the Analytical Scientist

MASS SPECTROMETRY TAKES FLIGHT

From quantifying pesticides to ensuring the perfect glass of whisky, there seems to be no limit to the applications of mass spectrometry. Here, we present nine application notes that highlight the versatility of MS techniques.

ANY MATRIX
ANY INTERFERENCE
ANY PARTICLE SIZE

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The Impressive Flight of Mass Spec

Mass spectrometry continues to spread its wings across a seemingly endless set of applications.

I feel certain that J. J. Thomson – and his protégé Francis Aston – would be shocked (and possibly somewhat smug) to learn of the omnipresence that mass spectrometry enjoys in analytical laboratories around the globe today. The power – speed, selectivity and sensitivity – of modern instruments has secured mass spectrometry's position as the 'gold-standard' detector in many fields (I've heard a certain analytical guru say more than once "if you're not using MS, you're wasting your time"...) Meanwhile, high-resolution and accurate mass measurements have made advanced non-targeted analysis possible, opening up new research directions and fields – consider for a moment any '-omics' discipline without mass spectrometry...

The annual conference of the American Society for Mass Spectrometry – better known simply as ASMS – is testament to the astounding growth of a technique born out of physics but embraced by (analytical) chemists and biologists. The diversity on show at ASMS each year – echoed by the range of applications included in this special supplement – demonstrates how flexible and applicable MS can be, but perhaps also highlights the underlying simplicity of the principle behind it – measuring the masses of chemical species.

ASMS 2017 (June 4–8 in Indianapolis) is no different – a quick glance through the program once again leaves me wishing for either a time machine or a clone, given the number of fascinating parallel tracks. And the 'bookend' plenary lectures – "Towards a Good Start in Life: Neonatal Screening and Beyond" and "Saving the Great Coral Reefs" once again reinforce the scope and scale of the technique. Likewise, our cover feature from Enrico Davoli in the main magazine (page 24–32) takes us on a mass spectrometric journey from the macro (city-wide environmental analysis) to the micro (three-dimensional chemical mapping of cancer tumors), as he explores "the space dimension," using data that affords him the utmost confidence.

In the following pages, instrument manufacturers – who have played a huge role in advancing the field of mass spectrometry (through determination, perspiration and finally innovation) – share the new applications they are most excited about. From identifying aroma compounds in whisky to online vehicle exhaust monitoring to proteomics to biopharmaceutical characterization, there's likely to be something for everyone. And together, they beautifully represent how easily "Mass Spec Takes Flight."

Rich Whitworth
Content Director

Contents

CONFIDENTLY IDENTIFYING AROMA COMPOUNDS IN WHISKY

Trace volatiles in whisky are separated and identified using flow-modulated GC×GC-TOF MS.

By Natasha Spadafora, Laura McGregor and David Barden

Over 1,000 volatile aroma components from a range of chemical classes have been reported from whisky, and confident identification of these is important for quality control and authentication purposes, as well as in the engineering of new aromas. Comprehensive two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOF MS) is ideal for the analysis of such complex samples, because the enhanced separation capacity allows analysts to screen the entire composition in a single analysis, with confident identification of compounds that would ordinarily co-elute.

Thermal modulation is the most commonly used GC×GC modulation technique, but this often requires expensive liquid cryogen and struggles to modulate whisky volatiles that have boiling points similar to, or lower than, pentane. This study investigates the application of flow-modulated GC×GC-TOF MS using a reverse-fill/flush modulator that allows separation of volatiles ranging from C_1 to C_{40} (and above) without the use of liquid cryogen.

The sample analyzed was an Islay single malt, which was diluted, treated with salt, and incubated at 35°C before sampling using solid-phase micro-extraction (SPME). For the GC×GC-TOF MS analysis, an INSIGHT™ flow modulator (SepSolve Analytical) and a BenchTOF-HD™ instrument (Markes International) were used; ChromSpace® software (Markes International) was used for data processing. For full experimental details and comparison with two other whiskies, visit **http://bit.ly/2p6VHXD**.

