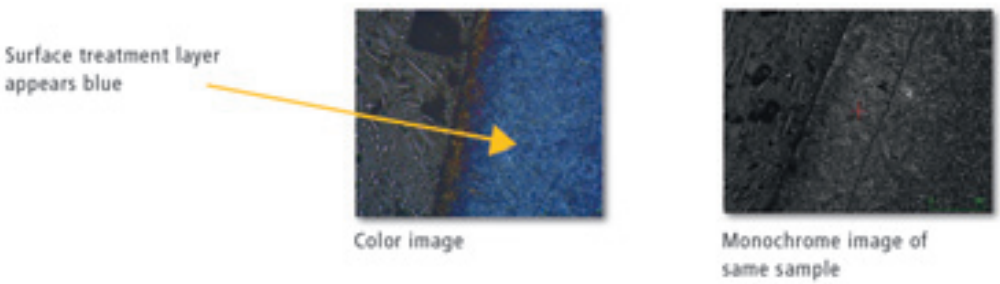
Improvement of the automatic reading function

Accordingly, in recent years, there has been a need for strengthened data management functions, improvements to operability and the enhancement of various functions to suit the application.

Compliant with ISO standards

As a standard, all Shimadzu HMV-G3 series models are now compliant with measurements at low test forces in accordance with ISO standards related to Vickers hardness tests. Protective functions to heighten data reliability, as well as report creation functions are also included as standard.

Additionally, a motorized micrometer for adjusting the position of the stage can be added optionally, and a model equipped with a 5 megapixel camera, the best in the industry, has been integrated into the series. The system can be configured according to application- and user-specific needs.



By comparison: Color vs. Monochrome image

MS^n analysis of nonderivatized and Mtpp-derivatized peptides

Two recent studies applying LCMS-IT-TOF instruments

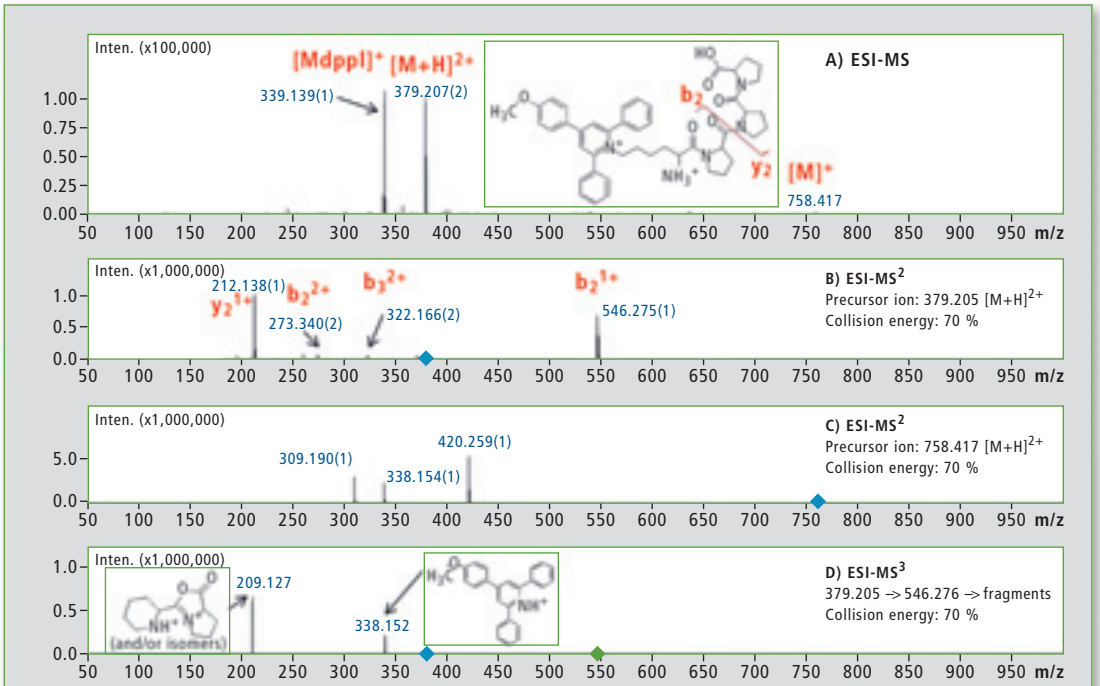


Figure 1: ESI-MS spectrum of the crude synthetic peptide Lys(Mdpp)-Pro-Pro-Pro (panel A), ESI-MS^2 fragmentation spectra of $[M+H]^{2+}$ (panel B) and the fragmentation spectrum of $[M]^{1+}$ (panel C) and ESI-MS^3 analysis of $[M+H]^{2+} \rightarrow b_2^+ \rightarrow$ (panel D). (Mdpp – 4-(4-methoxyphenyl)-2,6-diphenylpyridinium salt).

The LCMS-IT-TOF combines QIT (Ion Trap) and TOF (Time-Of-Flight) technologies. The QIT ion trap gives an efficient MS^n capability. After selecting the appropriate precursor ions, several MS^n experiments could be performed during one introduction of the sample, with product ions obtained serving as parent ions for the next stage of fragmentation. MS^n spectra with high mass accuracy offer improved reliability of signal assignment and structural analysis by fragmentation pathways. The IT-TOF instrument provides high resolution and accuracy for all MS and MS^n modes as well as mass stability and excellent resolution for LC-MS analysis. Such features make LCMS-IT-TOF the instrument of choice for advanced scientific studies in impurity analysis, metabolic profiling and biomarker research [1].

The research projects of the Group of Chemistry and Stereochemistry of Peptides and Proteins from the Faculty of Chemistry, University of Wrocław, Poland focus on the study of peptides and proteins by mass spectrometry. Professor Zbigniew Szewczuk and coworkers investigate the structure of natural and synthetic peptides, peptide conjugates and adducts, searching for posttranslational modifications and biomarkers. Due to low concentration of some peptides in biological samples, the increase in sensitivity of MS analysis is one of the important research goals of the group.

Two examples of recent studies are presented here which due to the required MS^n analysis were possible only by application of the LCMS-IT-TOF instrument.

1. MS^n analysis by LCMS-IT-TOF of derivatized peptides with 4-(4-methoxyphenyl)-2,6-diphenylpyrylium salt

Tandem mass spectrometry is a powerful tool in proteomic research. Despite the rapid development of this technique, insufficient sensitivity is still a problem due to poor ionization of some peptides present at low concentration in the biological sample. To overcome this problem, introduction of a functional group containing stable positive charge into peptide molecule, which increases the ionization efficiency, allows sensitive detection by electrospray mass spectrometry (ESI-MS).

Recently, researchers have developed several efficient methods for peptide derivatization by quaternary ammonium salt (QAS) synthesis [2]. The highest intensity increase (as much as 1,000 x) was observed for pyrylium ionization reagents, which react readily with the primary amino groups inside chain of lysine residue to form pyridinium salts bearing a stable positive charge [3]. The peptides derivatized with pyrylium reagent can be detected even at attomolar level (10^{-18}). A new kind of isobaric tag for peptides which is based on combination of $^{16}O/^{18}O$ exchange and derivatization by isotopologues of pyridinium salts was also proposed [4].

