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Milestones in development and technology

In every ocean, at every depth – microfibers and microplastics

Micro FTIR analysis of smallest particles from deep sea to polar ice

On all continents, scientists find polymeric microparticles in living organisms, the same in marine animals such as polar cod (*Boreogadus saida*) or deep-sea crustacea (*Lysianassoidea amphipods*), which are an important part in the food chain of fish, penguins, seabirds or seals. Two working groups (Newcastle University, UK and Wageningen Marine Research, Netherlands) investigated the stomach contents of the respective animal species and isolated microparticles of around 100 µm in size.

Polar cod from the North Pole

The Arctic Ocean is often imagined as being undisturbed and untouched nature. However, the Arctic is not a closed system but is influenced by man, for example through climate change or environmental pollution. In recent years, an additional sea gyre has been described near the Svalbard archipelago (Norway) in the Arctic Ocean, which collects marine plastic waste from southern, more urbanized areas. Plastic residues were also found in the sea ice itself. When the ice melts, the plastic particles trapped in the ice can be released and are available to organisms that live and feed under it.

One of these organisms is the polar cod, a species of fish that spends its first years under sea ice. It feeds on small crustaceans and is an important prey for larger animals such as sea birds and marine mammals. Polar cod

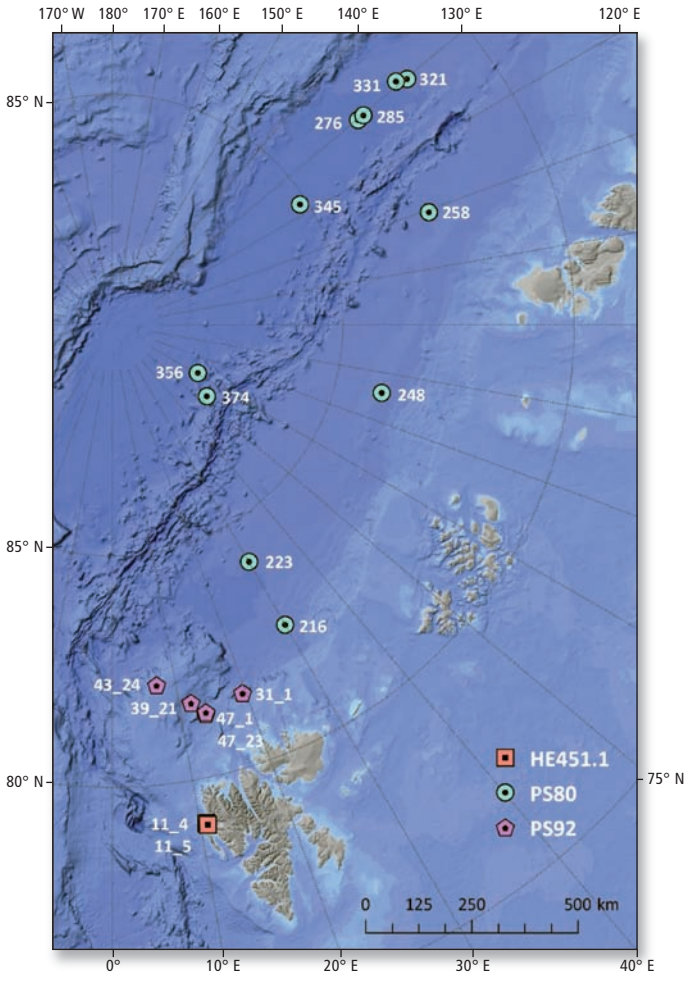

Figure 1: Locations of polar cod samples

is therefore considered a key species of the Arctic ecosystem. For this study, the fish were caught directly under the sea ice of the Eurasian basin and in open water around Spitsbergen [1].

Deep sea shrimps

The University of Newcastle used submersible probes to collect mobile marine organisms in the extreme depths of the sea. Samples were taken at a depth of 11,000 m in the Mariana Trench, a deep-sea channel in the western Pacific just east of the Micronesian Islands, and in other trenches in the Pacific with depths up to 7,000 m. Most organisms were Lysianassoidea amphipods, creatures adapted to the conditions of the deep sea. It has been found that these deep-sea organisms are even capable of develop-

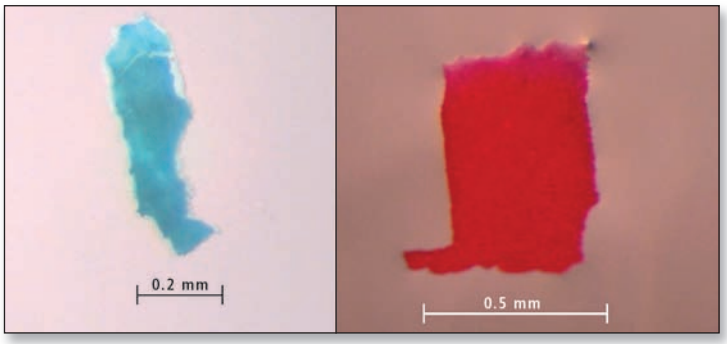

Figure 2: Two of the particles found in polar cod stomachs loaded with plastic

ing enzymes that digest wood fibers [2].

Sample preparation

The biggest problem in microparticle analysis is contamination of the samples by electrostatically charged particles flying at the sample or particles carried by humans (scales and fibres). Some plastic particles, so-called microfibers, for example from clothing, are not only absorbed by animals but also so light that they can float in the air. It is impossible to distinguish between fibers that get into the sample via intake by the animals themselves or via air pollution [1].

Purification of microparticles is another difficult issue. Should the samples be treated chemically to remove all protein-like residues or should they simply be washed with water? Using chemical cleaning may lead to loss of information about adhering chemicals; cleaning with water preserves this information, but the cleaning itself may be less thorough. The microparticles from amphipods were treated with a KOH (potassium hydroxide) solution that dissolves all organic adhesions without attacking the plastic itself. Particles from polar cod were cleaned with water.

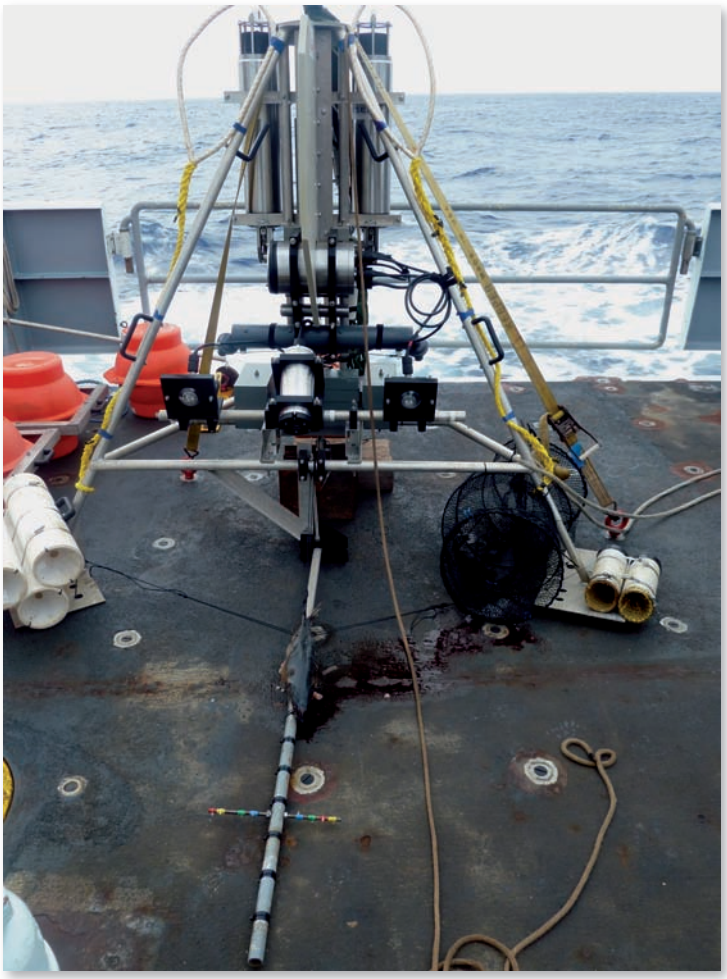

Figure 3: Submersible probes used in the deep sea

Analysis using infrared microspectroscopy

The definition of a microparticle varies from publication to publication. Particles in the µm range are the reason for developing a measuring technique that works well with the appearance of the samples. Infrared spectroscopy is the method of choice.

The IRTracer-100 FTIR instrument, the AIM-9000 infrared microscope and the DC3 diamond cell from Specac were used to examine the microparticles. Depending on their appearance, single point measurements or surface analyses (mapping) were carried out. The diamond cell presses small, uneven particles flat, up to the point where they become transparent. This way, particles can be analyzed using the transmitted light method. The infrared spectra measured were identified with spectral libraries from different sources (Shimadzu, Sadtler, STJapan-Europe and others).

Study of plastic intake

To investigate the potential correlation between plastic in sea ice and the ingestion of plastic by fish, plastic was sought in 72 polar cod stomachs. Of the 72 fish, two individuals (2.8 % of the collected sample) containing small plastic particles were found. This result is comparable to intake rates for fish from Norwegian and Canadian waters. The blue particle of 200 µm shown in figure 2 was analyzed using infrared microscopy. The result of the transmitted light measurement showed that the particle consists of a combination of inorganic (kaolin) and polymer-based material (PMMA) (figure 5, page 4).

The infrared spectrum (transmission) of the red particle in figure 2 is the result of a mixture of epoxy resin and acrylic based adhesive [1].

Figure 4: Hirondellea gigas as example for the family amphipode

Figure 5 shows an example of the analysis of the blue particle. The main components PMMA (polymethyl methacrylate) and kaolin of the particle were identified using the library function. The figure 5 shows the kaolin in the middle of the transmission spectrum of the microparticle and below the spectrum of PMMA.

The stomach of a deep-sea shrimp contained a particle composed of "polyethylene" partially surrounded by a mixture of organic and inorganic matter (figure 6, page 4). As a polymer, polyethylene is naturally very light with a density below 1 (table 1, page 4) and it actually floats in upper water layers. This particle has presumably reached the deep sea layers by the agglomeration of small aquatic animals and their skeletons or other external influences.

Characteristic	PE-LD	PE-HD	PE-LLD	PMMA	Sea water (temperature: -10 °C; Salt concentration: 35 g/L
Density in g/cm^3	0.915 - 0.935	0.94 - 0.97	0.87 - 0.94	1.18 - 1.19	1,027

Table 1: Density of sea water, polyethylenes (PE) and polymethylmethacryle (PMMA) [Wikipedia]

Figure 5: Analysis of the samples found in the polar cod

In the upper right section of the particle (figure 6) the measuring spot of 15 x 15 µm can be seen. It is a transmission image of a particle of 100 µm which was widened by smoothing using the diamond cell.

The figure clearly shows that the particle has a corona. Measurement determined that it was a ring of $CaCO_3$ (calcium carbonate) (figure 7). Due to the clear representation, the particle was scanned by way of screening and mapping (figure 8). In the mapping view, set to the carbonylic band of $CaCO_3$, this ring can be displayed very clearly.

Conclusion – Plastics as additional stress factor

The results show that the effects of plastic pollution have reached the Arctic Ocean and the deep sea and that fish and amphipods encounter plastic particles. The consequences of the intake of plastic particles remain unclear.

In combination with other factors such as climate change, increased shipping and the expansion of fisheries, plastic pollution can be an additional stress factor for the sensitive Arctic ecosystem [1].

Once plastic reaches the bottom of the deep sea, it is expected to remain there. The result is an accumulation of plastic on the seabed – an alarming conclusion. Isolating fibers from the stomach of an animal living at 11 km depth already shows the extension of the microplastic problem [3].

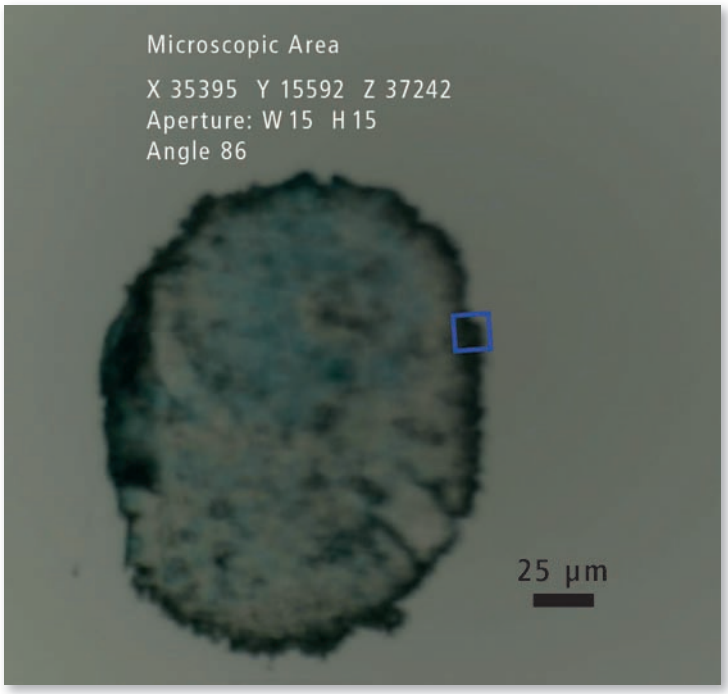

Figure 6: Image of the particle with the CCD camera of the AIM-9000 infrared microscope

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Literature

[1] Plastic ingestion by juvenile polar cod (Boreogadus saida) in the Arctic Ocean, Susanne Kühn, Fokje L. Schaafsma, Bernike van Werven, Hauke Flores, Melanie Bergmann, Marion Egelkraut-Holtus, Mine B. Tekman, Jan A. van Franeker; Polar Biology, 2018. https://doi.org/10.1007/s00300-018-2283-8
[2] National Geographic News August 30, 2012.
[3] https://news.sky.com/story/plastic-pollution-reaches-oceans-deepest-parts-11127144

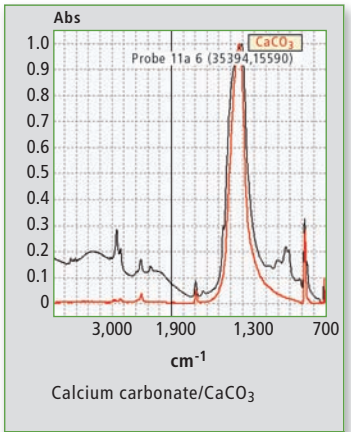

Figure 7: Spectra of the amphipods samples

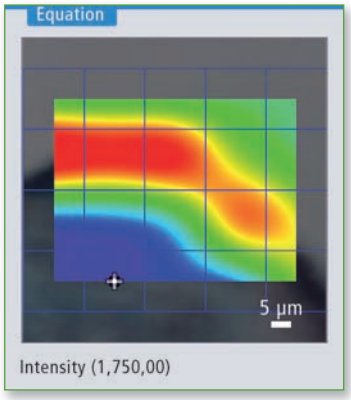

Figure 8: FTIR-Mapping of the amphipod sample

Figure 8a: Color-scale of the FTIR Mapping

High-value compounds in antibiotics

ALSACHIM supports pharmaceuticals players and their antibiotics R&D programs

ALSACHIM, a Shimadzu Group company, has introduced a full range of stable labeled internal standards of antibiotics to support the pharmaceutical industry and hospitals for developing Therapeutic Drug Monitoring. This covers specific labeled compounds such as Tazobactam, Avibactam or Ceftolozane and Ceftaroline.

As a world leader in the synthesis of stable labeled internal standards (^{13}C, ^{2}H, ^{15}N), stable labeled building blocks, APIs (Active Pharmaceutical Ingredient) and metabolites used in bioanalysis studies, ALSACHIM has joined the Shimadzu group in 2017. It completes Shimadzu's product and solution portfolio in the clinical market.