The ability of the TOF MS instrument to generate spectra that are a close match for those in commercial quadrupole-acquired libraries allowed a large number of compounds to be identified, and these were dominated by medium-chain esters (Figure 1A). The sample had a strong 'peaty' aroma that is in accordance with the presence of a range of phenols (Figure 1B), rapidly extracted from the dataset using an automated scripting function within ChromSpace. Another feature of the software is the ability to deconvolve co-eluting species (Figure 1C), which in such highly complex samples can occur even when using GC×GC.

In summary, this study illustrates the power of flow-modulated GC×GC to provide simple, robust and affordable separation of complex aroma profiles, without the restrictions imposed by thermal modulation. In addition, the quadrupole-matched spectra generated by the TOF MS instrument are complemented by rapid automated identification in the GC×GC software and reliable deconvolution of co-eluting peaks. This ability to comprehensively profile such complex samples and confidently identify components shows that this method has considerable potential for advancing research within the food and fragrance industries.

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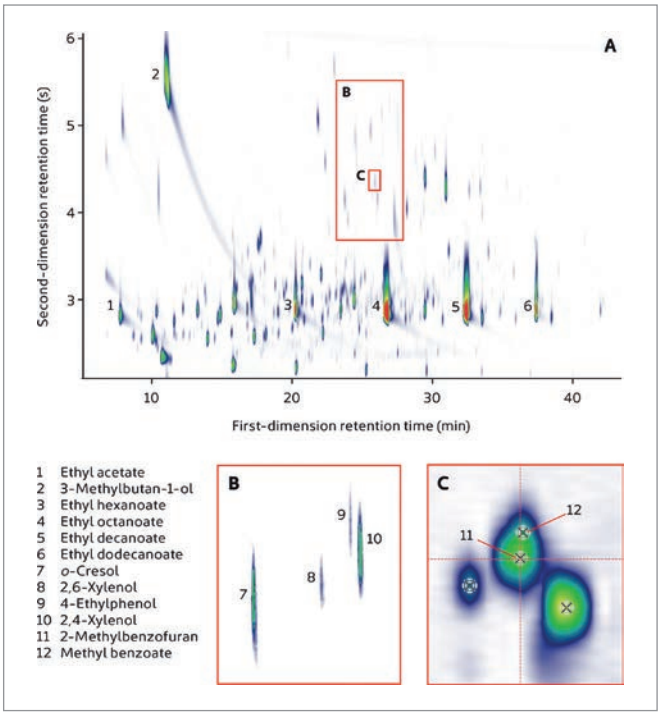
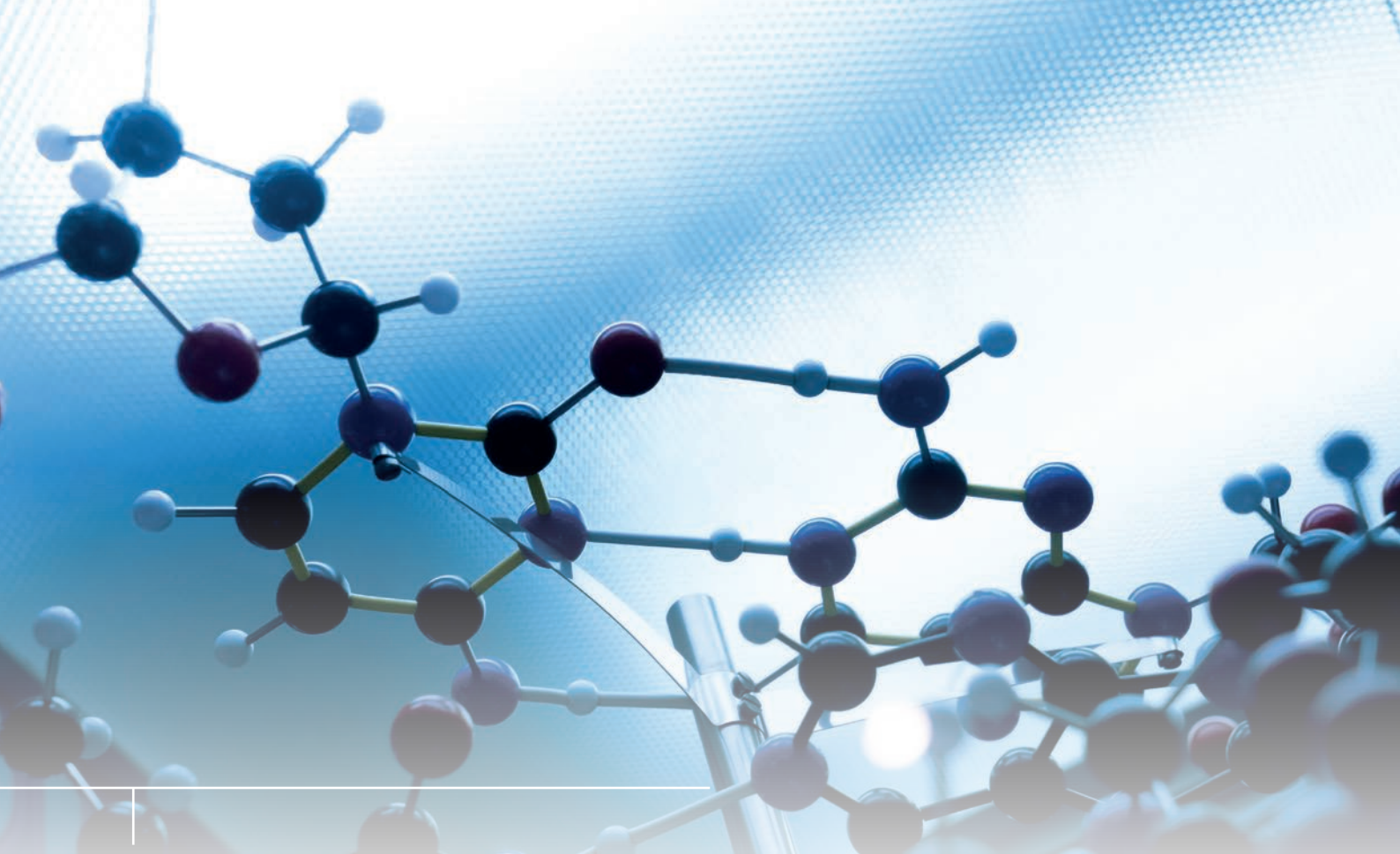