However, it is necessary to search for new ionization markers which will show a similar degree of ionization enhancement and have improved properties (higher solubility, sensitivity and precision of analysis). Researchers recently developed a new ionization reagent (4-(4-methoxyphenyl)-2,6-diphenylpyrylium salt, Mdppl) to improve the applicability in proteomic research (unpublished data). Specific MS^n transitions are used for unambiguous analysis of particular peptides derivatized with Mdppl.

The 4-(4-methoxyphenyl)-2,6-diphenylpyrylium salt was linked to the side chain amino group of a

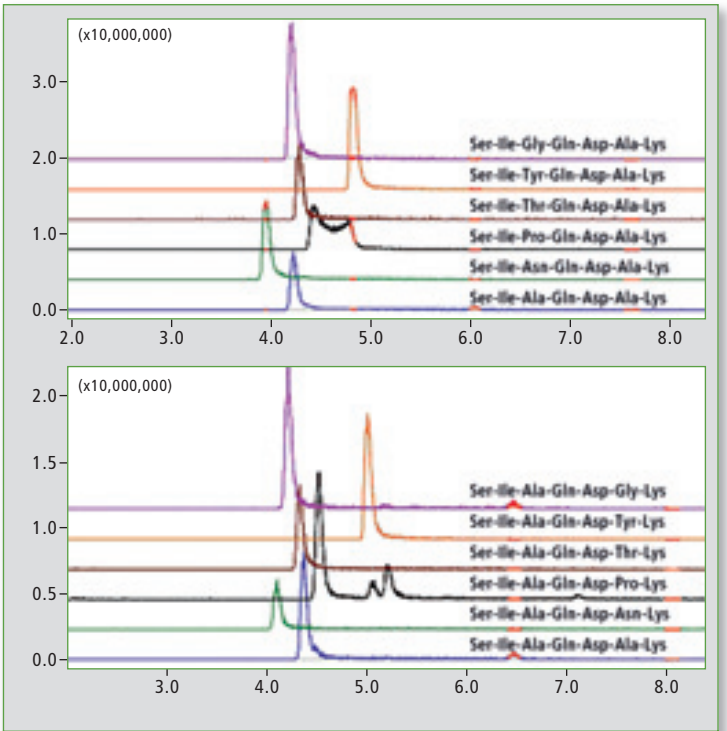


Figure 2: LC-MS chromatograms of peptide library subsets. Extracted ion chromatograms (XIC) for +2 ions represent library components.

model peptide (Lys-Pro-Pro-Pro). Figure 1 shows ESI-MS and ESI-MS^n analysis of Lys(Mtpp)-Pro-Pro-Pro.

In MS^2 experiments, a series of b and y ions were obtained. Due to the increase in collision energy, the intensity of m/z 338 signal grew although its intensity was still negligible. Peak at m/z 209 after MS^3 experiment corresponded to the internal fragment of peptide sequence. The signal is characteristic for pyridinium salts, but it appears mainly in the charge remote fragmentation mechanism [5].

To confirm this statement clearly, an MS^3 experiment was performed in which for the first fragmentation reaction the $[M+H]^{2+}$ was selected, containing one mobile proton responsible for charge directed fragmentation mechanism. The single-charged fragments resulting do not contain a mobile proton and may undergo fragmentation according to charge remote fragmentation mechanism. Combination of these two consecutive fragmentation reactions occurring according to different mechanisms may only be performed using instruments equipped with ion trap (IT).

Precise analysis of the resulting fragments requires high-resolution spectra which can only be guaranteed with IT-TOF instruments. The researchers noted that fragmentation of single-charged ions gave information about ionization tag whereas the same experiment for double-charged ion provided more information about peptide sequence. When combined, these experiments provide more reliable and comprehensive structural information. The m/z 338 fragment ion can be used in MRM or parent ion scan to study all peptides containing 4-(4-methoxyphenyl)-2,6-diphenylpyridinium groups.

As a result of MS^3, $[M+H]^{2+} \rightarrow b_2^+ \rightarrow$ fragments appear at m/z 209 and 338 as either $[M+H]^{2+}$ or $[M]^+$ (figure 1B, 1C). The MS^3 technique therefore leads to unique fragments, meaning that it can be used for the sensitive and unambiguous analysis of reporter ions in proteomic studies of compounds derivatized by Mtppl, for example: 379 → 546 → 338 and 379 → 546 → 209. This gives completely new possibilities for use of MS^n analysis in quantitative and qualitative proteomics.

To conclude, the MS^n method can be used successfully for sensitive peptide sequencing at high collision energies. Such fast confirmation of the structure of derivatized peptides based on the MS^3 method is extremely reliable. MS^3 analysis allows unambiguous identification of peptide fragments resulting from MS^2 experiments. The accuracy of the IT-TOF instrument enables determination of the molecular formula of the fragments, making it possible to propose structural formulae of the ions obtained. The IT-TOF instruments not only allow the determination of fragmentation pathways, but also offer new and fast methods for identifying trace amounts of potential biomarkers in the samples tested. MS^n experiments combine two fragmentation mechanisms: charge remote and charge directed.

2. Internal fragments formed during MS^n for analysis of isomeric peptides

The importance of mass spectrometry analysis in peptide science is obvious at several levels – from characterization of natural or synthetic products, sequence analysis to stability studies and quantification. There is also a growing interest in application of mass spectrometry to physicochemical studies including charge variants, molecular interactions and adduct stability studies.

The main advantages of mass spectrometry include high sensitivity and unambiguous identification of product, with excellent detection levels and the possibility of application to isotopically modified reference materials.

The lipophilicity of chemical compounds, represented as logarithm of the octanol-water partition coefficient (logP), is a very important parameter in medical chemistry. Typically, logP is measured in the traditional shake-flask method (SFM) which may be time-consuming, requires significant amount of material and is of limited use in case of highly hydrophobic compounds. Various chromatographic methods including RP-TLC and RP-HPLC supplement or replace the SFM, offering several practical advantages including reproducibility, insensitivity to impurities and degradation products, broader dynamic range and reduced sample handling and sample size [6]. One of the main advantages of HPLC in lipophilicity determination is the ability to compare directly the properties of a series of compounds by analyzing mixtures.

Lipophilicity is studied thoroughly in design and development of neuropharmaceuticals due to blood-brain-barrier issues. Several research groups investigate antinociceptive activity of peptides [7, 8], and there is a significant interest in application of RP-HPLC to lipophilicity comparison of the analogs proposed.

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Figure 3: MS^2 analysis of peptide library subsets, $[M+2H]^{2+}$ ions were selected as precursors

The LC-MS procedure seems ideal for this application, offering the additional benefits of indication of charged state of studied peptides. More complex tandem MS^2 approach is needed in case of isomeric peptides, to assign the peak order to proper sample components. In the recent project, the researchers encountered a problem which required a more advanced approach to differentiating of isomeric peptides.

To investigate the effect of sequence modification on chromatographic behavior of peptides and, subsequently, their lipophilicity, a peptide library was designed and synthesized based on podocin 290-296 fragment with a sequence Ser-Ile-Ala-Gln-Asp-Ala-Lys. The alanine residues were selected as diversity positions and replaced with tyrosine, threonine, proline, glycine and asparagine to provide a broad range of changes.