For 13 years, the France-based company has been supplying its products to customers all over the world, spread throughout the chemical discipline, including the pharmaceutical and biotech industries, clinical and bio-analytical CROs and research institutions in areas such as Health, Food and Environment.

Tazobactam-$^{13}C2$-$^{15}N2$, Avibactam-$^{13}C5$, Ceftolozane-d2-$^{15}N2$ – high-value compounds in antibiotics

In a constant R&D state of mind and to be close to market needs, ALSACHIM has developed major labeled compounds in fields of antibiotics which present a real health problem nowadays. All major players in the health area are working on issues related to antibiotics (antibiotic resistance, antibiotic residues in blood, combination of antibiotics etc.).

ALSACHIM has thus introduced stable labeled internal standards in the different families of antibiotics, such as in these ranges: Beta-lactams, Cephalosporin, Carbapenem, Penicillin etc. The range of labeled and unlabeled antibiotic molecules has expanded. Now, ALSACHIM offers its customers high-quality analytical isotope labeled standards such as Amoxicillin-d4, avibactam-$^{13}C5$, cefepime-$^{13}Cd3$, cefotaxime-$^{13}Cd3$, ceftaroline-$^{13}Cd3$, ceftazidime-d6, ceftolozane-d2-$^{15}N2$, cloxacillin-d5, fluocloxalilin-$^{13}C4$-^{15}N, meropenem-d6 or tazobactam-$^{13}C2$-$^{15}N2$.

UV-1900: Perfect instrument for all analytical challenges

UV-Vis spectroscopy has never been so fast and easy

UV-Vis spectrophotometer UV-1900

The new UV-1900 spectrophotometer integrates a progressive double-beam optical system with Shimadzu's patented Lo-Ray-Ligh® diffraction grating technology in a Czerny-Turner type monochromator. This technology ensures low stray light with a photometric repeatability previously unknown in its instrument class.

UV-1900 also guarantees maximum efficiency with the industry's fastest level scan function (29,000 nm/min); it takes just three seconds to measure in all wavelength regions. Controlled using easy-to-handle color touchscreen or the new LabSolutions UV-Vis software, UV-1900 is perfect for optimizing laboratory workflow.

Figure 1: Enhanced usability via touchscreen

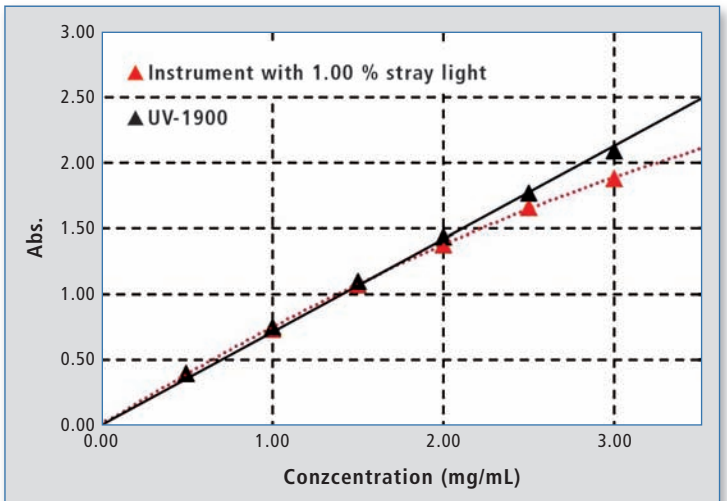

Figure 2: Calibration curve of UV-1900 (black line) is more linear than the curve of an instrument with 1 % stray light (red line)

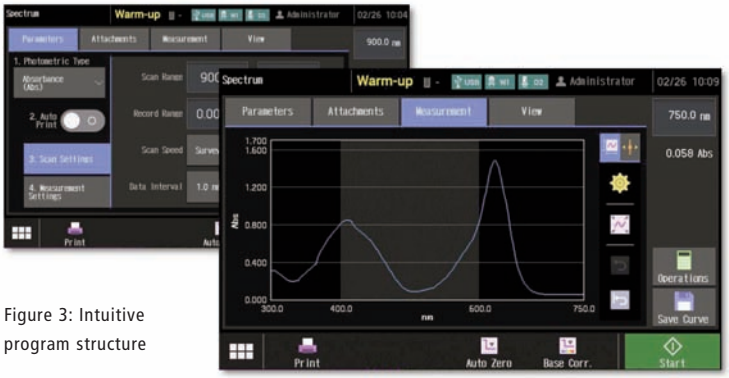

Figure 3: Intuitive program structure

High technologies combine for unprecedented abilities

Known from high-end models, the Lo-Ray-Ligh diffraction grating brings the lowest stray light level in its class (less than 0.5 % @ 198 nm) to the UV-1900. It ensures high photometric repeatability with high accuracy, offering one of the largest ranges of linearity (see figure 2).

The UV-1900's optical system also shows the highest resolution in its class (1 nm) which guarantees maximum measurement accuracy as required by Pharmacopeia. Moreover, the new ultra-fast scan function enables users to perform accurate measurement with an amazing scan speed of 29,000 nm/min. This reduces analysis time significantly and enables elucidation of fast chemical processes.

Perfect instrument for all analytical challenges

The color touchscreen (controlled by fingertip or included stylus) is

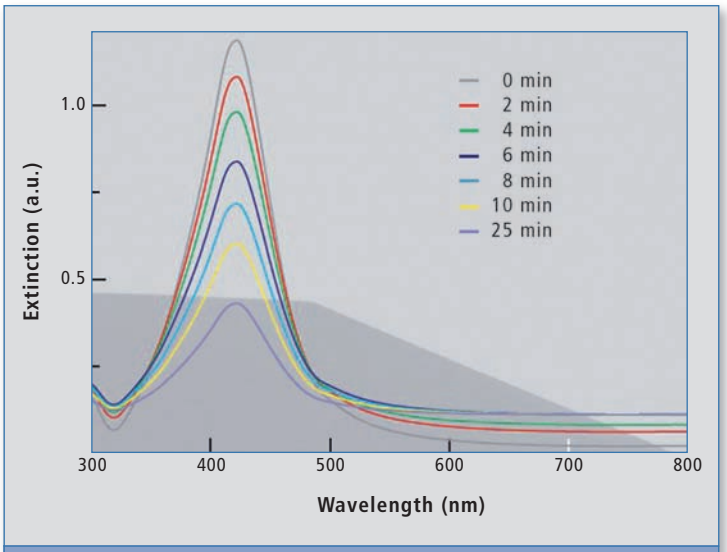

Figure 4: Kinetics study of silver nanoparticles agglomeration

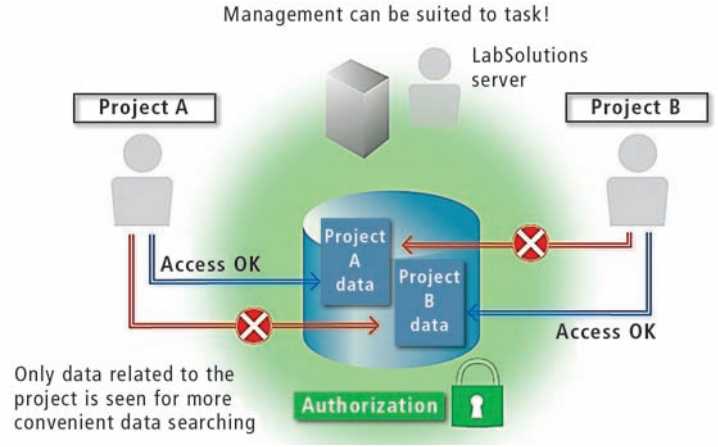

Figure 5: LabSolutions DB or CS project management

accompanied by an intuitive program design. All typical programs (photometric, spectrum, quantitation, kinetics, time-course and biomethod for DNA or protein quantification) are accessible without the need for a PC. The onboard validation and security functions enable full support for Pharmacopoeia, GLP/GMP, FDA and other regulations. Moreover, the sample compartment accepts many common accessories such as the sipper unit, temperature control and the Specular Reflectance Measurement Attachment.

This qualifies the UV-1900 as the perfect standalone instrument for many application fields such as food, pharma or chemistry. The entire spectral range can be scanned in a matter of seconds. Whereas until now only the intensity at one significant wavelength could be observed for time-course measurements, the change of the entire absorption spectrum can now be investigated.

LabSolutions UV-Vis for advanced capabilities

With the aim of improving work efficiency by exploiting UV-1900 maximum capacities, the new LabSolutions UV-Vis Software is intended for frequent users of UV-Vis spectrophotometers in various applications.

The clear and simple program layout fits into the LabSolutions family (LabSolutions IR, LabSolutions RF). System and measurement configuration workflows are optimized by easy to follow windows and self-explanatory icons.

Different programs are accessible via the LabSolutions Manager. From the start, spectrum, quantitation, photometric and time-course are available. The new Spectra Evaluation Function makes quality control easier than ever with automatic judgement for the samples analyzed. Additional programs, e.g. color analysis, can also be added as options.

Multiple data sets can be exported easily with just a click to other formats such as batch text or Microsoft Excel. Moreover, LabSolutions UV-Vis supports the user with convenient functions such as real-time display of concentration data or automatic determination of optimal parameters for peak detection.

Compliance with ER/ES regulations is assured with the Shimadzu LabSolutions DB or CS platform. These tools provide a project management function suited to tasks and system operations. These functions enable equipment and user management, security policy and data processing to be set on a project by project basis, thereby improving the efficiency of data searches and management tasks.

Conclusion

The new UV-1900 defines a new standard for compact UV-Vis spectrometers. Lo-Ray-Ligh diffraction grating technology in the double-beam instrument enables excellent photometric specifications while the color touchscreen and connection to LabSolutions UV-Vis provide an easy state-of-the-art workflow, both in standalone and PC controlled operation while still being fully compatible with UVProbe. Its broad range of measurement modes and compliance with international regulations open many application fields for this compact instrument.

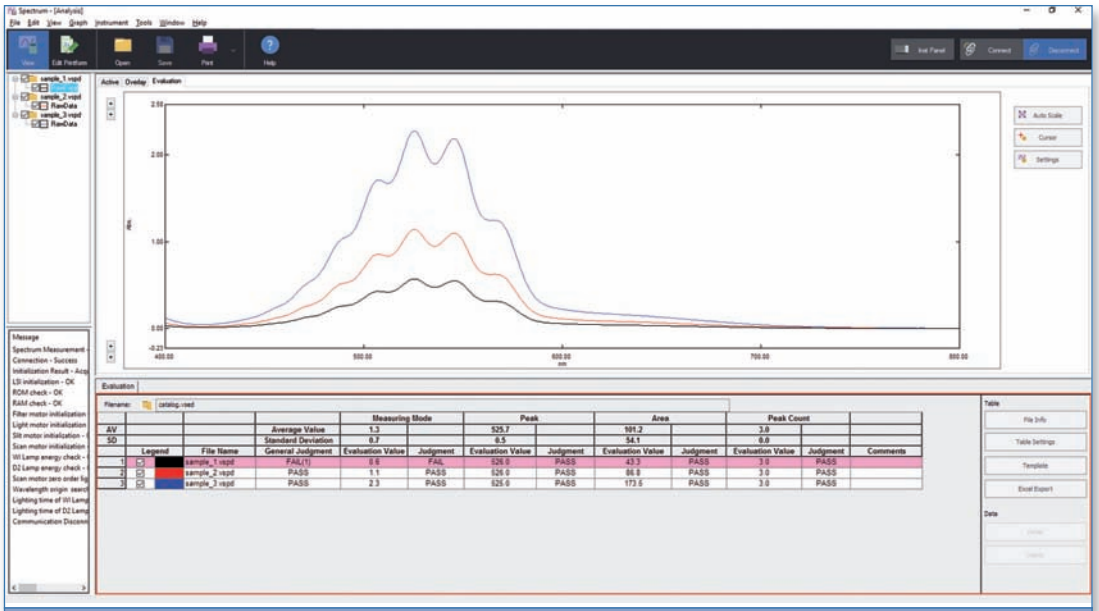

Figure 6: Layout of the new LabSolution UV software

Completely pure

TOC analysis for cleaning validation in the pharmaceutical industry

When manufacturing pharmaceuticals, highest purity and careful handling of substances and active ingredients are essential. It is especially important to remove all process residues from production effectively, because only a well cleaned plant prevents contamination and adulteration of the drug produced, thereby contributing to patient safety.

In 1988, for example, 200 million doses of a cholesterol-lowering drug had to be recalled – they were contaminated with pesticides. It is assumed that there was a cross-contamination, as solvent drums from the pesticide production were re-used in the manufacturing of the medication. For finishing, the active ingredient was delivered to another facility where normally no pesticides are manufactured, and whole product batches were contaminated. At that time, the drug manufacturer neither carried out adequate controls, nor validated cleaning procedures.

Regulatory requirements

As a result, key regulatory requirements for cleaning were specified in 1993 in the "Guide to Inspections – Validation of Cleaning Processes (7/93)" of the FDA (American Food and Drug Administration). Documented evidence is now required that demonstrates an approved cleaning process which consistently reduces active pharmaceutical ingredients (API), process residues, detergents and microbial contaminants on product contact surfaces of manufacturing equipment to an acceptable level for the processing of pharmaceuticals. This is particularly important in the production of compounds in batch processes, as the plant is used for various substances and contamination of the downstream products must be avoided.

When a compound batch leaves the facility, the equipment used, e.g. reactors or fermenters is cleaned before the next batch can be produced. The cleaning process to be used is usually strictly specified, and is then evaluated analytically and documented.

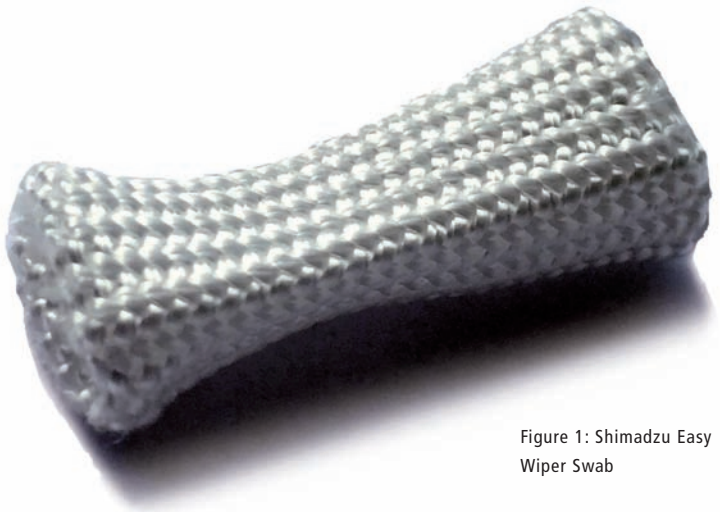

Figure 1: Shimadzu Easy Wiper Swab

A sample from the plant is examined for certain parameters. If a defined limit value is not exceeded, the system is regarded as having been cleaned and may be used again. This process is called cleaning validation. The cleaning method used has a great influence on the type of sampling technique required.

Cleaning procedure 'Clean in Place'

CIP (clean in place) cleaning occurs automatically without dismantling of the system. This means that the system must be build according to CIP design. This includes the use of rinsing heads, collection tanks, avoidance of dead spaces, and recycling possibilities of the cleaning agent. CIP cleaning is very effective because time and temperature, as well as cleaning agent and solvent consumption are optimized. Automatic cleaning also enables a standardized and easy to validate procedure. In the case of CIP cleaning, the rinsing liquid of the last rinse cycle (final rinse) is taken as a sample and analyzed. This is a particularly simple, easily automated and fast method.

Figure 2: TOC-L with SSM-5000A

Cleaning procedure 'Clean out of Place'

For COP (clean out of place) cleaning, the system must be disassembled and the components cleaned individually. This procedure is very time consuming and labor intensive. Due to the individual cleaning, this procedure cannot be standardized. However, the lower cost of the system and the possibility of visual assessment are beneficial.

In case of COP-cleaning, wiper testing (swab) is used to sample visible residues (figure 1). These include coatings, crusts, caking in angles and corners and substances that are particularly difficult to dissolve. The swab used can be extracted in a solvent and the extraction solution is then examined analytically. If water is used as an extraction solvent, TOC determination is used for subsequent analysis. Alternatively, the swab can also be analyzed directly (using a carbon-free swab) with a TOC solids module.

Applicable analysis methods

Several analytical methods are available to verify successful cleaning, but they differ in sensitivity and specificity. Common methods are shown in table 1.

Using specific methods such as HPLC or GC, agents and detergents can be selectively detected. However, it is possible in some chemical cleaning processes that initially existing components can no longer be detected due to

decomposition reactions. If acids or alkalis are used as additional cleaning agents, non-specific parameters such as pH or conductivity are often used for detection. However, it is not possible to detect active ingredients and excipients in this way.

To determine quickly whether the concentration of active substances and residues of cleaning agents and auxiliary substances is low enough after cleaning, the sum parameter TOC can be applied.

TOC analysis in cleaning validation

TOC (Total Organic Carbon) analysis captures all carbon from organic compounds in one analysis and is therefore particularly suitable for determining contamination by organic components. Carbon content of the sample is oxidized to CO_2 and analyzed with an NDIR (non-dispersive IR) detector.

The TOC value reflects the total organic contamination caused by the precursor, the additives used and the remaining cleaning agents. Aqueous samples (final rinse or swabs eluted in ultrapure water) can thus be analyzed quickly and easily (analysis time: approx. 4 min).

Precondition required is good water solubility of all substances. For water-insoluble substances, direct combustion of the carbon-free swabs is recommended. Table 2 gives examples of recovery rates of various water-soluble and water-insoluble substances in the sample types mentioned.

Two methods, one device

For cleaning validation, a mixture of swab method and final rinse method is usually used. This allows the entire plant as well as special critical locations to be examined with maximum sensitivity. Rinse water and swab samples obtained by extraction and by direct combustion can be tested using a single TOC analyzer.

Modern analyzers such as Shimadzu's TOC-L series handle the preparation of liquid samples automatically (acidifying and sparging). With a high volume of samples, this can be done in an autosampler to save time.

The systems operate with a highly efficient platinum catalyst at 680 °C combustion temperature. A special injection unit enables automatic dilution of the sample if the calibration range is exceeded. Standards are also automatically diluted to create calibration curves at equidistant concentration intervals.

Method	Specific	Detectable components	
		Active ingredients	Cleaning agents
TOC	No	x	x
pH	No		x
Conductivity	No		x
UV-VIS-Spectroscopy	Depending on wavelength		
Chromatography	Yes	x	x

Table 1: Methods of analysis

For direct combustion of swab samples, the analyzer is combined with the SSM-5000A solid sampling module (figure 2). Swabs are combusted directly in an oxygen environment at 900 °C with a catalyst. Quantification of the CO_2 combustion product subsequently enables determination of TOC.

Conclusion

In the pharmaceutical industry, various cleaning methods are used, for example "clean in place" or "clean out of place", whose validation is bound to certain techniques. TOC analysis is especially suitable for determination of contamination caused by organic compounds. This is done with the final rinse method or the swab method, each with advantages and disadvantages. Both are therefore used in cleaning validation. With the TOC-L series systems, cleaning of production plants in the pharmaceutical industry can be documented quickly and easily using both methods.

Further information on this article:

- SCA-130-202 TOC determination in Final Rinse cleaning validation File "202_cleaning_validation-final_rinse_12.pdf"
- SCA-130-203 TOC determination in cleaning validation Swab method File "203_203_cleaning_validation-_swab_methode_12k.pdf"

Substance	Water solubility	Recovery rates		
		Rinse	Swab eluted	Swab direct
Tranexamic acid	Soluble	105.0 %	107.0 %	101.0 %
Anhydrous caffeine	Soluble	108.0 %	109.0 %	100.0 %
Isopropylantipyrine	Insoluble	109.0 %	92.2 %	105.0 %
Nifedipine	Insoluble	107.0 %	89.9 %	106.0 %
Gentashin ointment	Insoluble	4.4 %	1.7 %	100.0 %
Rinderon ointment	Insoluble	15.2 %	7.5 %	104.0 %

Table 2: Recovery rates for different substances and sampling types

SteBLife – a new short-time evaluation procedure for fatigue life calculations of materials, specimens and components

SteBLife reduces test effort by up to 95 % using a servo-hydraulic testing system by Shimadzu

The availability of reliable fatigue data is of continuous and often urgent need. In the following article, it is shown how to use the potentials of non-destructive and destructive testing methods in order to achieve a significant gain in information concerning fatigue behaviour combined with a reduction of experimental effort and cost required.

The new SteBLife approach is an enhanced short-time calculation method developed at the Chair of Non-Destructive Testing and Quality Assurance at Saarland University, Germany which takes into account that a material's elastic-plastic reaction and hence relationship is non-linear.

With respect to a test strategy, the number of fatigue experiments required to determine a material's complete S-N-curve can be limited to just a few tests with a special step-shaped specimen. This leads to a significant improvement in efficiency when compared to conventional determination of an S-N-curve where a minimum of 15 fatigue tests is required. Within the work to be presented the SteBLife method is demonstrated for normalized SAE 1045 (C45E) steel.

Experimental setup

Stress-controlled constant amplitude tests (CATs) were carried out on C45E (SAE1045) at ambient temperature with a frequency of f = 5 Hz on a servohydraulic type EHF-L testing system by Shimadzu with a maximum cyclic/quasistatic load F = 21/25 kN using a sinusoidal load-time function at a load ratio of R = -1.

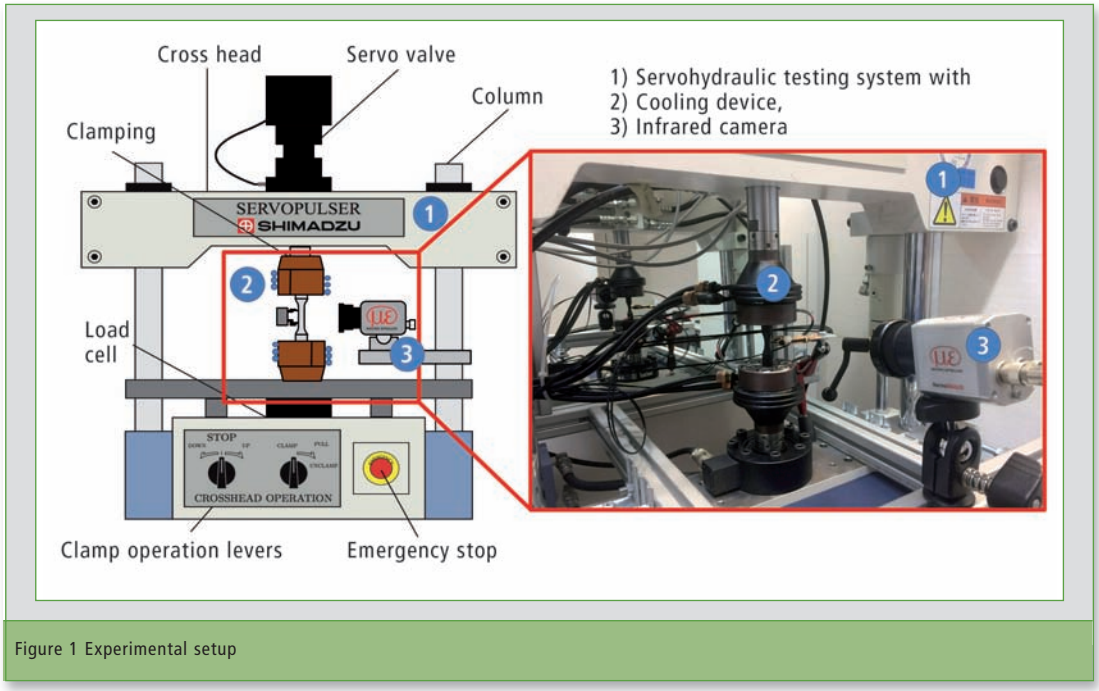

Figure 1 Experimental setup

The fatigue tests were performed to a maximum number of cycles N_{max} of 2 x 10^6 or until specimen failure. To characterize fatigue behavior, the change in temperature ΔT on the surface of the specimen was measured continuously during testing along the gauge length(s) by an infrared camera. This change in temperature is directly related to deformation-induced changes of the microstructure in the bulk material and can be considered to represent the current fatigue state. The experimental setup is shown in figure 1 [1, 2].

To achieve thermal stability of the specimen grips, a cooling device based on a Peltier-element-cooled water circuit was developed for use with the Shimadzu standard grips in order to reduce the temperature difference between the upper and the lower grip during the test to less than 1 K [1].

Results

For temperature measurements, five measurement fields and two reference fields with a size each of 5 x 5 pixels were defined along the specimen within the infrared camera software, one in each gauge length and two at each shaft.

Each gauge length is of a cylindrical cross section and has the same volume. Transition from one gauge length to the next is realized

through a radius of r = 4 mm, which results in a mild notch only and has to be taken into account. For homogeneous materials, just one test is needed to obtain a trend information regarding the S-N-curve.

For this application, SteBLife$_{stc}$ (s: single test, tc: trend curve) was developed. In the case of inhomogeneous materials, scatter bands for different failure probabilities become of high interest as additional information in the S-N-dataset. SteBLife$_{msb}$ (m: multiple tests, sb: scatter bands) provides this information, and the scatter distribution of five fatigue tests is plotted according to a Gaussian distribution [3].

For SteBLife$_{msb}$, a total of five specimens were loaded by a sinusoidal alternating axial force with an amplitude of F_a = 9.170 kN (R = -1) on the Shimadzu servo-hydraulic testing system. Due to the symmetry of the specimen geometry, one cyclic deformation curve for the diameter of 6 (ΔT_1) and two for the diameters of 6.2 (ΔT_2) and 6.4 mm (ΔT_3) each were generated resulting in local stress amplitudes of 360 (ΔT_1), 337 ($\Delta T_{2o/u}$) and 318 MPa ($\Delta T_{3o/u}$) respectively. The five temperature-based cyclic deformation curves for one SteBLife test are shown in figure 2a.

Specimen failure occurred during the test (figure 2a) at N_f = 52,320 cycles, which corresponds with conventionally determined fatigue lifetimes. The cyclic deformation curves provide the database for σ_a-ΔT-curves. As data input for the SteBLife$_{msb}$ calculation, ΔT-values are taken from N_f/2 and plotted as σ_a-ΔT-curves as shown in figure 2b.

In order to calculate the parameters for one single S-N-dataset of the material, a combination of Morrow [4] and Basquin [5] equations is used in the following.

In accordance with SteBLife$_{msb}$, results of the five tests performed were analyzed with respect to lifetime as well as their σ_a-N_f-relation in the LCF/HCF regime. N_f as well as the σ_a-N_f-relation show scatter, which is allocated to material inhomogeneity. Different failure probabilities can be calculated from the Gaussian distribution.

In this example, P_f = 5, 50 and 95 % has been chosen for plotting of the scatter bands, while others can be calculated accordingly. Based on this approach, S-N-curves for P_f = 5, 50 and 95 % are determined. The results are shown in figure 3 together with lifetimes of 11 CATs evaluated on conventional hourglass specimens. Figure 3 underlines that conventionally determined lifetimes can be described reliably by the scatter bands calculated on the basis of SteBLife$_{msb}$.

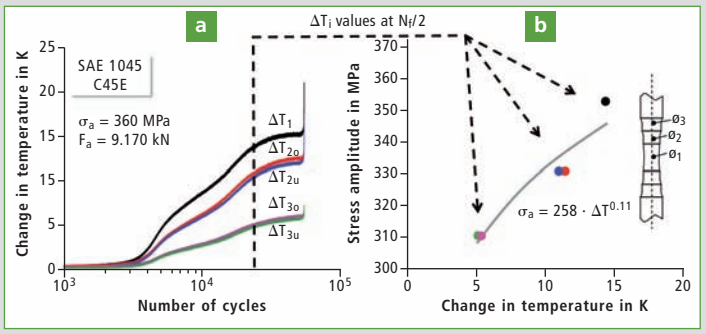

Figure 2a) Temperature based cyclic deformation curves for different diameters of the step-curved specimen, b) stress amplitude-temperature-curve in a constant amplitude test with σ_a = 360 MPa (related to 6 mm) for normalized SAE 1045 (C45E)

Résumé

With the new SteBLife fatigue life calculation method, a stepped specimen geometry with five gauge lengths has been designed

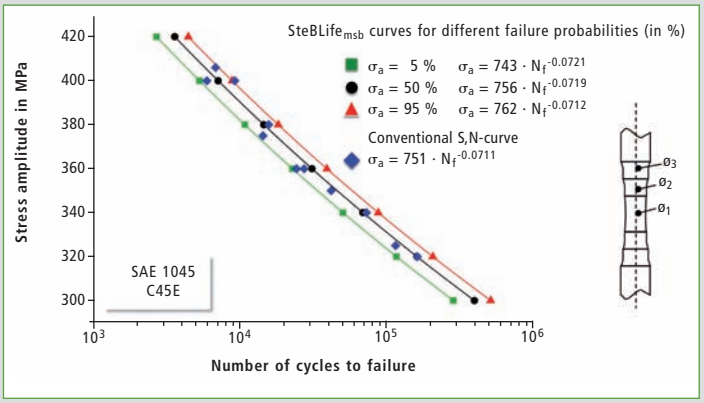

Figure 3: Experimental S-N-data and SteBLife$_{msb}$-curves with calculated scatter bands for different failure probabilities from five SteBLife tests for normalized SAE 1045 (C45E)

that can be used to determine a complete S-N-curve. This has been made possible by using non-destructive testing methods such as thermography, allowing the material response to be monitored in each of the sections. This short-time procedure enables reductions in experimental determination of the S-N-curve of more than 90 %, thereby providing the opportunity to take more fatigue relevant parameters into account and enabling the S-N-curve to be determined on a broader database.

With respect to test strategy, the number of fatigue tests required can be limited to four to five tests only when utilizing SteBLife$_{msb}$. Using SteBLife$_{stc}$ reduces the effort for determination of the S-N-curve without scatter bands to one single CAT only, being much more efficient when compared to the approx. 15 fatigue tests required in the conventional way.

Moreover, SteBLife$_{msb}$ offers the possibility to calculate scatter bands for different failure probabilities, an important advantage particularly with regard to inhomogeneous materials with scattering properties.

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Mineral oil residues in food

Determination of MOSH and MOAH using LC-GC-online technique and comprehensive chromatography

Figure 1: LC-GC-FID-online system with GC-2010 Plus, LC-20ADXR, SPD-20A and CBM-20A LITE

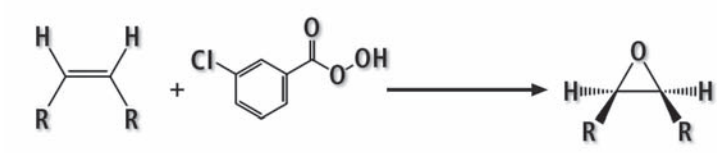

Figure 2: Reaction scheme of epoxidation

Mineral oil residues in food have attracted much public concern in recent years. Whether rice, noodles, olive oil or chocolate, there have been numerous examples of mineral oil contamination of food. At the moment, the challenges to face are the ubiquitous presence of mineral oils from the raw material to the finished product, and the lack of information concerning composition, health effects, analysis and regulatory limits.