Subset Library 1:
Ser-Ile-**Xxx**-Gln-Asp-Ala-Lys
Subset Library 2:
Ser-Ile-Ala-Gln-Asp-**Xxx**-Lys
where **Xxx** represents Gly, Tyr, Thr, Pro, Asn or Ala

The two resulting subset libraries (modified in position 3 or 6, respectively) were analyzed using LC-MS. The peptides in each subset differ in molecular weight and MS analysis was sufficient to assign the signals. The elution profiles represented by extracted chromatograms (XIC) are presented in figure 2. The retention time differences between the isomeric peptides were minimal and the effect of position of the modifying residue on retention time could be studied only after a method to distinguish isomeric peptides could be established for analysis of sets.

The researchers subjected the subset libraries to MS^2 analysis using $[M+2H]^{2+}$ peptide ions as precursors (figure 3). High mass stability of TOF allowed conducting of a long series of LC-MS experiments without recalibration. The parent ions in overlapping chromatographic peaks were successfully selected and fragmented due to high resolution and accuracy of the IT-TOF instrument. In practically all cases the fragmentation produced b_2 and y_5 ions, which are identical in pairs of isomeric peptides and cannot be used to assign the peaks in chromatogram of a mixture of libraries. Some observed differences were identified as +2 fragment ions (Thr and Pro peptides) or were rather weak (Gly analogue).

Analysis was performed using a Shimadzu IT-TOF instrument, and it was decided to use the MS^n feature and subject the y_5 ions to further fragmentation (MS^3). The Tyr analogues were selected for preliminary studies, and the results shown in figure 4 reveal interesting differences in MS^3 fragment patterns. The characteristic MS^3 transitions will be used in further lipophilicity studies of podocin fragment peptide libraries.

Based on individual properties of the peptide sequence studied, regular MS^2 experiments could not confirm which Ala residue was replaced in a given peptide. The isomeric peptides could be identified only after obtaining deeper

insight into their fragmentation using MS^3 function of the IT-TOF instrument.

Conclusion

Scientific challenges could be solved quickly, successfully and efficiently due to the MS^n capability and high mass accuracy of the Shimadzu LCMS-IT-TOF instrument.

Methods:

Pyrylium salt synthesis: Synthesis of pyrylium salts involves two steps. The first step involves synthesis of a specific chalcone based on condensation reaction with different commercially available aldehydes and ketones. The reaction is carried out in alkaline environment, in the presence of 10 % NaOH in ethanol. In the second step, cyclization reaction is performed with obtained chalcone and proper ketone. The reaction requires the presence of trifluoromethanesulfonic acid (TFMSA) as a counterion.

Derivatization procedure: Sample of model peptide was dissolved in dimethylformamide (DMF) and excess proper pyrylium salt and trimethylamine (TEA) was added. The mixture was incubated at 60 °C for three hours. After this time, solvent was evaporated under nitrogen and the residue was lyophilized.

Peptide library synthesis was performed on solid support using Fmoc chemistry, and equimolar mixtures of peptide derivatives were used at diversity steps. Crude products were applied in the LC-MS experiments.

MS and MS^n experiments: MS and MS^n was performed on a Shimadzu IT-TOF mass spectrometer. For the experiments using precursor ion mode, a fragment ion corresponding to pyridinium was chosen.

LC-MS conditions: UHPLC Nexera equipped with an Aeris Peptide XB-C18 column (50 x 2.1 mm, 3.6 µm) was used applying a gradient separation from 5 to 60 % B in 15 min, A: 0.1 % HCOOH in water, B: 0.1 % HCOOH in acetonitrile, flow rate 0.2 mL/min. The IT-TOF instrument was operated in positive ion mode, and the 150 - 1,000 m/z range was analyzed.

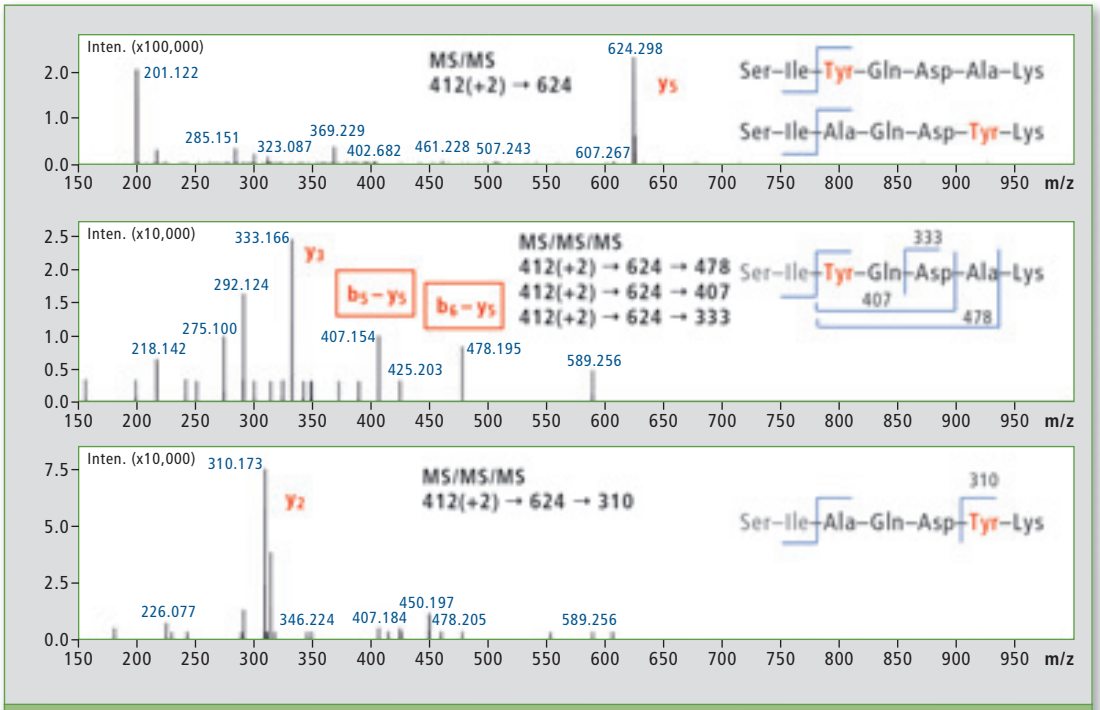

Figure 4: MS^n analysis of Tyr-containing library elements. MS^2 results were not sufficient to produce characteristic ions, whereas MS^3 experiments could be used to unambiguously identify the isomers.