To start from the beginning: Mineral oil hydrocarbons (MOH) are divided into the two groups of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH). MOSH consist of branched and unbranched open chain hydrocarbons called paraffines and cyclic hydrocarbons with at least one saturated ring called naphthenes. They can accumulate in the human body and cause granulomas.

Mineral oil aromatic hydrocarbons (MOAH) consist of aromatic substance classes that can be highly alkylated and have 1 - 4 rings. They make up 15 - 30 % of the whole mineral oil fraction and contain potential mutagenic and carcinogenic substances [1]. The levels of mineral oil hydrocarbons found in food (e.g. in table 1) are therefore not tolerable.

Analysis of MOH is a big challenge

So far there are no EU-regulations and no approved analysis method for mineral oil residues (excepting edible oil [3]), but MOSH concentrations of up to 2 mg/kg and MOAH levels below 0.5 mg/kg are considered to be acceptable [1]. Analysis of MOH is a big challenge due to its highly complex composition of unresolved and unidentified substances. As method of choice, online-coupled HPLC-GC-FID is used [4].

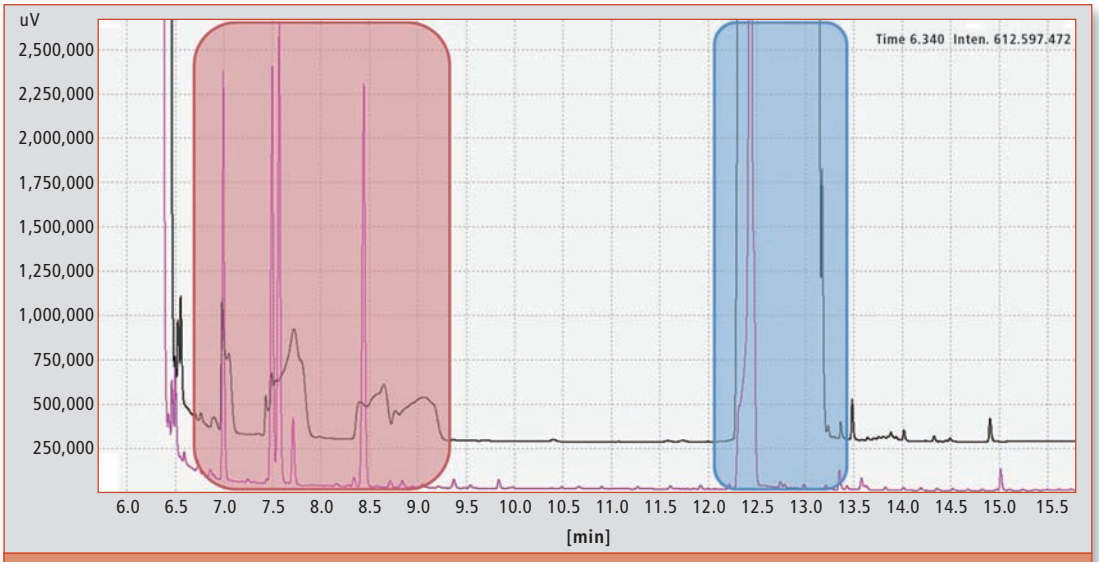

Figure 3: MOAH fraction of extra virgin olive oil sample before (black trace) and after epoxidation (violet trace). Retention range of internal standards marked in red, squalene in blue.

The concept of this method is the pre-separation of MOSH and MOAH fractions using normal phase high pressure liquid chromatography (HPLC), high volume injection (450 µL per fraction) onto a dual column gas chromatograph to improve sensitivity and to further separate the two fractions (e.g. to identify interferences), then flame ionization detection (FID) as non-selective detector, providing virtually the same response for compounds of interest. This results in a fully automatized, closed system without the possibility of sample contamination during pre-separation. To control elution from LC, transfer to GC and for quantification, a set of nine internal standards is added to the sample prior to analysis.

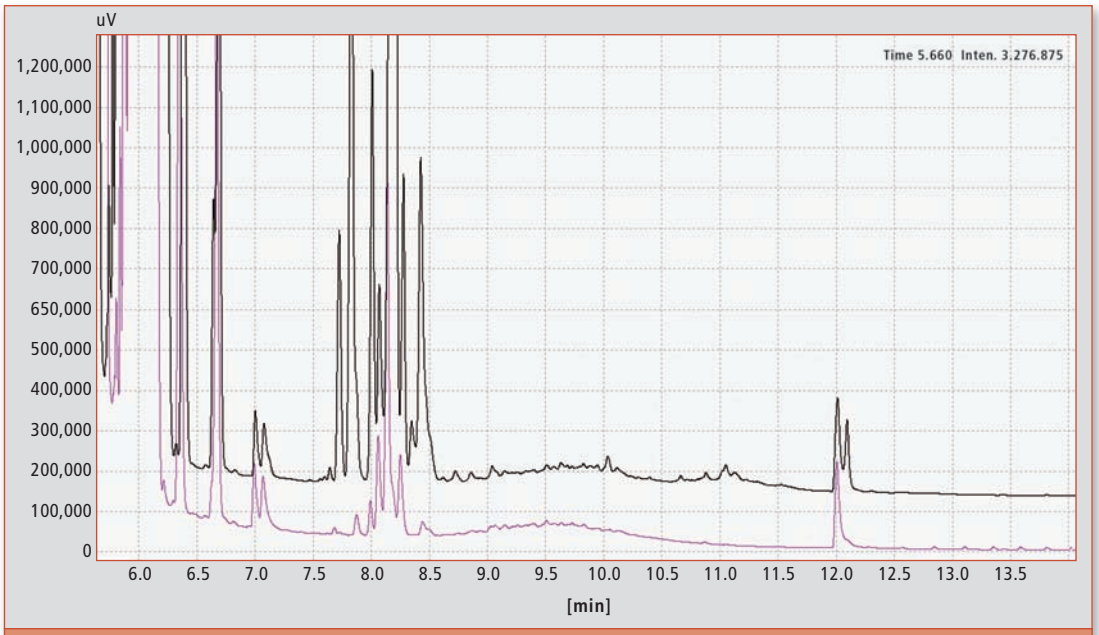

Figure 4: MOAH chromatograms of a cumin extract before (black trace) and after (violet trace) epoxidation

Due to the complex food matrix, sample preparation and analysis can be tricky. Dry foods like rice, noodles or spices are homogenized and extracted in Hexane/Ethanol. The resultant extract is washed with water, dried with sodium sulphate and the volume adjusted to 1 mL. Aliquots of 50 µl extract are injected into the LC, and from there 450 µL are transferred directly onto the pre-columns for the MOSH and MOAH fraction respectively.

Analysis of native oils

Analysis of native oils is more challenging since they can contain many native olefins (eg. squalene, sterenes, carotenoids...) which interfere with the MOAH fraction. These interferences can be removed by epoxidation with 3-chloroperbenzoic acid (mCPBA). The peracid attacks double bonds in the olefins forming epoxides, thereby changing the polarity of the resulting substance in a way that it shows a different retention behavior and is removed from the MOAH transfer window [5].

Fully automatic epoxidation is possible. The oil sample is weighed into a 10 mL glass vial, and internal standard and n-hexane are added. The vial is placed in the autosampler rack of the LC-GC system and a 2 mL autosampler vial with Na_2SO_4 is prepared. The autosampler transfers the 10 mL vial into the agitator which is heated to 40 °C, and adds the epoxidation reagent. The reaction takes place at 40 °C for 15 min in the agitator.

Afterwards, Na_2SO_3 (sodium sulphite) solution and Ethanol are added to stop the reaction and to enhance phase separation prior to centrifugation. After centrifugation, the autosampler transfers the vial back to the tray, and an aliquot of the upper hexane phase into the prepared 2 mL autosampler vial with Na_2SO_4 (sodium sulfate). After 5 min drying time, an aliquot of 50 µL of this phase is injected into the LC, and 450 µL are transferred directly on the pre-columns for the MOSH and MOAH fractions.

Rice	1.8 - 160 mg/kg
Fish (related to fat content)	10 - 1,200 mg/kg
Cocoa and chocolate	5 - 1,300 mg/kg
Baby food (packed in paperboard)	up to 33 mg/kg
Products stored in jute bag (rice, coffe, cocoa)	up to 500 mg/kg
Bakery products	up to 2,800 mg/kg
Edible oil	up to 6,000 mg/kg

Table 1: Levels of mineral oil hydrocarbons detected in food [2]

Figure 3 shows a chromatogram of the MOAH fraction of an extra virgin olive oil sample. The black trace shows the sample before epoxidation. The red marked area indicates the retention range of the internal standards. Due to the high concentration of squalene (marked in blue), the chromatography is so severely influenced that the internal standards cannot be used for quantification. The pink trace shows the same sample after successful epoxidation. Over 90 % of squalene was removed, the internal standards show perfect peak symmetry and no MOAH contamination masked by squalene could be found in the blue marked area.

"Fract & Collect": a powerful tool for separation and identification

Nevertheless, epoxidation of some samples is not satisfactory in terms of elimination of interferences and correct data evaluation. Figure 4 shows an example of a cumin extract before and after epoxidation. It was not possible to remove all interferences and to create an evaluable chromatogram, although harsh epoxidation conditions were used.

In such cases, a more powerful tool for separation and identification is used: The MOSH and MOAH fractions are pre-fractionated on the LC and collected using a method called "Fract & Collect". Afterwards, comprehensive GCxGC-MS is used to further separate the fractions obtained from the LC and to identify and differentiate between interferences from MOSH and MOAH. In the chromatogram shown in figure 5 (page 14), the red marked compounds refer to the internal standards, the yellow marked ones being mono- and diterpenes. The green marked area clearly shows MOAH which was difficult to identify in the LC-GC chromatograms.

For the analysis of chocolate or candy bars, fat and naturally occurring n-alkanes have to be removed. This is done using flash chromatography with silica and aluminium oxide prior analysis [4].

According to a proposed method published by the German Bundesinstitut für Risikobewertung (BfR), quantification is done by integration of the curve for different molecular weight regions [6]. For food contact materials, three ranges are proposed for the MOSH fraction (C_{10}-C_{16}, C_{16}-C_{25} and C_{25}-C_{35}) and two ranges for the MOAH fraction (C_{10}-C_{25}, C_{25}-C_{35}). For dry food, only the ranges up to C_{25} are used.

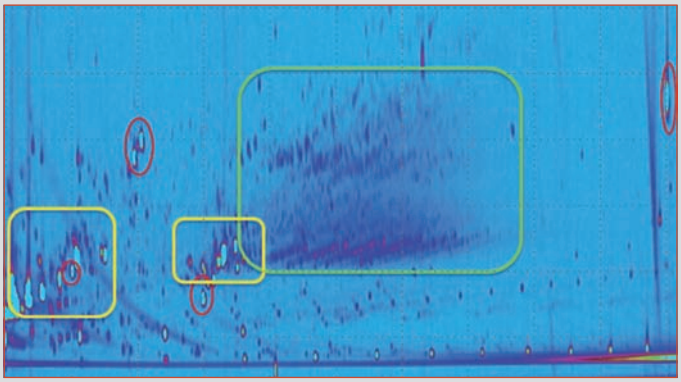

Figure 5: Comprehensive GCxGC-MS chromatogram of the MOAH fraction of an epoxidized cumin extract

Figure 6a (page 15) shows the MOSH (black trace) and MOAH (purple trace) of a spaghetti sample with a MOSH concentration of 12.7 mg/kg from C_{16}-C_{35}. The internal standards are marked with symbols (black squares internal standards MOSH, purple stars internal standards MOAH).

The rice sample in figure 6b also shows additional peaks in the rear part of the chromatogram. These are naturally occurring odd-numbered alkanes with a chain length of C_{21} to C_{35}.

Summary

Analysis of mineral oil residues in food is a challenge. Due to the complex food matrix, auxiliary methods like epoxidation and flash chromatography are often necessary. Nevertheless, online coupling of HPLC with GC-FID provides a valuable tool for quick and easy analysis with automatization as an important key factor.

Read for you in chrom+food FORUM 4-18

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50 years of Shimadzu in Europe

Milestones in development and technology

It all began in **1968** with five employees, and developed over 50 years into a large European network with offices and trade partners in 81 cities from 47 countries, employing more than 700 people in Europe: Shimadzu now celebrates the 50th anniversary of its presence in Europe.

The first 25 years were dedicated to developing the business and expanding the network. In the beginning, West European states were in the business focus, but first approaches were also made to East German, Russian and Yugoslavian markets. The business grew steadily, and an important milestone occurred in **1987** with the move from Düsseldorf to Duisburg, Germany to new premises hosting the European Headquarters, at the time with 38 employees.

Own R&D as well as production facilities built the foundation for evolution in the 25 years, enabling the company to meet market needs faster and more flexibly. 6,300 sqm of offices and production space provided options for future expansion. In **1992**, another 6,000 sqm were added to expand the analytical and medical operations. Furthermore, the ShimCAT Center for Application and Training has been opened, utilizing application tasks on-site.

Development of Shimadzu as an organization

Since the 1990s, when Shimadzu had already been present in Europe for 25 years, political and societal changes on the continent opened up new market opportunities in Eastern Europe.

Shimadzu started to expand its base in Europe with additional technical offices in Germany. In the following years, many branch offices and subsidiaries have been established. In **1990**, Shimadzu founded its first subsidiary in Austria, covering the additional sales regions of CSFR, Hungary, Romania and Bulgaria. One year later, Shimadzu Italia commenced business in Milan, Italy's industrial and financial heart. In **1992**, Shimadzu Benelux was founded. From the Dutch city of s'Hertogenbosch, Shimadzu can easily serve the economic centers of Amsterdam, Rotterdam and Brussels. Strategically located in Milton Keynes, Shimadzu UK was established in **1996**, half way between the economic centers of London and Birmingham. Zagreb, the capital of Croatia and an international trade and business center, is Shimadzu's hub along the Adriatic east coast, starting in **1997** and adding Albania, Bosnia-Herzegovina, Bosnia, Macedonia, Montenegro and Serbia in **2001**. At the end of the decade, Shimadzu Switzerland was launched in the outskirts of Basel where the Swiss, French and German borders meet, one of the most dynamic economic regions of Switzerland.

In **2002**, Shimadzu France, located east of Paris, has been established. From there the whole French market is served up to Réunion in the Indian Sea.

To integrate Shimadzu Germany into the new European organizational structure, it was established in **2007** as an own entity, although Shimadzu since its market entry in 1968 has of course been very active in Germany as the largest market-place in Europe. Due to a fast growing workforce of over 110 employees, the German subsidiary in **2017** has moved into an own office space close to the European headquarters.

Simultaneously with the expansion of subsidiaries, Shimadzu has increased its network of distributors in almost all other European countries, so that Shimadzu meanwhile operates all over the continent.

The concept of running subsidiaries, i.e. own entities, in selected European markets is part of Shimadzu's "Strategy of the Future", a European business restructuring process establishing independent companies in major markets and countries in order to better support new trends and markets.

The latest highlight in development of the European homebase was in **2017**, when Shimadzu acquired ALSACHIM, a France-based specialist company for stable isotope-labelled compounds, metabolites and pharmaceutical related substances, and analytical purposes. ALSACHIM is promoted under its own brand, but is a "Shimadzu Group Company". Together with ALSACHIM, Shimadzu is able to provide the clinical market with complete solutions consisting of hardware and software as well as application kits.