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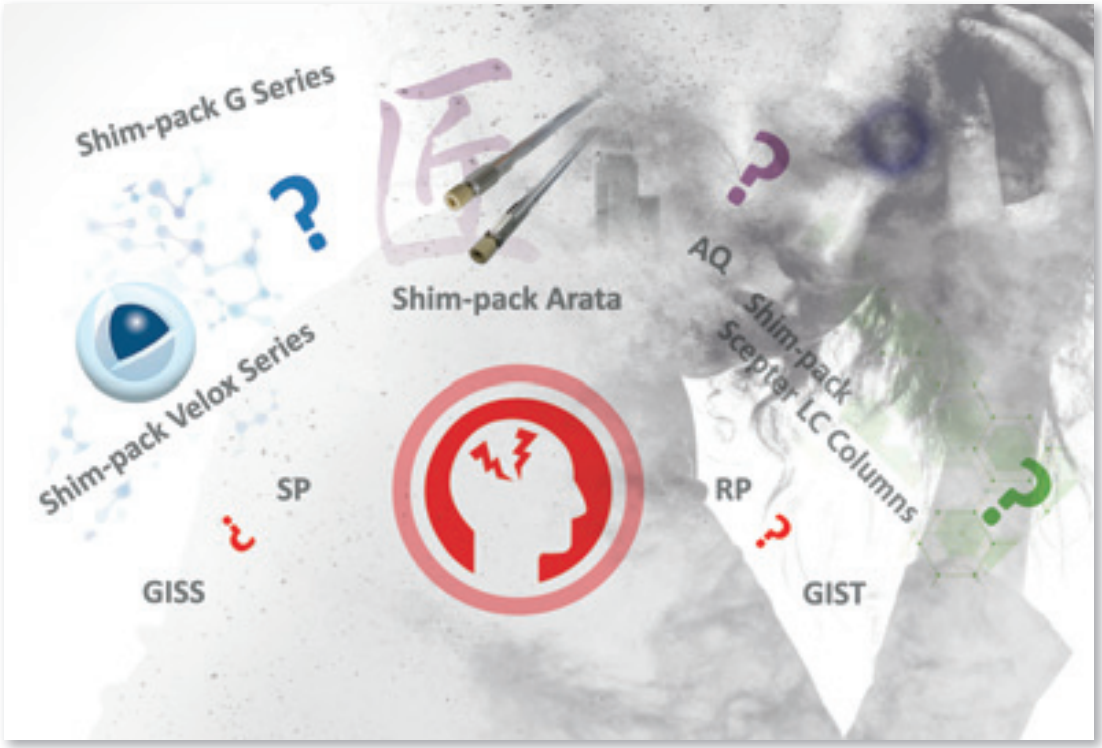
No more headaches!

A guide to choosing the perfect C18 column



The all-rounder in liquid chromatography: C18-columns are broadly used in pharmaceutical, food, clinical and environmental industries for reversed-phased chromatography. With thousands of options in the market, it can be overwhelming to choose the right C18 column. Shimadzu therefore offers a guide to its portfolio of C18 columns to simplify the selection process (figure 1). No more headaches through information overflow.

Shim-pack Velox: Maximize separation performance with core shell technology

Designed to maximize performance of LC systems, Shimadzu's Shim-pack Velox columns with core shell technology enable users to achieve increased separations and faster analysis on any LC platform. Whether developing a high-efficiency LC separation method, transferring an existing method for increased throughput while maintaining resolution or trying to improve the resolution of a complex separation, Shim-pack Velox columns satisfy any users' needs.

Due to their robustness, excellent column lifetimes are obtained, even for the most challenging sample matrices.

The Shim-pack Velox SP-C18 (Sterically Protected) series was designed specifically for applications under low pH condition, offering a well-balanced retention profile with long lifetimes even under the harsh acidic conditions needed for LC-MS(/MS) analysis.

Shim-pack Scepter: excellent stability and performance under a wide range of conditions

These next generation organic silica hybrid-based columns achieve excellent stability and performance over a broad pH range. With different chemistries, Shim-pack Scepter columns are effective for method development/scouting and are suitable for a wide variety of applications. Due to their different particle sizes (1.9 µm, 3 µm, 5 µm) and different column dimensions, Shim-pack Scepter LC columns are fully scalable between UHPLC, HPLC and preparative LC, making method transfer seamless between different laboratory instrumentation.

The Shim-pack Scepter HD-C18 with its high carbon loading and therefore high hydrophobicity, is especially suited for the analysis of isomers. This is particularly useful for pharmaceutical applications where medical substances need to be separated from structurally similar impurities.

Shim-pack Arata LC columns: unprecedented resolution and peak shape of basic compounds

LC columns claiming to be designed for basic compounds often fail to achieve adequate resolution of certain compounds. This problem is overcome successfully with the Shim-pack Arata columns, which solve issues such as fronting of highly polar basic compounds, peak shape deterioration of acidic compounds or long equilibration times required for low ionic strength acidic mobile phase.

The Shim-pack Arata Peptide C18 columns offer an excellent separation performance of peptides, even under weak ion pairing acidic mobile phases, and are therefore suitable for MS detectors. Since the adsorption of peptides to particles is minimized, the Shim-pack Arata Peptide C18 column ensures high recovery of peptides and provides excellent peptide analysis.

Shim-pack G series: for general applications

The Shim-pack G series consists of GIST, GISS and GIS, designed not only for conventional analysis but also for ultra-high speed and preparative methods due to their various particle size ranges. The Shim-pack GIST series includes standard columns for general applications, while the Shim-pack GIS columns are especially suited for preparative chromatography and the Shim-pack GISS for MS analysis.

Shim-pack GIST series: ultra-high inertness and high durability

Shim-pack GIST series columns are packed with newly developed high-purity porous spherical silica. Superior inertness not only improves analytical precision but also increases the column lifetime. It provides symmetric peaks even for strong ionic compounds, and undesired adsorption and peak tailing are reduced to a minimum. It is capable of covering a wide pH range (1 - 10) and is therefore suitable for many applications.

Furthermore, the Shim-pack GIST series is designed with various particle size ranges, making it easy to transfer any analysis from ultra-high-speed to conventional methods.

Compared to general C18 columns, Shim-pack GIST C18-AQ achieves strong retention of hydrophilic highly polar compounds while maintaining high inertness and durability in highly or 100 % aqueous mobile phases. Further-

more, it reduces the absorption of basic and acidic compounds and achieves superior peak shapes in the analysis of metal complexes.

Shim-pack GISS Series: high-speed analysis with ultra-high inertness and high durability

The Shim-pack GISS series maintains the same ultra-high inertness and wider pH range as the Shim-pack GIST series while providing rapid separations with symmetric peaks. The optimization of surface area, pore size and chemical bonding delivers superior peak shapes, making these columns ideal for LC/MS/MS analysis. Shim-pack GISS series also includes the 1.9 µm and 3 µm HP series for UHPLC-analyses.

Shim-pack GIS Series: high retentivity, lower column back pressure, high inertness

These columns are packed with high purity silica gel and are ideal as generic HPLC columns. Highly uniform particles ensure stable mobile phase delivery and outstanding low pressure. The Shim-pack GIS columns not only help reduce solvent costs, but also relieve system load. In addition, the uniform silica surface and stable chemical modifications ensure high analysis reproducibility.

Shim-pack GIS C18-P is designed with a polymerically bonded octadecyl group, providing high steric selectivity for separation of planar and non-planar compounds, such as vitamin D2/D3 or PAHs.