New solutions for tomorrow

Shimadzu's Laboratory World was launched in **2013** with over 1,500 sqm of floor space, and the European

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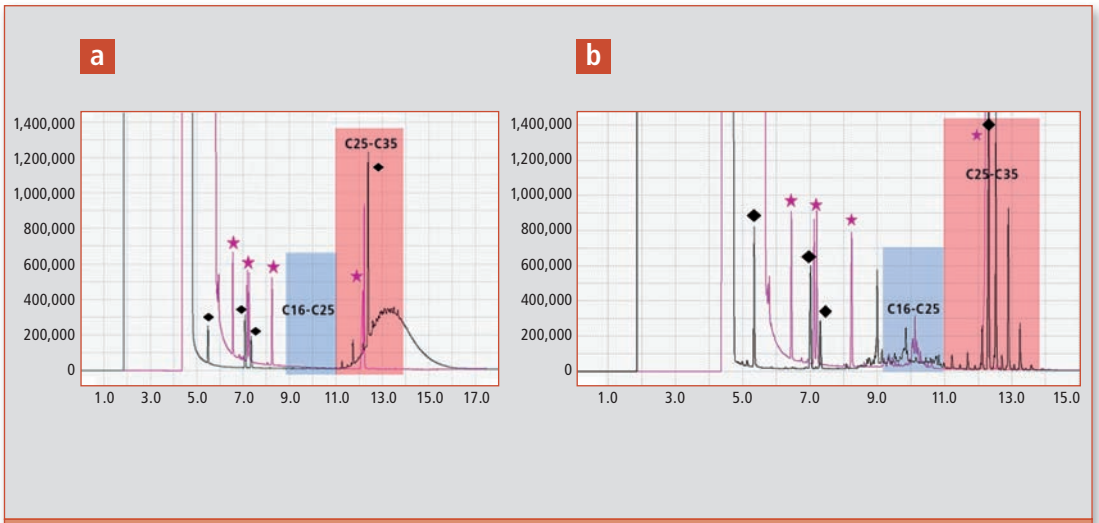

Figure 6: a) Spaghetti sample with MOSH concentration of C16-C35 and b) rice sample with naturally occurring odd-numbered alkanes

Innovation center (EUIC) in 2017. The cutting-edge Laboratory World focuses on the growing needs of industries and science, while its demonstration and testing facilities help customers to experience daily operation as realistically as possible. Shimadzu's entire product range is available for testing and for professional development of applications, and provides a new home for the ShimCAT Center for Application and Training.

The EUIC with its unique approach combines academic-scientific know-how of universities with Shimadzu's technological expertise to provide even more customer-focused service on the next level and to create new solutions for tomorrow. It merges the cutting-edge analytical technologies of Shimadzu with game-changing topics and expertise in markets and science covered by opinion leaders, strategic thinkers and scientific experts in order to create new solutions for tomorrow.

Technological progress and continuous digitalization

In the last two and a half decades, Shimadzu Corporation has celebrated the 60th anniversary of GC technology and IR spectroscopy, the 40th anniversary of HPLC and the 140th anniversary of the company. Shimadzu introduced many industry firsts in analytical instrumentation as well as in medical technology, many of them driving miniaturization of instruments, and also automatization, e.g. from pretreatment of the sample to analysis. These firsts are pushing efficiency and productivity in clients' labs and hospitals. In particular, coupling technologies focus on sensitivity and accuracy of analysis and measurements.

The "Excellence in Science" slogan embraces all these activities and progress, replacing the earlier "Solutions for Science" approach. "Excellence in Science" claims outstanding quality in technology and services and in every single aspect of working with clients. It summarizes Shimadzu's approach towards ensuring better consumer, patient and environment protection as well as product safety.

Analysis of VOC and SVOC emissions in vehicle interiors

Materials analyzed according to VDA 278 with thermodesorption

GCMS-System with TD-30R Thermal Desorption System

In recent years, measures to reduce the use of organic components in the automotive industry have made remarkable progress. In Germany, specialized VDA 278 regulations for volatile organic compounds (VOC) and semi-volatile organic compounds (SVOC) have been defined for non-metallic automotive materials. Automobile manufacturers use the VDA 278 regulations for molded parts in the passenger compartment; they allow estimation of the effects of emissions while improving products.

New cars should smell as neutral as possible, but large-area odor carriers such as carpets, headliners or seat covers influence customers' perception. Odors in the car interior should not be annoying or even harmful, but should ideally contribute to the purchase decision.

For analysis, samples are extracted thermally using thermodesorption analysis (TD), separated in GC and detected in a mass spectrometer. The samples are transferred to the TD tube and then introduced into the GCMS through defined desorption programs. This allows for a convenient and rapid analysis of VOC and SVOC. Since some of the desorbed organic components are semi-volatile compounds, the analysis system used needs to adhere to important quality requirements to prevent the possible "carryover" of sample components between measurements.

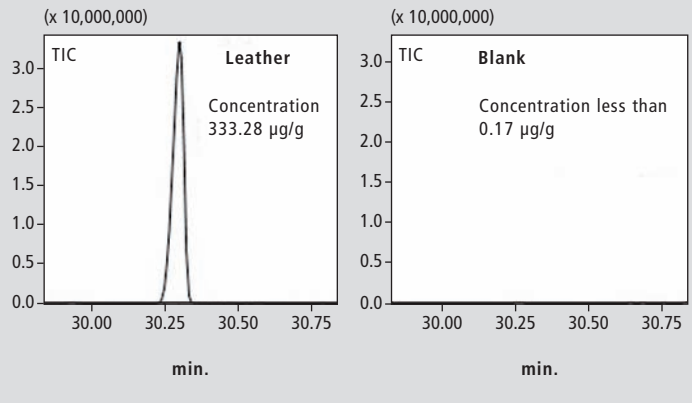

Figure 1: Chromatogram of a leather sample

TD-30: All requirements of VDA 278 are met and exceeded

Shimadzu has introduced a new solution for gas and material analysis. Its flexible extensibility supports a wide range of quantitative analyses, for example in research or quality control. The new TD-30 desorption system offers several features, e.g. particularly inert lines, shortest possible connections between GCMS and TD, and fast heating and cooling of the TD tubes. A built-in overlap function significantly increases sample throughput. The TD-30 thus fulfills and even exceeds all requirements of the VDA 278 regulation.

According to VDA 278, the samples (rubber, plastic, leather) are cut into thin strips and approximately 30 mg of each sample is placed in the TD tube. The tube is provided with about 5 mg of glass wool on either side and sealed with lids. VOC and SVOC components are desorbed at different temperatures for 30 min each, and fractions collected are then introduced into the GCMS. Analysis conditions are summarized in table 1.

Results: Evaluation of calibration and recovery rates

Standards for the calibration curves are prepared by diluting toluene and n-hexadecane with methanol to 0.5 µg/µL. 4 µL of the solution are then added to the Tenax® TA tube and measured with GCMS to determine the response factor (Rf). The Rf s are later used to quantify the components in the samples. The formula used to calculate Rf is as follows:

$$R_{f(Toluene/C16)} = \frac{m_{Toluene/C16}\ [\mu g] \times 1{,}000{,}000}{Peak\ area}$$

Emission of the components examined is calculated as:

$$\text{Emission } [\mu g/g] = \frac{R_{f(\text{Toluene/C16})} \times \text{Peak area (Component)}}{1{,}000 \times \text{Sample weight [mg]}}$$

In addition, recovery rates of the analysis system are calculated by preparing a mixture of typical VOC components (concentration of about 0.11 µg/µL) and adding 4 µL of the solution to the Tenax TA tube. Calculated recovery rates are summarized in table 2.

Analysis results for non-metallic automotive materials

Table 3 shows the results of quantitative analysis (in µg/g) for VOC components in rubber, plastic and leather. Among the calculated values from the leather sample is a very high concentration of bis(2-ethylhexyl)phthalate of 333.28 µg/g (table 3, page 18 and figure 1), which is used as a softener in plastics.

Directly after this sample measurement, an empty TD tube was tested to determine the possible carry-over of analytes into the subsequent measurement. After this measurement, a value of 0.17 µg/g bis(2-ethylhexyl)phthalate was calculated, representing only 0.05 % of the concentration of the previous measurement (figure 1). The high quality of the system and its suitability for complex samples of the VDA 278 standard was therefore proven.

Configuration of used instruments	
GCMS	GCMS-QP2020
Thermodesorber	TD-30R
Software GCMS	GCMSsolution™ Ver.4.45
Software TD-30	TD-30 Control Software
GC column	SH-Rxi™-5Sil MS (60 m x 0.25 mm I.D., df = 0.25 µm) (SHIMADZU)
TD-30 settings	
Desorption temperature of TD tube	90 °C for 30 min (VOC) / 120 °C for 30 min (SVOC)
Desorption flow rate of TD tube	60 mL/min
Trap cooling	-20 °C
Desorption temperature of trap	280 °C for 10 min
Joint temperature	280 °C
Valve temperature	250 °C
Transfer line temperature	280 °C
GC settings	
Control mode	Pressure
Pressure	200 kPa
Injection mode	Split 1:100 (Column flow rate 1.99 mL/min)
Temperature oven	40 °C (3 min) – (10 °C/min) – 300 °C (13.5 min)
MS settings	
Ion source temperature	200 °C
Interface temperature	250 °C
Scan mass range	m/z 35-400
Scan event time	0.5 sec
Scan Speed	769 u/sec

Table 1: Configuration of used instruments

Component	Recovery rate [%]
Benzene	106.5
Toluene	93.5
p-Xylene	99.9
o-Xylene	75.4
2-Ethyl-1-Hexanol	101.3
2.6-Dimethylphenol	94.2
Dicyclohexylamine	89.2

Table 2: Calculated recovery rates

Conclusion

The TD-30 is a revolution in the market of thermal desorption systems. The TD-30R high-end model in particular sets new ➧

standards in this area. This system offers the highest sample capacity (120) on the market. Additionally, horizontal alignment of the TD tubes ensures analysis without carry-over.

An overlap function increases sample throughput. The unique design of the transfer connection – without cold spots and with the shortest path between TD and GCMS – enables highly sensitive analyses over a very wide temperature range.

Further information on this article:

- www.ssi.shimadzu.com/sites/ssi.shimadzu.com/files/Products/literature/GCMS/C146-E349.pdf

Component	In VOC fraction [µg/g]			In SVOC fraction [µg/g]		
	Rubber	Plastic	Leather	Rubber	Plastic	Leather
C8	0.00	0.00	0.00	0.00	0.00	0.11
Toluene	0.35	0.54	0.53	0.44	0.44	0.24
C9	0.00	0.00	0.00	0.00	0.00	0.13
C11	0.00	0.00	0.00	0.00	0.00	0.31
1.3-Dichlorobenzene	0.00	0.00	0.00	0.00	0.00	0.08
2-Propyl-1-Pentanol	0.36	0.52	0.73	0.18	0.18	0.78
C12	0.00	0.00	0.17	0.03	0.03	0.06
N/A	0.00	0.00	0.43	0.06	0.06	0.87
C13	0.20	0.14	0.26	0.13	0.13	0.13
C15	0.14	0.12	0.36	0.16	0.16	0.14
C16	0.31	0.00	0.60	0.16	0.16	0.86
C18	0.14	0.00	0.73	0.00	0.00	2.02
C19	0.00	0.00	0.30	0.00	0.00	1.37
Dibutylphthalate	0.00	0.00	2.92	0.00	0.00	17.53
C20	0.00	0.00	0.18	0.00	0.00	1.28
C22	0.00	1.09	0.17	0.00	0.00	0.82
C23	0.00	0.00	0.15	0.00	0.00	0.82
C25	0.00	0.00	0.00	0.00	0.00	1.78
Bis(2-ethylhexyl)phthalate	0.41	1.6	33.67	0.00	0.00	333.28

Table 3: Results of the quantitative analysis

Nexis GC-2030, a high-end GC system

Oil in water?

Fast and efficient analysis of mineral oil in environmental samples according to H53

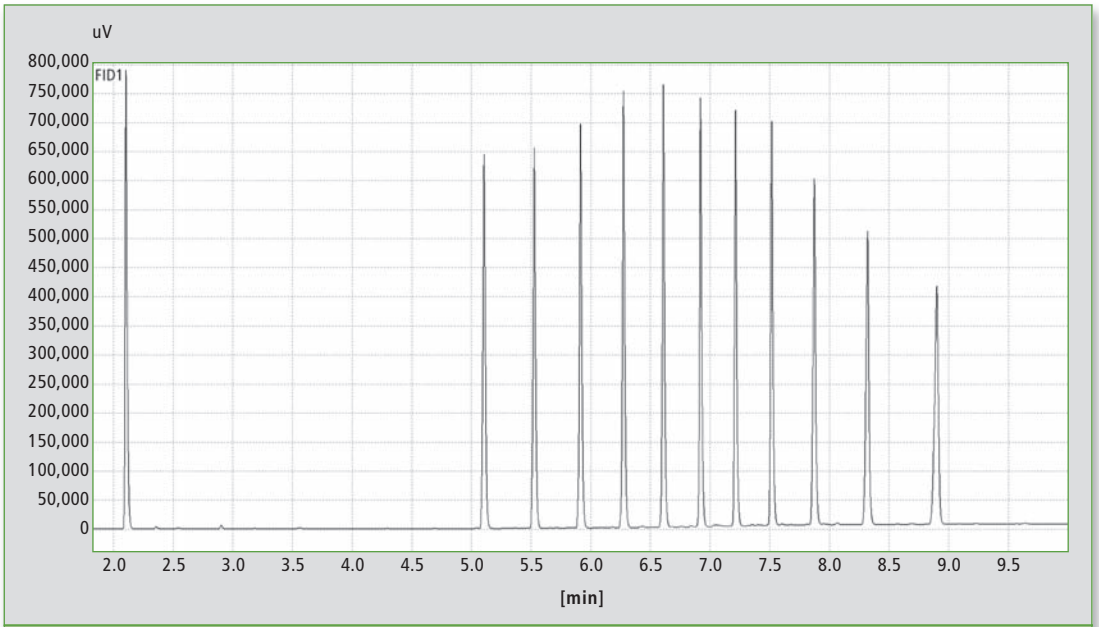

Figure 1: Chromatogram of an alkane standard (C10, C20 to C40, even number of C atoms) in heptane; C40/C20 > 0.98

Every day worldwide, huge amounts of crude oil are processed into various mineral oil products such as petrol, kerosene, diesel, heating oil and lubricating oil. Mineral oil products are generally composed of mostly saturated hydrocarbons, so-called mineral oil hydrocarbons (MOH). During the production as well as the commercial and private use of mineral oil products, water and soil are repeatedly contaminated.