Shim-pack GIS RP-Shield contains a polar functional group embedded between silica surface and an octadecyl group, making it stable in 100 % aqueous mobile phases without phase collapse. The embedded polar functional group is also extremely base-deactivated, enabling the column to provide superior peak shape for acids. It also provides unique selectivity due to hydrogen bonding interactions, making it suitable for separations that cannot be achieved by other modes, such as hydrophobic interactions or π-π interactions.

Shim-pack MC: the perfect column for Micro LC

The Shim-pack MC columns are compatible with a range of mobile phase flow rates, from one microliter per minute to dozens of microliter per minute, making them the ideal columns for micro LC.

More information on Shimadzu's C18 column range can be found at www.shimadzu.eu/hplc-columns

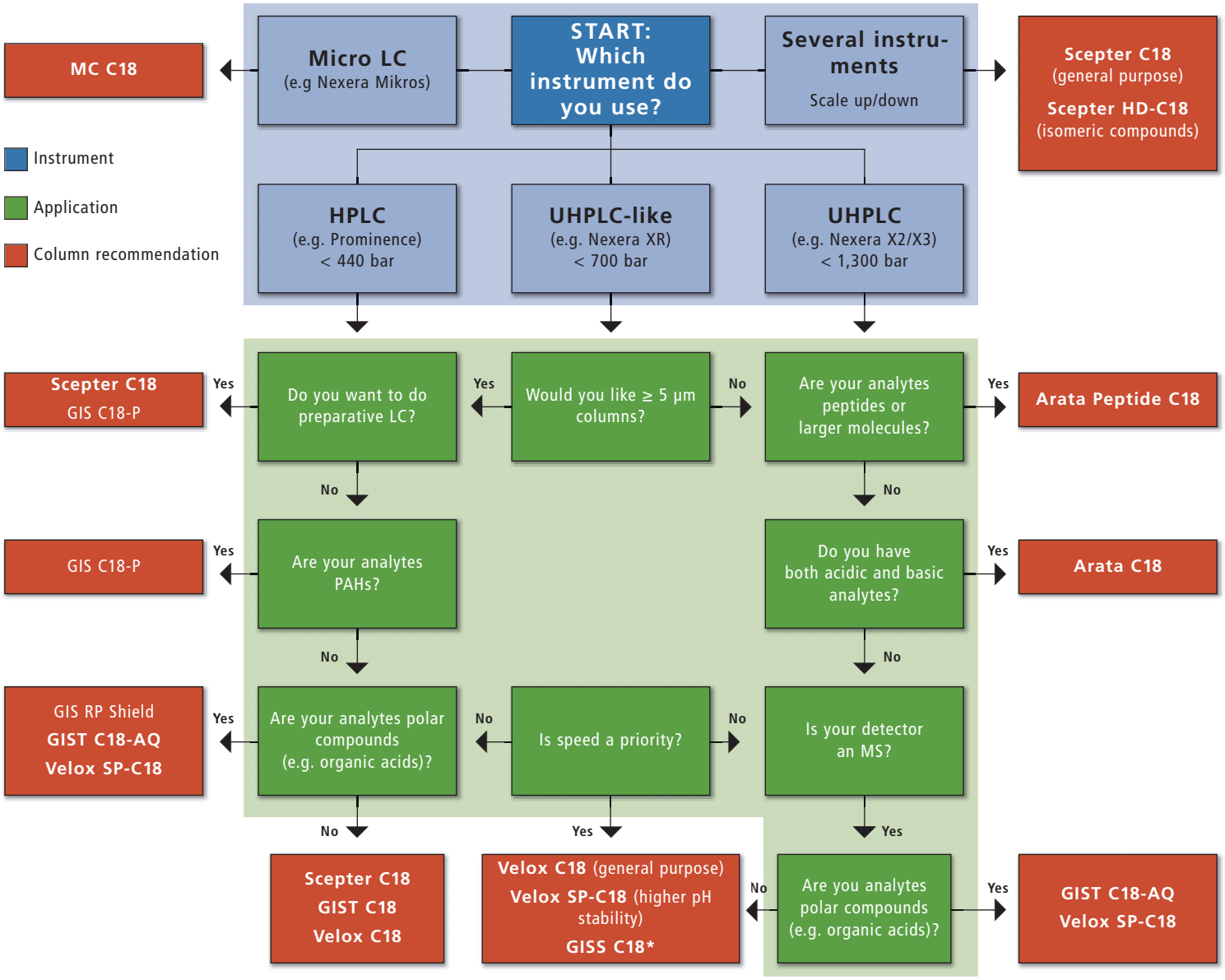
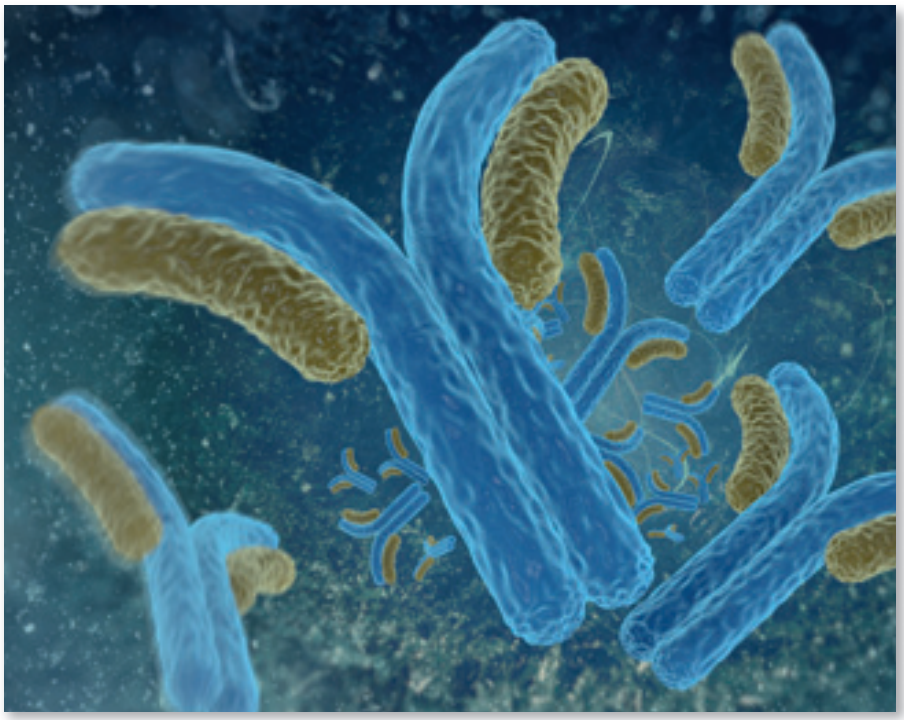


Figure 1: Selection guide to choosing the right column. *The Velox series, with core shell technology, is the first choice for high speed analysis.
The columns recommended for MS are shown in **bold**.

Validated method for monoclonal antibody drugs

Assessment of the nSMOL methodology in the validation of bevacizumab in human serum



3D illustration of antibodies

Development and validation of bioanalytical methods for proteins can be hindered by the availability of assay reagents. LC-MS/MS hybrid assays enable an approach capable of using more generalized reagents. For these assays, the capture and subsequent sample processing steps are highly critical for optimal sensitivity and selectivity.