Since mineral oil hydrocarbons are difficult to biodegrade, it is very important to control contamination of environmental samples

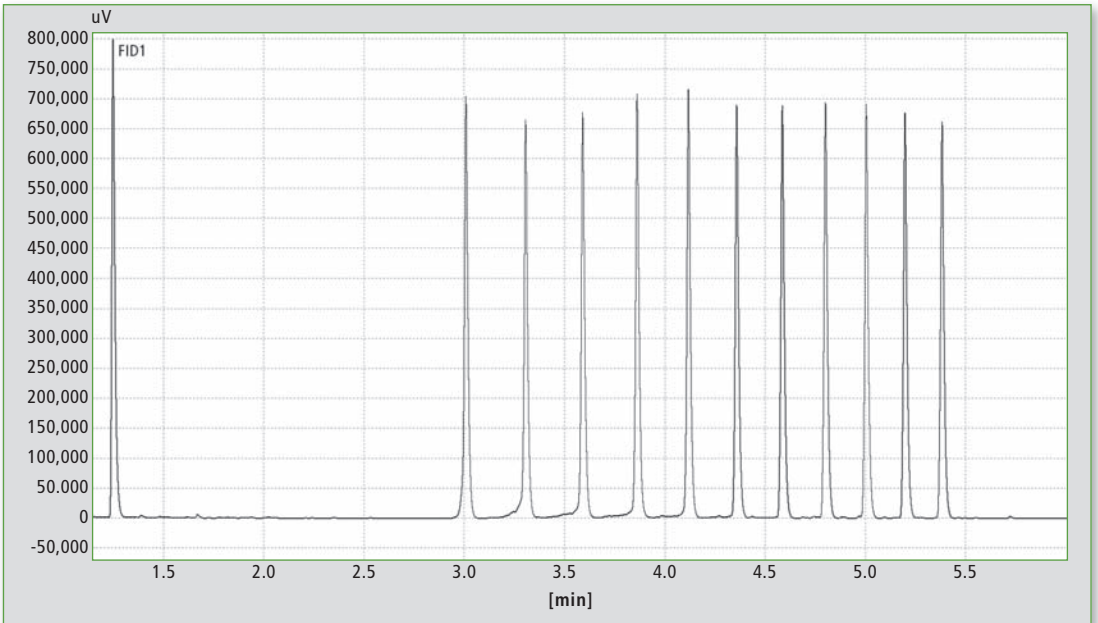

Figure 2: Chromatogram of an alkane standard (C10, C20 to C40, even number of C atoms) in heptane; fast-GC with hydrogen as carrier gas; C40/20 > 0.90

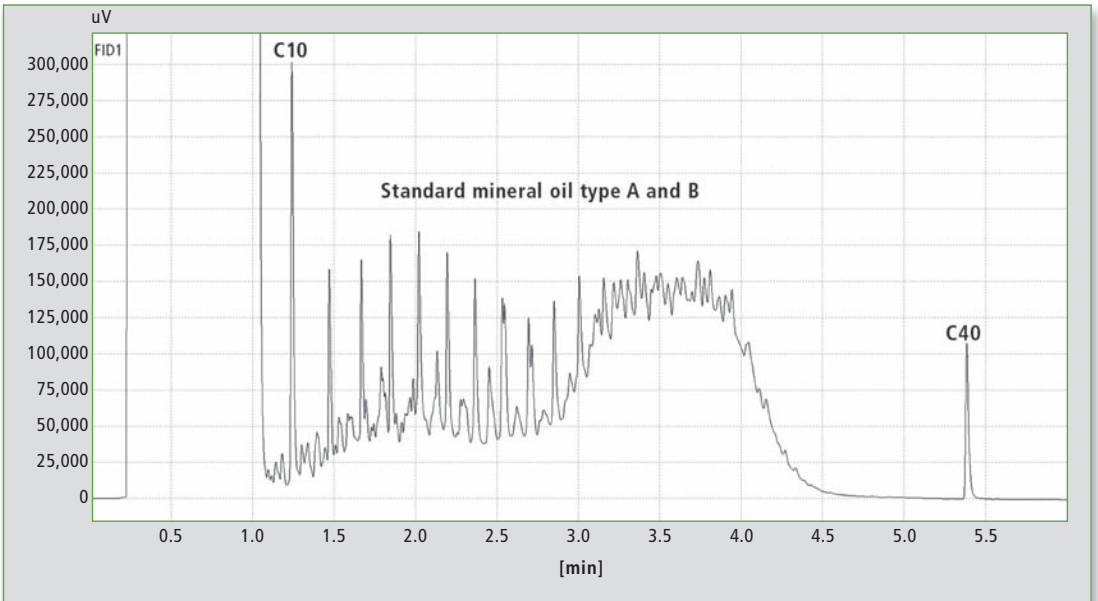

Figure 3: Chromatogram of the calibration standard 1 mg/mL, mineral oil type A and B in extraction solution with retention time markers C10 and C40

by MOHs. The analysis of mineral oil hydrocarbons in drinking water, surface water and waste water is defined by the European standard EN ISO 9377-2, colloquially referred to as H53 [1].

A water sample is extracted and then purified with Florisil to remove polar substances. The purified extract is analyzed by gas chromatography with flame ionization detection (GC-FID). It is not possible to assign individual substances due to the complexity of the hydrocarbon mixtures. Quantification is therefore performed by integration of the total peak area between the marker substances n-decane (C10) and n-tetracontane (C40) [1].

In this way, the boiling point range lies between 175 and 525 °C [2]. For determination of mineral oil concentration, a mineral oil mixture (diesel lubricating oil mixture, mineral oil type A and type B) is used as an external standard [1]. Soil and sludge samples can be determined analogous to H53, as defined by the European standard ISO 16703:2011.

Column-friendly and easy to maintain: the split/splitless injector

Since determination of mineral oil hydrocarbons covers a wide boiling point range, analysis is carried out conventionally using an on-column injector (OCI) to ensure non-discriminatory sample introduction. ISO 9377-2 prescribes a ratio of the alkanes n-tetracontane (C40) to n-eicosane (C20) of at least 0.8 [1]. Disadvantages of on-column injection are the high matrix load of the column and the use of a retention gap.

A more column-and maintenance-friendly alternative is to use a split/splitless injector (SPL), provided it meets the above requirement.

Repeated measurements of an alkane standard using Nexis GC-2030, a high-end GC system, achieved a ratio C40/C20 > 0.98 (figure 1). The split/splitless injector of GC-2030 is therefore sufficiently discrimination free. A conventional column (Rtx-5, 15 m, 0.25 mm ID, 0.25 µm film thickness, Restek) and a method optimized for baseline separation of alkanes and chromatogram runtime were used (table 1, page 20). Retention time of C40 was 8.9 min, meaning analysis up to C40 was completed in less than 10 min.

Shortening analysis time: hydrogen as carrier gas

Using a greatly increased linear velocity, the analysis could be further accelerated to achieve chromatograms with runtimes of less than 6.5 min. Further acceleration with simultaneous use of less extreme linear velocities was achieved by using hydrogen as carrier gas.

Subsequent reoptimization of the column dimensions led to a further reduction of analysis time: The change to MXT-1 with dimensions of 15 m, 0.25 mm ID, 0.1 µm film thickness (Restek) and C40 retention time of 5.4 min finally enabled a chromatogram runtime of less than 6 min (figure 2, table 2, page 20).

Gas chromatograph	GC-2030
Injector	Split/Splitless
Injector temperature	280 °C
Injection volume	1 µl
Split ratio	Splitless
Column	Rtx-5 15 m, 0.25 mm ID, 0.25 µm df (Restek)
Carrier gas	He
Carrier gas velocity	60 cm/sec
Oven program	60 °C, 1 min, 45 °C/min, 340 °C, 3 min
Detector	FID
Detector temperature	340 °C

Table 1: Method parameters for conventional GC on Rtx-5 column

Gas chromatograph	GC-2030
Injector	Split/Splitless
Injector temperature	320 °C
Injection volume	1 µl
Split ratio	Splitless
Column	MXT-1 15 m, 0.32 mm ID, 0.1 µm df (Restek)
Carrier gas	H2
Carrier gas velocity	80 cm/sec
Oven program	50 °C, 0.7 min, 95 °C/min, 115 °C, 65 °C/min, 200 °C, 55 °C/min, 370 °C, 0.22 min
Detector	FID
Detector temperature	370 °C

Table 2: Method parameter for fast GC on MXT-1 column

This leads to injection cycles of 10 min including cooling and equilibration time. Sample introduction was still free of discrimination with a ratio of C40/C20 > 0.90. Furthermore, due to the increased temperature stability of the MXT-1, maximum temperature of the furnace program could be raised. This shortens the chromatogram runtime for real samples that include compounds greater than C40.

Based on these optimization results, real samples were measured as examples. To produce a calibration series, a mineral oil standard type A and type B as well as an extraction solution already mixed with C10 and C40 were used, both are commercially available (Sigma Aldrich, figure 3, page 19). This reduced the wet-chemical work involved but also meant that no background subtraction of pure solvents was possible without retention time markers. Instead, the unspiked extraction solution was included in the calibration series as concentration level 0.

Since the concentration of the real samples was not known, two calibrations were performed: a 6-point calibration in a range of 0 to 1.5 mg/ml and a 5-point calibration in a larger range of 0 to 10 mg/ml (figure 4). For both calibrations regression coefficients greater than 0.9998 were obtained. The real samples tested showed different mineral oil impurities (figure 5). Most of them were in the smaller calibration range, but one sample was slightly outside in the larger calibration range.

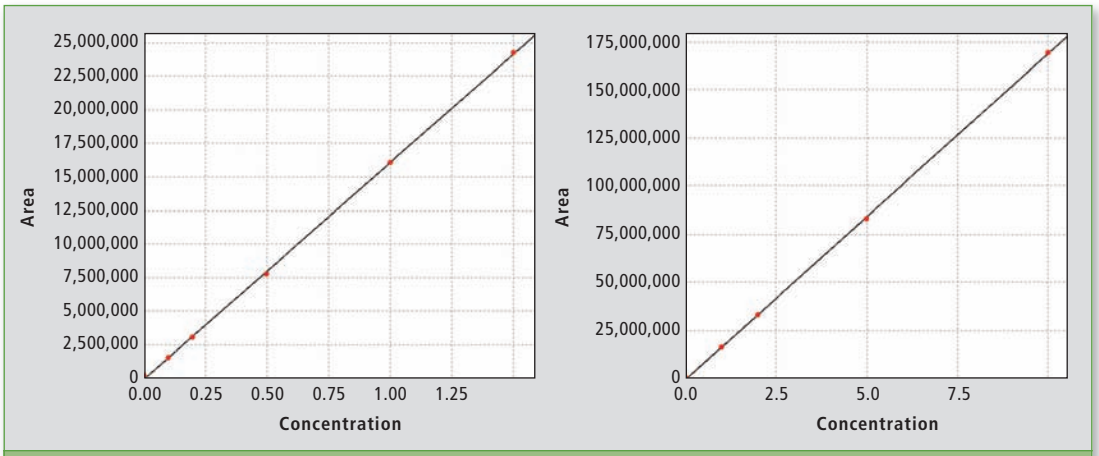

Figure 4: Calibration curves in the range 0 to 1.5 mg/mL (left) and 0 to 10 mg/mL (right)

Résumé

A split/splitless injector simplifies and accelerates H53 measurements as long as it meets the requirements of the ISO 9377-2 standard. The area ratio C40/C20 must be above 0.8, and the Nexis GC-2030 meets this with a ratio greater than 0.9. With the use of hydrogen as carrier gas and by selecting a suitable column, chromatogram runtimes can be reduced significantly (down to six minutes) while maintaining reliability of the results.

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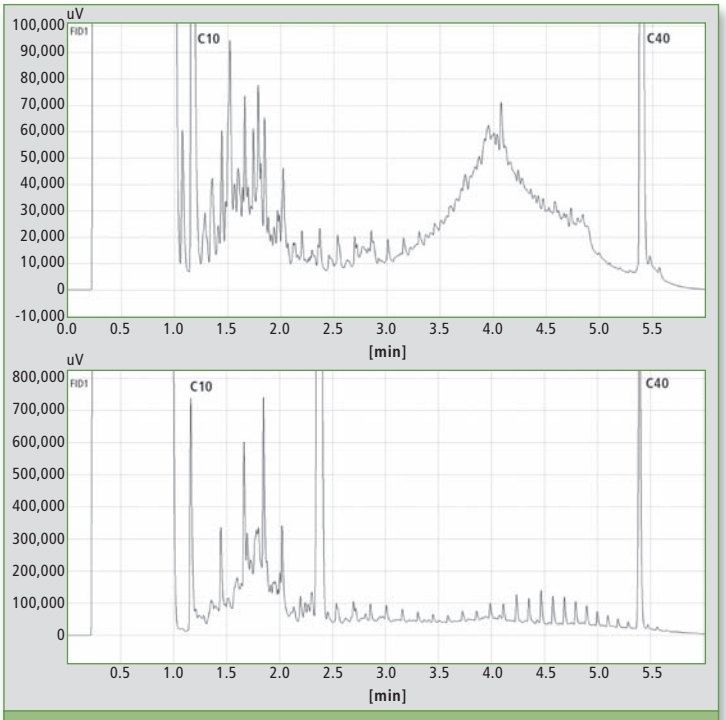

Figure 5: Chromatograms of two real samples

Spoilt for choice

Not all C18 columns show the same separation behavior

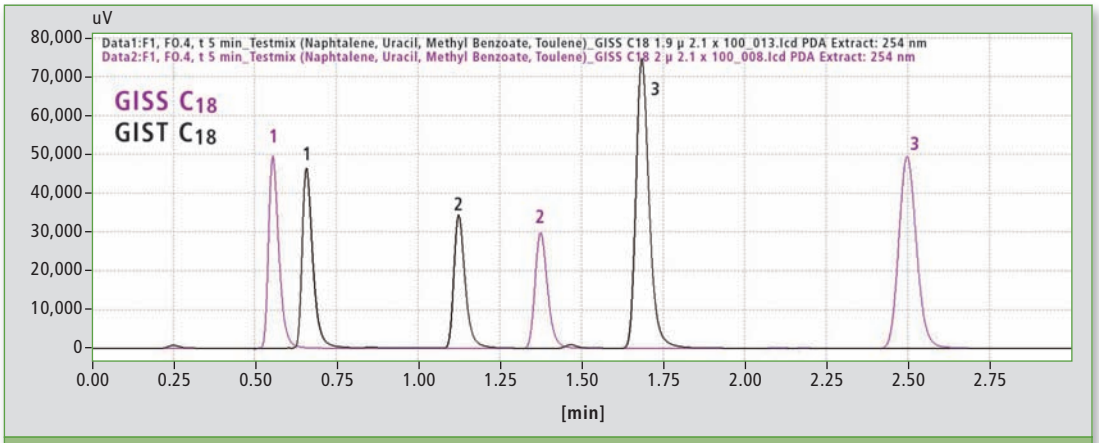

Figure 1: Comparison of two different stationary phases packed with C_{18} modified silica gel (GISS and GIST). Dimensions: 2.0 x 100 mm, 2 µm particle size; mobile phase: 35 % water, 65 % acetonitriles. Samples: 1) uracil, 2) methyl benzoate, 3) naphthalene.

A wide range of different liquid chromatography columns available, with many stationary phases to choose from. Even if the stationary phase used is clearly defined, there can be many differences. This article refers only to silica gel based stationary phases with C_{18} modification, also called octadecyl silanes or ODS columns, which are the general standard for reversed phase chromatography. To illustrate the problem with an example, figure 1 shows two chromatograms of different Shim-pack C_{18} columns.

Sample, method used, and column dimensions are identical. So, from a purely chemical point of view the separation principle is the same, but the retention times are nevertheless very different. Peak 3 (naphthalene) shows a retention time of 1.68 minutes on GISS C_{18} while with GIST C_{18}, it elutes after 2.50 minutes.

The difficulty of selecting a suitable C_{18} column is great, because various columns can behave differently. This depends on the type of silica gel and its production as well as on the modification and packing technique of the column.

Support for comparing and characterizing of C_{18} columns

Due to the huge selection of columns, it is important to compare the chemical and physical properties and differences of the various stationary phases in order to characterize the selectivity. Different approaches exist, as published by Engelhardt, McCalley or Tanaka [1, 2, 3]. The latter will be discussed below.

The Tanaka test provides characteristic values that can be used to assess the separation behaviour of a column more precisely. They are calculated under standardized conditions from the retention times of suitable analytes. It is therefore possible to compare stationary phases accordingly and to make an informed choice more easily. Since the methods and all other parameters are prescribed for the Tanaka test, comparable values can be obtained – even if the measurements are carried out in different laboratories. The Tanaka test is therefore a useful tool for comparing different columns.