Nano-surface and molecular-orientation limited proteolysis (nSMOL) provides a unique sample processing approach to quantification of monoclonal antibody (IgGs) drugs in biological samples. The nSMOL technique provides selective proteolysis of the Fab region of the antibody using a specialized protein A capture resin and trypsin-immobilized nanoparticles for protein digestion.

Evaluation of the method with a cancer-treating medication

Shimadzu scientists have tested this technology and successfully developed and validated methods for several monoclonal antibody drugs [1, 2]. As part of an independent evaluation of the methodology, Intertek Pharmaceutical Services (a contract research organization based in San Diego, CA) has evaluated the technique and validated an LC-MS/MS method for bevacizumab using a validation protocol consistent with industry and regulatory standards for pharmaceutical drug candidates [3, 4]. Bevacizumab is a medication applied to treat a number of types of cancer.

To assess the utility of the nSMOL technique, an LC-MS assay was developed for the analysis of bevacizumab in human serum with a standard curve range of 100 ng/mL to 10,000 ng/mL. Initially, the standard Shimadzu sample processing protocol using the kit analogue internal standard was followed and yielded results with acceptable accuracy and precision. However, for the chromatography developed internally, the analogue internal standard gave atypical results at high analyte concentrations in frozen QC samples.

Applicable for the rapid development of methods with minimal assay optimization?

The method was subsequently optimized to a 96-well plate method with stable labeled peptide internal standard for improved throughput, selectivity and assay performance. No substantial changes were made to the critical Shimadzu protocol steps to test whether or not the methodology can be used to rapidly develop methods with minimal assay optimization. Using 10 µL of serum, a LLOQ (lower limit of quantification) with strong peak response (>20 : 1 S/N) was observed. Conversion of the assay to a 96-well format required a change in the aliquot size of the method due to an unknown drop in sensitivity, but 10 µL was deemed to be a more than acceptable assay volume.

Assay performance during the validation was acceptable and well below the ±20 % acceptance criteria (table 1) with inter-assay %Dev values ranging from -5.89 % to -1.66 % (2.17 % at the LLOQ) and inter-assay precision values at ≤ 7.05 % (13.8 % at the LLOQ). Analyte peak response in blanks of all individual human serum lots evaluated was <20 % of the LLOQ peak response, showing acceptable selectivity.

No substantial matrix effects

A lack of substantial matrix effects is highly important for any LC-MS assay. In this validation, low and high QCs were prepared in six individual lots of human serum as well as in a lot of hyperlipemic serum and a lot of hemolyzed serum. Choice of evaluation of QCs over matrix factor was to test a more stringent approach in evaluating nSMOL as these QC lots could have differing characteristics in assay capture and trypsin digestion, as well as the typical lot to lot variation in ion suppression/enhancement caused by endogenous compounds present with chromatographic elution of the analyte.

For the matrix effects experiments, at least two-thirds of the individual lots were within 20 % of nominal concentration and the overall mean concentration was within 20 %, indicating that there were no substantial matrix effects (table 2). In addition, both hemolyzed and hyperlipemic lots were within 20 % deviation from nomi-

Run #	Quality Control Concentrations (ng/mL)							
	100		300		3,000		7,500	
	Amount found (ng/mL)	Dev. (%)	Amount found (ng/mL)	Dev. (%)	Amount found (ng/mL)	Dev. (%)	Amount found (ng/mL)	Dev. (%)
1	97.1	-2.90	276	-8.00	2,540	-15.3	7,110	-5.20
	91.2	-8.80	264	-12.0	2,910	-3.00	6,960	-7.20
	88.1	-11.9	306	2.00	3,030	1.00	8,370	11.6
	105	5.00	276	-8.00	2,910	-3.00	7,660	2.13
	90.2	-9.80	238	-20.7	2,860	-4.67	7,850	4.67
	79.1	-20.9	292	-2.67	3,110	3.67	8,030	7.07
2	a	a	302	0.667	2,800	-6.67	6,970	-7.07
	107	7.00	311	3.67	3,170	5.67	7,370	-1.73
	132	32.0	271	-9.67	2,720	-9.33	6,990	-6.80
	117	17.0	286	-4.67	2,720	-9.33	7,020	-6.40
	111	11.0	297	-1.00	2,910	-3.00	6,580	-12.3
3	108	8.00	281	-6.33	2,740	-8.67	6,740	-10.1
	118	18.0	289	-3.67	2,750	-8.33	7,610	1.47
	104	4.00	292	-2.67	2,500	-16.7	7,780	3.73
	86.4	-13.6	a	a	3,070	2.33	4,560 b	-39.2 b
	98.5	-1.50	254	-15.3	2,840	-5.33	7,590	1.20
Mean	102		282		2,850		7,380	
sd	14.1		19.9		190		515	
% CV	13.8		7.05		6.65		6.98	
% Dev	2.17		-5.89		-5.04		-1.66	

Table 1: Quality control results for Bevacizumab in human serum.
a = No analyte response detected. Sample is rejected.
b = Outlier value per the Dixon Test, value is not included in statistical calculations.

Lot #	Quality Control Concentrations (ng/mL)			
	300		7,500	
	Amount found (ng/mL)	Dev. (%)	Amount found (ng/mL)	Dev. (%)
Lot 1	340	13.3	8,160	8.80
Lot 2	393	31.0	8,680	15.7
Lot 3	351	17.0	8,620	14.9
Lot 4	225	-25.0	5,540	-26.1
Lot 5	326	8.67	7,350	-2.00
Lot 6	342	14.0	8,110	8.13
Mean	330		7,740	
sd	56.0		1,180	
% CV	17.0		15.2	
% Dev	9.83		3.24	
A. R.	4/6		5/6	

Table 2: Matrix effects for bevacizumab in individual lots of human serum.
A.R. = Acceptance Ratio.

Sample Type	Quality Control Concentrations (ng/mL)			
	300		7,500	
	Conc. (ng/mL)	Dev. (%)	Conc. (ng/mL)	Dev. (%)
Hyperlipemic Serum	373	24.3	8,670	15.6
	342	14.0	8,550	14.0
	346	15.3	8,450	12.7
Mean	354		8,560	
sd	16.9		110	
% CV	4.77		1.29	
% Dev	17.9		14.1	
Hemolyzed Serum	301	0.333	7,810	4.13
	252	-16.0	7,610	1.47
	276	-8.00	7,420	-1.07
Mean	276		7,610	
sd	24.5		195	
% CV	8.87		2.56	
% Dev	-7.89		1.51	

Table 3: Matrix effects for bevacizumab in hemolyzed and hyperlipemic lots of human serum

nal concentration (table 3). Since some individual lots were outside 20 % and since there was a bias in the hyperlipemic lot measured concentrations, some chromatographic adjustment or sample cleanup modification might be useful to improve assay performance in this area.

Conclusion

Overall, the nSMOL methodology resulted in acceptable assay performance in the validation of bevacizumab in human serum with minimal adjustment of standard assay conditions. As individual laboratories use nSMOL, some adjustment of assay conditions might be warranted and as with any LC-MS assay, use of a stable labeled internal standard is highly recommended.

nSMOL is a trademark of Shimadzu Corp.

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How much fluorescence does a polymer show during quality control?

Polymers and fluorescence – Part 2: Fluorescence spectroscopy of industrial base polymers

Microplastics and synthetic materials in the environment are some of the greatest challenges worldwide for mankind, animals and the environment. The example of PET bottles demonstrates that their fluorescence intensity provides information on the material composition. This Part 2 of a series of articles expands the focus on different polymers. In Part 3, which will be released in the next issue of Shimadzu NEWS, particle size and its effect on fluorescence will be examined.

As described in Part 1 of the last issue, polyethylene terephthalate (PET) can be examined easily with fluorescence spectroscopy, simply by applying a piece of the PET bottle wall in order to analyze the fluorescence. This revealed fluorophores that might not have been suspected in the transparent, colorless or slightly bluish-clear polymer pieces.

In the next step, polymers are examined that originate not from the consumer sector, but rather as "new" or "recycled" directly from industrial production. For this purpose, crystal clear and colorless polymers were selected, such as polycarbonate (PC), polystyrene (GPPS), styrene acrylonitrile (SAN) and a recyclate of polycarbonate (PCrec).

The industrial polymer variants listed here are used for example as "glass replacements" in industry due to their hardness (impact resistance) and durability. Some of the granules have a cylindrical or slightly cloudy lenticular shape. The biopolymer granules examined, such as polylactides (PLA) and polybutylene succinate (PBS) were transparent and colorless, with slight turbidity. The non-aromatic polymers such as polypropylene (PPhomo), polyethylene (PE) and polyvinyl chloride (PVCsoft), also appeared as colorless, opaque and soft granules in lenticular form.

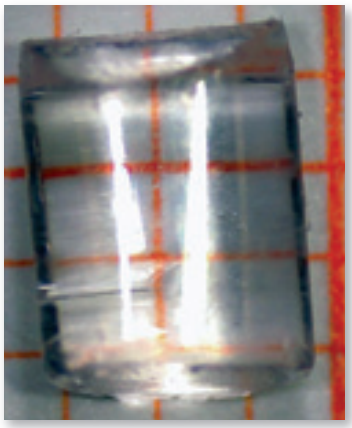


Figure 1: A typical polymer granulate in crystal clear and colorless cylindrical form on graph paper. The orange grid in the background of the granulate corresponds to 1 mm per square.

In this application, "industrial" polymers and recycled polymers are all colorless, transparent or turbid. It is expected that all polymers would show their natural fluorescence.

Fluorescence can occur when the molecule absorbs much energy. Considering the molecules of PC, GPPS, SAN and PET, all structures contain high-energy ring systems.

Fluorescence and phosphorescence

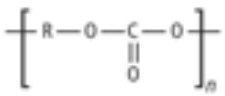
When an electron in the ground state S0 is exposed to light, the energy of the electron in the molecule is changed. This elevates it to the excited state S1 with a higher energy level (see figure 2). This excited state is not stable in the long term and changes rapidly as it approaches the ground state. The excited electron decays by radiating the energy through heat or light. The radiative transition from state S1 to S0 that emits a photon is called fluorescence. The alternative excitation from T1 to S0 is called phosphorescence. In the T1 state, three electrons are at a higher energy level (excited triplet state). S1 is the excited singlet state (1 excited electron in higher energy levels).

Polycarbonates contain several phenyl-based molecular groups, which are very energy-rich due to the π electrons of the double bond in the ring. In fluorescence, electrons can be lifted into higher

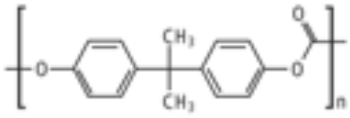
energetic orbitals (S1) from a high-energy ring system using hard radiation. This energy level is unstable, and the electron leaves this orbital emitting photons (luminosity = fluorescence).

The importance of the phenyl group

The general structure of the PC is



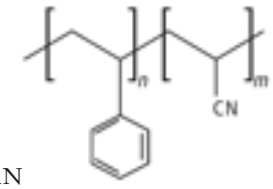
where R is a placeholder for hydroxyphenole. The best known is the PC type, which is produced with bisphenol A using phosgene, whereby phenol is a waste product.



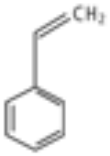
The molecular structure shows 2-ring systems (phenolic) and a carbonyl group with the usual -C=O double bond, which is softened electronically by the position in the ester group R-CO_2-. There are enough π electrons available for fluorescence.

In the polycarbonate used in this application, the PCmonomer is based on BPA (bisphenol A). Recycled PC samples should be identifiable for quality control purposes, as these traces may contain fluorescent additives.

Chemically speaking, SAN consists of styrene and acrylonitrile. The dominant molecular structure is again the phenyl ring from the styrene component.



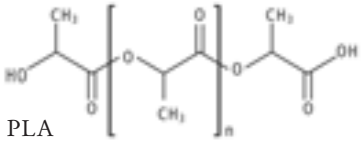
SAN



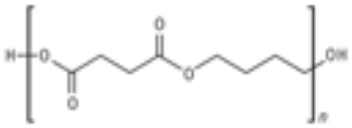
mono-PS

Since the phenyl group plays an important role in the molecular structure of all polymers under consideration, it can be assumed that the analytical fluorescence wavelength pairs are found in the same regions.

Polylactides and polybutylene succinate are chain molecules containing an ester group, which are equipped with high-energy electrons as shown in the structures.



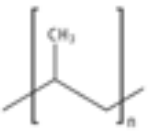
PLA



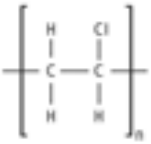
PBS

PE, PP, and PVC on the other hand are chain molecules in which no surplus energy-rich π electron compounds or aromatic elements are available for fluorescence.

PE



PP



PVC

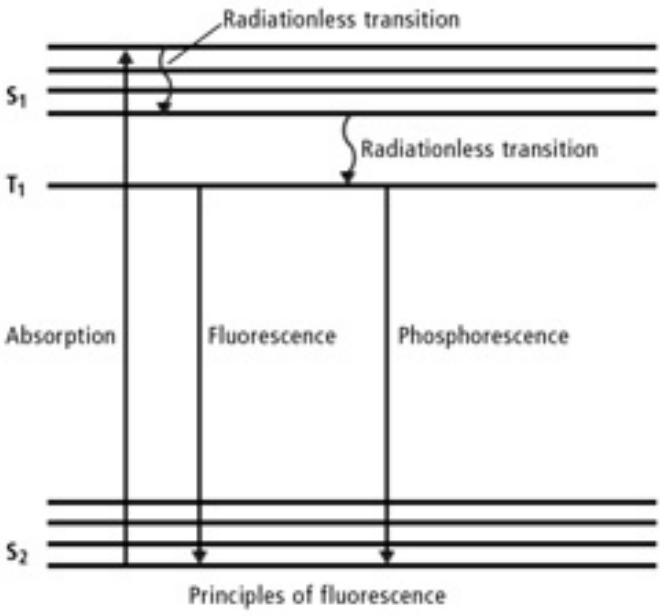


Figure 2: The imaginary axes would be seen as follows: The X axis corresponds to time and wavelengths; the Y axis represents energy. The excitation by light (arrow upwards) at the time 00:00 at a given wavelength leads after a short time (dwell time in S1 and release of first energy) to a wavelength offset to longer wavelengths. Therefore: Fluorescence needs an excitation/light source and appears shortly after excitation with higher wavelengths. Another conclusion is that if hard radiation is reduced by energy, it becomes softer or changes from short wavelength (hard) to long wavelength (soft) radiation.