Parameters of the Tanaka test

The test describes various column characteristics including capacity, hydrophobicity, steric selectivity and silanol interactions. These parameters (table 1, page 22) are determined using the retention times of suitable standard substances measured under specified analytical conditions.

Results from many columns based on the Tanaka test are helpful in finding out which columns are shortlisted. The freely accessible "Column Selector" from ACD/Labs contains Tanaka values of many columns for comparison [4]. However, other parameters and specifications that are important for selection of a suitable separation column for a particular application are also described and explained below.

Important specifications and parameters for column selection

To select a suitable stationary phase for a separation problem, the column parameters and specifications given in table 2 (page 22) are very helpful. Unlike the Tanaka test, these values come from the column manufacturer and are directly accessible.

Column efficiency is also important. This is measured in theoretical plates (N). The higher the number of plates, the narrower ➧

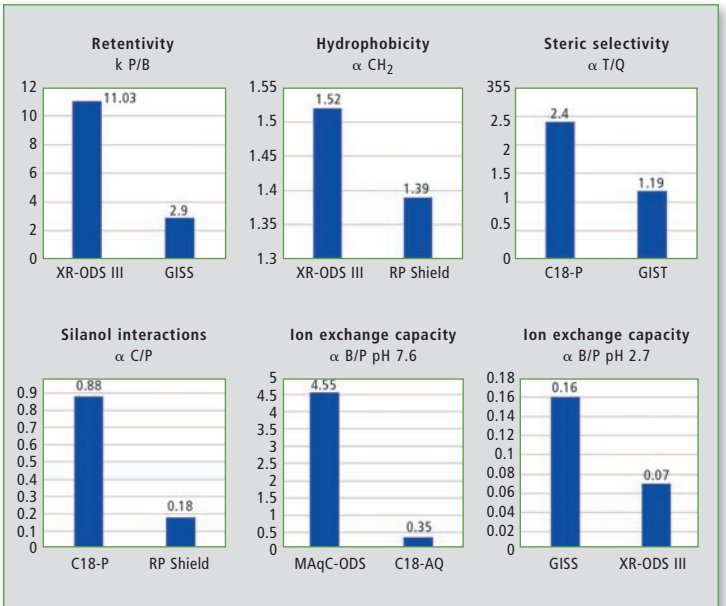

Figure 2: Display of the lowest and highest Tanaka parameters from the complete Shim-pack C_{18} range

the peak and/or the more it retains on the column.

$$N = \frac{L}{H} = 5.55 \cdot \left(\frac{t_R}{W_h}\right)^2$$

L = length of column
H = plate height
t_R = retention time
W_h = peak width at half height

A narrow peak and thus a higher plate number, is achieved by a small particle size, a small particle size distribution and a good packing procedure. A high N value therefore reflects a good packing quality of the column.

A second factor is the quality of the silica gel. Nowadays this point is negligible as all modern columns contain high-quality silica gel. In the past, high particle size distribution, poorly packed column beds and a high proportion of acidic silanol groups resulted in a broad peak shape, not only for basic analytes.

In addition to these general specifications, other modifications also influence the retention behavior of various C_{18} columns. These are described below with the example of the columns used for this article.

Columns used

The following Shim-pack C_{18} columns were used for this study: XR-ODS III and MAqC-ODS, and from Shim-pack G series GIST C_{18}, GIST C_{18}-AQ, GISS C_{18}, GIS RP-Shield and GIS C_{18}-P. The Shim-pack G series offers a complete portfolio with many different dimensions. This benefits the development of separation methods for phases with very small dimensions (UHPLC) and can be scaled up to very large columns for preparative applications.

XR-ODS III belongs to the XR Series; it is packed exclusively with small particle sizes. This makes it well suited for high-pressure applications with high resolution and sensitivity.

MAqC-ODS is a C_{18} column containing additional metal. Due to the metal content, the column gives cation exchange effects, retarding basic substances more strongly. The column is very well suited to analysis of water-soluble vitamins and basic components.

Shim-pack LC columns from Shimadzu

The GIST C_{18} column shows superior inertness. Even ionic components can be measured with symmetrical peaks and high reproducibility.

Compared to conventional C_{18} columns, the GIST C_{18}-AQ column shows very strong retention to hydrophilic polar analytes. It can also be used in 100 % aqueous mobile phase without loss of retention.

The GISS C_{18} column has the same inertness and stability range as the GIST C_{18}. It is used for fast separations and is ideal for LC-MS applications. With 20 nm, the GISS has the largest pore size in the Shim-pack range and is therefore the ideal choice for larger analytes with molecular weights up to 20,000 Da.

The GIS RP-Shield column has a polar functional group embedded between the silica surface and the C_{18} groups. This makes it stable even under 100 % aqueous conditions. The polar functional group is base deactivated, so acidic analytes in particular show good peak form. This results in a weak interaction to basic analytes and a strong interaction to acidic analytes. The additional interaction of hydrogen bonds gives the column a different selectivity compared to other C_{18} columns.

In the GIS C_{18}-P column, the C_{18} modification is polymerically bonded instead of monomeric as in most other ODS phases. This way, it offers high steric selectivity for separation of planar and non-planar analytes. Ideal applications are, for example, the separation of PAHs or analysis of structure-like components (vitamins D2 and D3).

Measurement Parameters and Methods

Instrument: LC-2040C 3D (Shimadzu)
Column: Shim-pack XR-ODS III (75 mm x 2.0 mm I.D., 1.6 µm)
All other Shim-pack C_{18} (150 mm x 4.6 mm I.D., 5 µm) Shimadzu
Mobile phase:
A: 20 % water, 80 % methanol

Tanaka Parameter	Column Characteristic	Explanation
K PB K Pentylbenzene	Retentivity	Amount of alkyl chains / carbon loading / surface area
α CH_2	Hydrophobicity	Surface coverage. The smaller the value the less hydrophobicity
α T/O k Triphenylene, o-terphenyle	Steric selectivity	
α C/P k Caffeine, Phenol	Silanol interactions (Hydrogen bondings)	Endcapping; if endcapping is not sufficient / lots of free silanols or polar groups are present, the value is high
k Benzylamine, Phenol pH 7.6	Ion exchange capacity pH 7.6 (Silanol pH 7.6). Number of free silanols on the column	A high value mirrors a high Ion exchange capacity at pH 7.6
k Benzylamine, Phenol pH 2.3	Ion exchange capacity pH 2.3 (Silanol pH 2.3). Number of free silanols on the column	A high value mirrors a high Ion exchange capacity at pH 7.6

Table 1: Listing and explanation of the individual Tanaka parameters

B: 30 % water, 70 % methanol
C: 60 % water, 30 % methanol, 10 % 200 mM KH_2PO_4; pH 2.7
D: 60 % water, 30 % methanol, 10 % 200 mM KH_2PO_4; pH 7.6
Oven temperature: 40 °C

Flow rate and injection volume are adapted to the column dimensions.

Results

The Tanaka test results show interesting differences between the various C_{18} columns in the Shim-pack range. The most divergent values are compared in figure 2 (see also table 1).

The k P/B value shows retention capacity of the column for nonpolar substances. Columns providing low retention times for amylbenzene have small values. For quick analyses, the GISS column can be used; if a column with high retention is required, the XR-ODS III is a good choice.

The CH_2 parameter highlights hydrophobicity and selectivity for substances of a homologous series which, like the test substances, differ only by the number of C chain links. Here, too, the hydrophobic XR-ODS III phase shows a high value. The RP-Shield, however, with the embedded polar group has significantly less retention and selectivity for the nonpolar test substances, and the α-value obtained for CH_2 is much lower.

The next parameter, α T/O, illustrates the column's ability to separate planar and non-planar molecules (steric selectivity). The C_{18}-P column is very good for separating structure-like analytes differing only in their spatial arrangement.

Silanol interactions, i.e. the ability of the column to form hydrogen bonds, are described by the α C/P value. This also evaluates the quality of end capping. This value should be taken with caution, as here the RP-Shield shows a very low value even though it contains a polar group. However, this embedded functionality has the opposite polarity of the silanol groups – hence the reduced α C/P value.

The last two parameters reflect the ion exchange capacity of the column at pH 7.6 and pH 2.3, i.e. in a mobile phase with pH above and below the pKa of the silanol groups, in which ionization to O^- is promoted once (pH 7.6) and suppressed once (pH 2.3). Here, the MAqC-ODS also shows cation exchange effects at pH 7.6 because it contains clearly acidic groups thanks to the metal.

			Example Column
Carbon loading	High values	High retention for nonpolar analytes	XR-ODS III
Carbon loading	Small values	Low retention for nonpolar analytes	GISS
Surface area	High values	High retention for nonpolar analytes	XR-ODS III
Surface area	Small values	Low retention for nonpolar analytes	GISS
Pore size	Up to approx. 10 nm	Usable for molecules up to approx. 5,000 Da	All Shim-pack Columns (except GISS)
Pore size	Up to approx. 20 nm	Usable for molecules up to approx. 20,000 Da	GISS
Pore size	30 nm and more	Good peak shape for macromolecules (Peptides and proteins)	Not available in the Shim-pack Portfolio
Pure silica	All new columns	Better Peakshape for basic analytes	
	Some old columns	Bad Peakshape for basic analytes due to many interactions	

Table 2: Listing of relevant column specifications with their effect on separation and a column example from the Shim-pack range

Conclusion

Selecting a suitable C_{18} LC column is a challenging task. There are many different C_{18} types available, and some of these differ highly in their separation properties. Classification according to the parameters of the Tanaka test protocol can help to make the right choice.

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Future of mass spectrometry in toxicology screening

European Innovation Center – interview with Prof. Saint-Marcoux, Limoges University Hospital (France)

Franck Saint-Marcoux (PharmD, Ph.D) holds the position of full Professor of Toxicology and is currently responsible for the Clinical and Forensic Toxicology Unit of the Pharmacology and Toxicology Department of the Limoges University Hospital (France). He obtained a Master's degree in analytical chemistry, a Master's degree in Advanced Studies in Pharmacy and a PhD in Pharmacokinetic Modelling in 2004. He is author or co-author of over 70 papers in peer-reviewed international journals and has presented at about 50 conferences and international events.

Since April 2017, he has worked with the Shimadzu European Innovation Center in the clinical division to develop tools for implementing tandem LCMS in toxicology.

Prof. Saint-Marcoux, Limoges University Hospital, France

Prof. Saint-Marcoux, can you outline the research you are conducting in general? What is the current state-of-the-art?

We have clinical toxicology and forensic toxicology activities in the lab. This covers numerous fields: accidental exposures to drugs or to toxic compounds, self-poisonings, suspicions of overdose, monitoring of addicts, driving under the influence of drugs (DUID), doping controls... In each situation, it is the responsibility of the toxicologist to ensure an unambiguous identification of xenobiotics involved (when indications are absent), to be able to measure toxicologically high concentrations or to have very low limits of detection, and to provide quantitative results. Most of the time, this has to be possible on a 24/7 basis, with a rapid sample analysis.

However, targets are continuously changing! There are always news drugs for which Therapeutic Drug Monitoring is necessary, i.e., individual dose adjustment based on measurement of the drug in blood. There are also always new illicit drugs to survey. This explains why we are continuously conducting research to develop pertinent, modern, practical and efficient analytical methods, mainly using LC-MS or GC-MS.

Can you describe the research you are doing at the European Innovation Center with Shimadzu?

Globally, the research we are doing at the European Innovation Center consists of the development of innovative analytical methods in the field of toxicology. Since the beginning of our collaboration, our efforts have focused on the development of screening procedures using the LCMS-8060 system.

In a first approach, a spectral library has been developed for the Shimadzu LC-MS/MS platforms. It contains over 8,000 MRM transitions for 1,280 certified reference standards (including 37 deuterated internal standard compounds) with 6,084 registered spectra including positive and negative ionization modes. This library uses product ion spectrum data that can be used in routine library searching and compound verification using reference library match scoring.

One originality of the approach is that we use a novel spectral acquisition method that allows "reconstruction" of a spectrum containing all the specific transitions of a molecule. It is the so-called "MRM spectrum mode". Unlike other approaches previously published where two or three collision energies were applied to all molecules in a method using product ion scanning, we have optimized collision energy for up to 15 transitions from a molecule. This approach makes it possible to obtain extremely specific and rich spectral information.

Currently, we are conducting research to evaluate the quantitative performances of a screening procedure based on this library. More precisely, we are developing a method for most commonly observed compounds, including antidepressants, anxiolytics, drugs of abuse, analgesics and antipsychotics, and testing its performances at infra-therapeutic, therapeutic and toxic concentrations in a quantitative approach. First results are promising and we are

close to be able to propose a first procedure.

Meanwhile, we are developing fully automated extraction methods carried out by the CLAM-2000 programmable liquid handler, coupled directly to an LCMS-8060 system. A first method has been developed for the determination of 42 amphetamines, cocaine and opiates. These works have been recently submitted for publication.

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

A screening is the first analysis performed when the nature or the presence of a drug is totally unknown, which is often the case in clinical and forensic toxicology. Usually, a screening precedes more specific analyses allowing the quantitation of detected compounds. Procedures that allow both detection and quantitation are awaited.

Additionally, the implementation of automation for all or part of the analysis process eliminates technical errors made by manual preparation and saves time in the laboratory enabling technicians to perform other manual tasks while the system performs the analysis automatically.

How are Shimadzu instruments helping you in your research?

The LCMS-8060 system is a powerful tool with high intrinsic performances. Whatever the application we have yet developed, no loss in sensitivity by acquiring data in MRM Spectrum mode has been observed compared to conventional 2 MRM per compound. For example, with the method for the determination of illicit drugs, the dwell time and pause time for data acquisition are typically 3 ms and 1 ms, meaning maximum sample loop time is less than one second even at the most intense region of overlapping compound elution. At this point in time, 220 MRM can be measured simultaneously while still achieving an average of 20 data points measured across a peak.

The CLAM-2000 is the first system that having procedures where no human intervention is necessary when the primary tube is loaded on board the system. Sample preparation is synchronized with the LC-MS/MS system resulting in no time being lost whilst maintaining the ability to prepare the sample on-line and to inject directly immediately after preparation.

What are Shimadzu's strengths compared to other vendors, not limited to instruments?

Through the European Innovation Center, Shimadzu is the first company who proposes an actual and effective collaboration with us. This is a win-win and exciting partnership!

All works are under the supervision of both the Department of Pharmacology and Toxicology of the Limoges University Hospital and Shimadzu Corporation, namely Stephane Moreau, Duisburg, Germany, and Alban Huteau, Marne-la-Vallée, France. The overseas Mass Spectrometry Business Unit of Shimadzu Corporation, with Neil Loftus and Alan Barnes, is also greatly involved in these developments. An important point is that we have with Tiphaine Robin a PhD student who is fully dedicated to the project.

Could you share any requests for Shimadzu for support or technology that would help improve your processes or research, even if these requests may be difficult to fulfill at this moment?

We have multiple projects in mind, such as the development of analytical methods on an upcoming QTOF system, or the development of analytical methods based on microsampling Wing devices. Unfortunately, we need more people. One potential solution would be to share a second Ph.D student with Shimadzu.

Take a look into the future: What will happen in the clinical and forensic fields and how will the change influence analytical instruments such as Clinical MS, and clinical procedures in ten years?

In a near future, we will face multiple challenges at the pre-analytical, analytical and post-analytical steps.

Firstly, devices that are less invasive than usual blood samplings, or that allow patients to sample themselves, will become more and more common. This implies that we will increasingly face new kinds of samples: dried blood spots (DBS), oral fluids, microvolumes of blood... Nowadays, blood samples are usually not less than 50 to 100 µL. If we anticipate that modern sampling strategies will systematically lead to samples of less than 5 µL, you can imagine the gain in sensitivity which we will need.