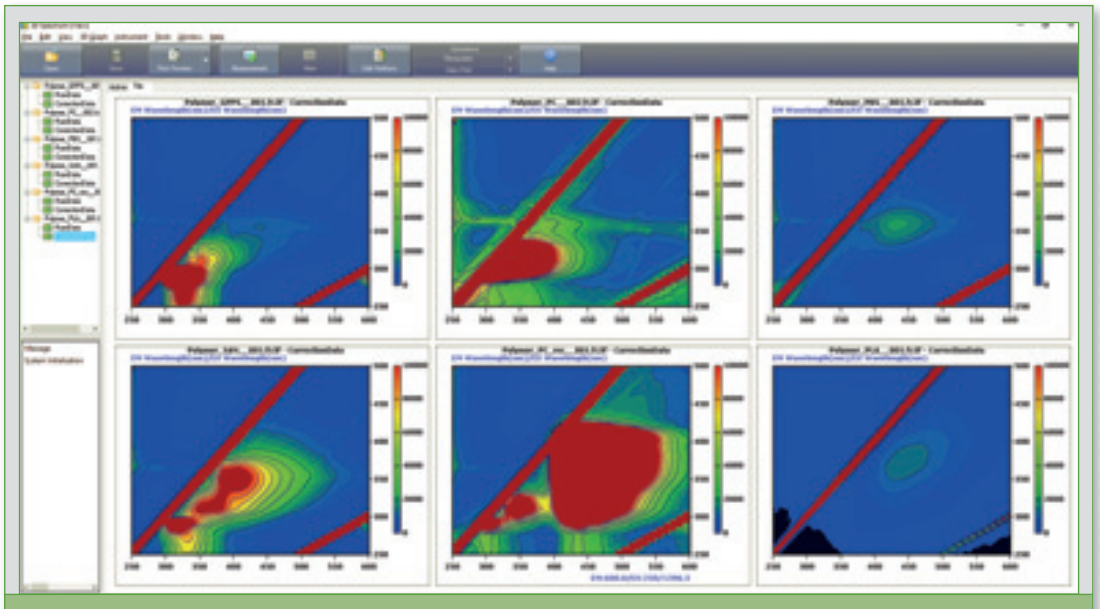


Figure 3: Fluorescence spectra of six different polymers: top left to right – GPPS, PC and PBS; bottom left to right – SAN, PCrec and PLA (for positions of the spots in the EEM see Table 1 [page 28]).

Sample preparation

The sample size and position within the barium sulphate ($BaSO_4$) bed in the holder for solid samples must be taken into account in the measurements. The experiment with the granules showed that the slit geometry of the light spot plays an essential role. The projection of the slit (rectangle) aims exactly at the center of the round holder. The inside diameter of the $BaSO_4$ bed is 2.5 cm.

Polymer	Range [nm]	Shape [µm]
Industrial	**Hotspot (EM/EX)**	**Pellet**
PC	350/310	Cylindric
PC rec	305/290	Lens shape
	350/310	
	435/375	
	435/400	
SAN	315/290	Cylindric
	370/315	
	400/350	
GPPS	310/290	Cylindric
Biopolymer		
PLA	370/275	Lens shape
	440/360	
PBS	430/360	Lens shape
Non-aromatic		
LDPE	285/260	Opaque lens
	330/290	
PP homo	300/280	Opaque lens
	330/300	
PVC soft	405/325	Opaque lens
	445/360	

Table 1: Active fluorescence zones (hotspots) of the polymer granules in different forms – cylindrical and lenticular granules

Depending on the selected slit, this round surface is illuminated in a more or less rectangular fashion. The granulate was embedded in the center of the holder and sealed with a quartz plate. This is important to note because the granules are not flat and cannot be homogenized with $BaSO_4$ due to their size and strength. $BaSO_4$ is used here as a fixing powder.

Evaluation of the measurements

Depending on the properties described, the different polymers also show different fluorescence. Figures 3 (page 27) and 4 show the EEM (excitation emission matrix) matrices obtained.

Remarkably, fluorescence activities could be found in all polymers. The hypothesis "no fluorescence with LDPE and PP" was not met. It appears that additives are present that promote fluorescence. Comparison between PC and PCrec for example, demonstrates that by using an additive (for bleaching), the recyclate is supposed to appear colorless and transparent (table 1).

Conclusion

Each polymer used here has its own fluorescence spectrum. In the industrial plastics PC, GPPS and SAN, fluorescence is determined by the fluorescence activity of the phenyl ring. In contrast, the chain molecules of the plastics PLA and PBS fluoresce due to the presence of the ester. According to the molecular structure, steric constraints and different excitation positions in the compact PLA molecule, the PLA molecule has hard radiation corresponding to the energy demand, whereas the PBS with long CH_2 chains requires less hard radiation. The non-aromatic polymers such as LDPE, PPhomo, and PVCsoft show fluorescence, although none is expected in these π electron-poor materials.

Fluorescence spectroscopy can therefore be used easily for quality control in order to make the "invisible" visible.

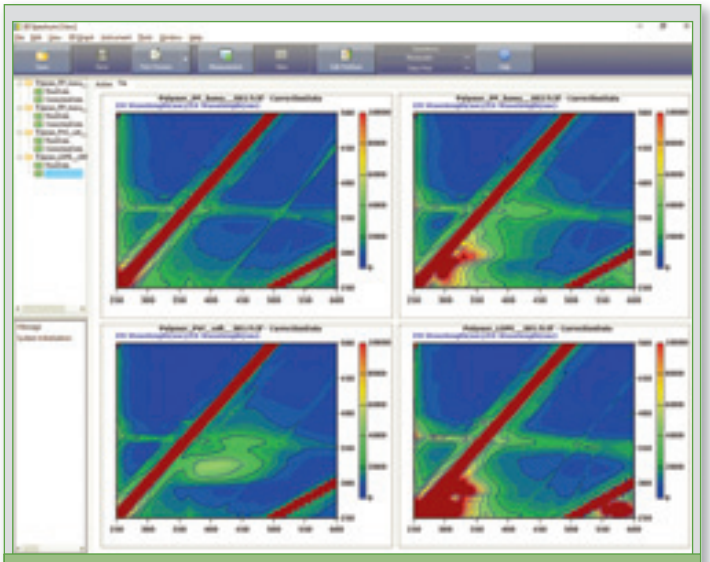


Figure 4: Spectra for long-chain non-aromatic polymers with the same scaling of intensity. Also at top left is the control EEM from the quartz plate above the $BaSO_4$ bed. Top right PPhomo, bottom left PVCsoft and bottom right LDPE (low density polyethylene).