Secondly, more and more drugs will belong to the field of "personalized" or "targeted" medicine. This will imply the development of sophisticated analytical methods such as the nano-surface and molecular-orientation limited (nSMOL) methods that Shimadzu has developed for the measurement of monoclonal antibodies therapeutics.

In the meantime, we have to imagine highly efficient laboratories where the simplicity of operation and the minimization of user involvement in the sample preparation process will be our watchwords. For this, the CLAM-2000 will be very helpful. But, considering my previous remarks, the system will have to be flexible enough to treat multiple kinds of samples. I will need to provide it with DBS or with microtubes, and to ask for different kinds of extraction procedures.

Very often, the time dedicated to treat/check the data is not considered, although it is a key point. You can't transform any high throughput lab into an efficient lab if you don't have software helping you to rapidly deliver results. Most of the time, I see in my lab pretty nice methods with automated extraction procedures and extremely shortened analytical runs, but for which hours and hours are still spent checking the data. Are the calibration curves ok? Are the internal standards areas ok? Are all peaks well integrated? Are the internal quality controls ok? We definitely need tools that answer these questions automatically and drive us safely, just as new cars can drive us safely on a highway!

CLAM-2000

Team from the left to the right: Piotr Stefanowicz, Remigiusz Bąchor, Zbigniew Szewczuk, Monika Kijewska, Mateusz Waliczek

Derivatization with pyrylium salts

A way to improve sensitivity of analysis of peptides using LC-MS/MS

Protein and peptide studies are essential for understanding many biological processes at the molecular level. Tandem mass spectrometry (MS/MS) has become the method of choice for both qualitative and quantitative analysis of proteins and peptides due to its high sensitivity, accuracy and applicability in analysis of complex mixtures. However, analysis of trace amount of peptides may be problematic due to insufficient ionization efficiency of some peptides. One way to overcome this problem is to introduce an ionization enhancer into the analyte molecule. The team at University of Wrocław developed a new ionization tag based on 2,4,6-triphenylpyridinium moiety, which enables LC-MS analysis of peptides at attomole level using MRM (multiple reaction analysis) mode.

Introduction

Advanced mass spectrometer technology does not always ensure successful analysis of trace amount of peptides due to the insufficient ionization efficiency, resulting in limited sensitivity of analysis. The affinity of the proton for the peptide molecule, the ionization efficiency, is a crucial parameter affecting ionization yield, hence the abundant signal of arginine (the most basic amino acid) containing peptides [1].

One method for increasing ionization efficiency of a peptide without arginine is to introduce a fixed charge group into the analyte molecule. The Wrocław University researchers recently proposed the application of 2,4,6-triphenylpyrylium salts for the derivatization of peptides [2].

Figure 1: Reaction of 2,4,6-trisubstituted pyrylium salt with peptide

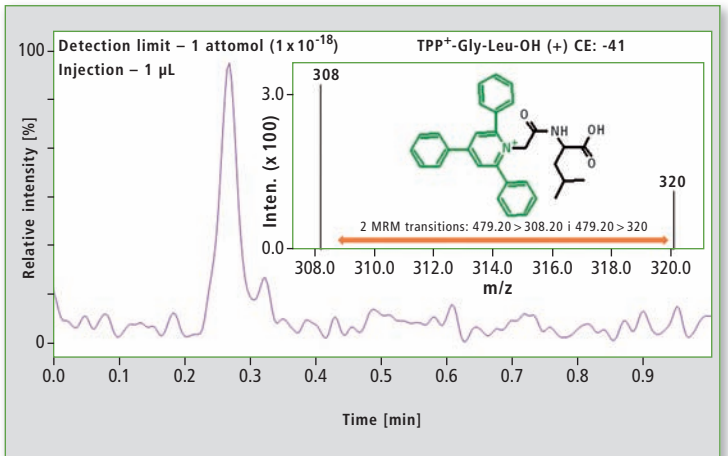

Figure 2: MS spectrum obtained for one attomole of the derivatized dipeptide TPP+-Gly-Leu-OH using Shimadzu LCMS-8050

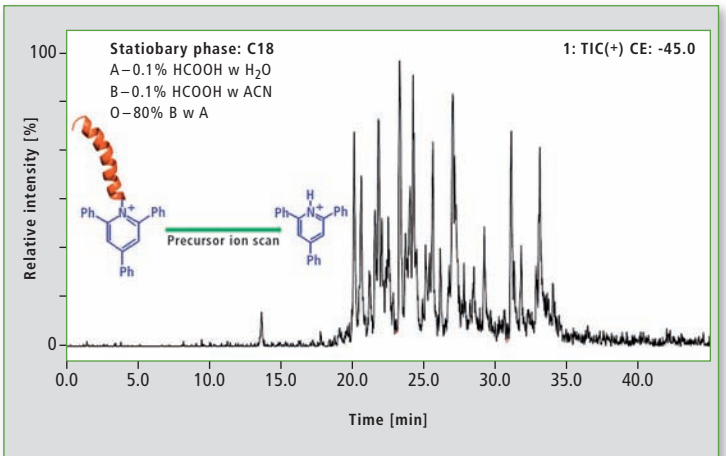

Figure 3: LC-MS analysis of derivatized BSA tryptic digest performed in precursor ion scan mode

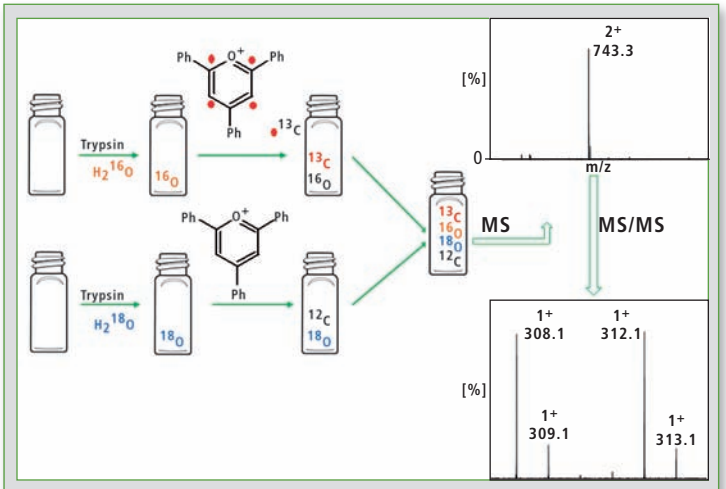

Figure 4: Schematic of the procedure to obtain isobaric peptides (duplex) from two samples

Pyrylium salts react readily with sterically unhindered primary amines to form pyridinium salts with a fixed charge. This reaction is particularly selective toward the ϵ-amine group of lysine. Thus, the 2,4,6-triphenylpyrylium salts as well as a tryptic digested protein for derivatization of synthetic peptides were applied. The peptides derivatized generate an abundant protonated 2,4,6-triphenylpyridinium ion during CID (collision induced dissociation) fragmentation, which has proved to be useful in analysis using selective mode e.g. MRM, or precursor ion scan.

The research team also proposed a novel method for obtaining isobaric peptides by combination of ^{18}O enzymatic labeling [3/4] and modification of peptides by ^{12}C and ^{13}C isotopologues of 2,4,6-triphenylpyrylium salts. This combination leads to the formation of isobaric duplexes. The subsequent fragmentation experiment results in formation of reporter ions, which differ by 4 Da. Their comparison provides quantitative evaluation of the peptides in the compared samples.

Currently, this approach is being tested on urine samples. The aim of this study is to develop a method allowing early detection of preeclampsia (PE) state based on podocin tryptic fragments, which was recognized as a PE biomarker. PE is a multisystem disorder which remains a leading cause of maternal and neonatal mortality and morbidity.

LC-MS analysis

LC-MS of derivatized bovine serum albumin (BSA) tryptic digest was performed on Shimadzu's LCMS-8050 triple quadrupole mass spectrometer. For the experiment using precursor ion mode, the researchers chose a fragmentation ion at m/z 308.2 which represents the protonated 2,4,6-triphenylpyridinium cation at the collision energy of 45 eV. The same LC-MS mass spectrometer was used to analyze the model isobaric peptides (duplex) by MRM mode as well as Q1Q3 scan. Separation was carried out on an RP-Aeris Peptide (50 x 2.1 mm, 3.5 µm) column with a gradient elution of 5 - 60 % B in A (A, 0.1 % HCOOH in water; B, 0.1 % HCOOH in MeCN) at room temperature over a period of 45 min for digested BSA (flow rate: 0.1 mL min^{-1}) and 15 min for model isobaric peptides (flow rate: 0.2 mL min^{-1}). The samples were dissolved in 400 µl of water: acetonitrile mixture.

Derivatization Procedure

The sample was dissolved in dimethylformamide (DMF), and three times excess of 2,4,6-triphenylpyrylium salt and the same excess of triethylamine (TEA) was then added. The resulting mixture was vortexed and incubated at 70 °C for 20 min. Finally, the solvent was evaporated under nitrogen stream and lyophilized.

Results

Detection limit

The derivatized dipeptide H-Gly-Leu-OH was used to check the limit of detection of the analysis. For this purpose, a selective MRM mode was used and a limit of detection for the examined dipeptide of 1 attomole could be shown (figure 2).

Analysis of BSA tryptic digest

For the analysis of derivatized BSA precursor, the ion scan mode was used. Protonated 2,4,6-triphenylpyridinium cation at 308 m/z and 45 eV collision energy was chosen as product ion. This resulted in observation of peptides containing ionization tag only (see figure 3).

2,4,6-triphenylpyridinium salt based isobaric peptides (duplex)

The $^{16}O/^{18}O$ exchange is widely used in comparative proteomics. Tryptic digestion of a protein was carried out in $H_2{}^{18}O$ resulting in incorporation of two heavy oxygen atoms into the carboxyl group of lysine. Tis approach meets some inconveniences, e.g. back-exchange or overlapping of the isotopic peaks. The research team proposed the modification of this approach by additional usage of 2,4,6-triphenylpyrylium salts.

One sample is digested in $H_2{}^{18}O$ and unlabeled 2,4,6-triphenylpyrylium salt, while the other sample is digested in unlabeled H_2O and labeled 2,4,6-triphenylpyrylium salt (figure 4).

Afterwards, the samples are pooled and analyzed together. The fragmentation of the ions corresponding to isobaric peptides generate reporter ions with a mass difference of 4 Da (labeled and unlabeled 2,4,6-triphenylpyridinium). Comparison of their intensity enables determination of relative concentration of peptide in two samples. In this way, there is no risk of overlapping of isotopic peaks.

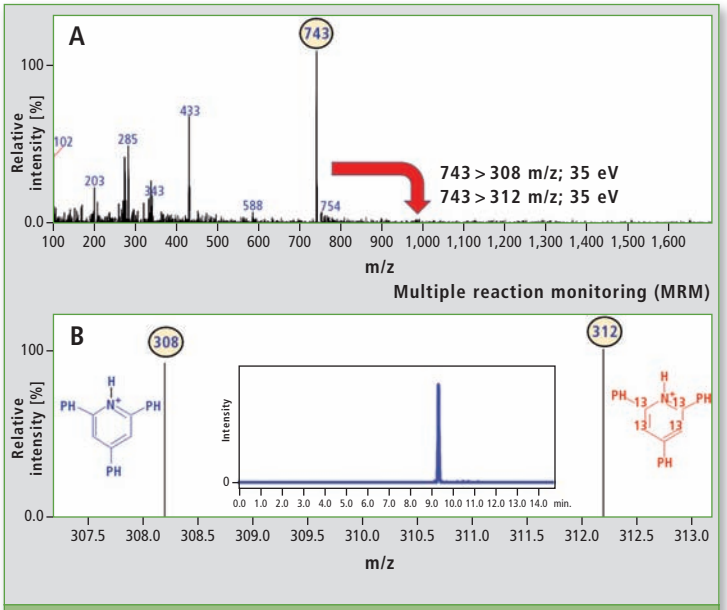

Figure 5: A) ESI-MS spectrum obtained for the model isobaric peptides formed from derivatized Ac-Leu-Val-Asn-Glu-Val-Thr-Glu-Phe-Ala-Lys-OH B) ESI-MS spectrum obtained for the model isobaric peptides (duplex) in the MRM mode using 743 > 308 and 743 > 312 m/z transitions. The inset shows LC-MS chromatogram obtained in the MRM mode.

Moreover, back-exchange was not observed since the pyridinium modified lysine residue was not recognized by the enzyme.

Analysis of peptides in multiple reaction monitoring (MRM) mode allows for detection of peptides with high sensitivity. This results from the improved signal-to-noise ratio. Thus, the analysis of the model isobaric peptides (Ac-Leu-Val-Asn-Glu-Val-Thr-Glu-Phe-Ala-Lys-OH; 66-75 fragment of BSA) using the MRM mode was performed. The research team used the diagnostic reporter ions (m/z 308 and 312) to the MRM transitions. Results of the analysis are presented in figure 5.

The full scan spectrum of the model isobaric peptides is presented in the upper part of figure. The abundant peak at m/z 743 corresponded to the duplex. In this experiment, two equimolar samples were analyzed, thus two diagnostic peaks with comparable intensity were observed. The presence of 2,4,6-triphenylpyridinium moieties in peptides increased the ionization efficiency. Analysis of modified isobaric peptides in MRM mode may therefore result in highly improved limit of detection.

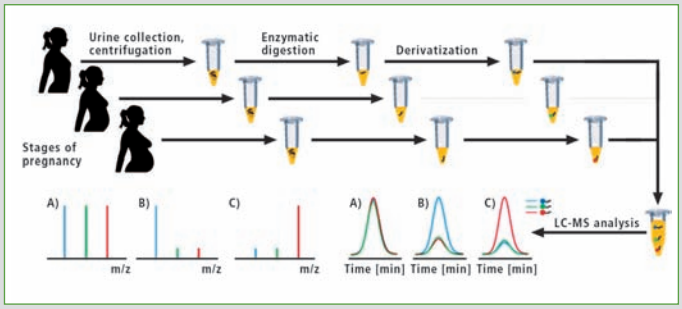

Figure 6: Schematic representation of the sensitive shotgun proteomics analysis of urine proteins derived from the same patient at different stages of PE.

The tryptic peptides isolated from samples collected at different time will be derivatized using ionization enhancers labeled with different number of stable isotopes. The derivatized samples will be combined and the changes in relative concentrations of podocin and other biomarkers will be analyzed by isotopic dilution approach by LC-MS.

Real samples analysis

The synthesized isotopically labeled and unlabeled 2,4,6-triphenylpyrylium salts were used to analyze a tryptic digest of podocin obtained from urine of pregnant women. The urine analyzed derives from different stages of PE. The schematic representation is presented in figure 6. Application of pyrylium salts allows both increase of analysis sensitivity and the introduction of stable isotope to the peptides, which in turn enables LC-MRM-MS analysis using multiple isotope dilution technique.

Conclusions

A new ionization tag based on 2,4,6-triphenylpyridinium scaffold was designed, enabling highly sensitive analysis of peptides by LC-MS at attomole level. The advantage of this ionization tag is convenient derivatization procedure and high selectivity toward ϵ-amino group of lysine. Moreover, the reaction of pyrylium salts does not require active groups with low stability in water solutions. The adequate combination of $^{16}O/^{18}O$ exchange and derivatization using 2,4,6-triphenylpyrylium salt or its $^{13}C_4$ isotopologue enables obtaining of isobaric peptides. The comparison of signal intensity of the reporter ions derived from the isobaric peptides allows for quantitation of the peptides. Recently, pyridinium salts in LC-MRM-MS analysis of podocin as a biomarker of PE stage was applied.

Authors

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