Figure 1. (A) Color plot indicating major compounds (1–6) identified in the headspace of an Islay single malt. (B) Aroma-active phenols (7–10) found using a scripting function. (C) Deconvolution of 2-methylbenzofuran (11, 'burnt/phenolic') and (B) methyl benzoate (12, 'cherry/phenolic'), both identified with match factors >890 by comparison against the NIST 14 library.



A NEW ERA IN PROTEOMICS: SPECTRAL LIBRARY FREE DATA INDEPENDENT ACQUISITION (DIA)

A new workflow promises the simplicity of shotgun proteomics and the quantitative precision and reproducibility of data independent acquisition.

Discovery proteomics aims to understand global proteome dynamics in a cell, tissue or organism. The key to gaining a comprehensive picture of the biology lies in the quantitative precision, reproducibility and the unbiased nature of analysis.

Modern discovery proteomics workflows mainly rely on high-resolution LC-MS/MS instruments. At present, shotgun proteomics (data dependent acquisition or DDA) is the most widely used discovery proteomics technique. A major limitation of shotgun proteomics is the semi-stochastic peptide selection for sequencing peptides. Therefore, the same peptides will not be identified reproducibly even when analyzing technical replicates. Despite the possibility of performing MS1 alignment, this results in missing values in the data matrix, problems in statistical analysis and an incomplete biological picture.

Recently, data independent acquisition (DIA) has emerged as a new alternative, where in a single measurement, all detectable peptides can be quantified with high sensitivity, quantitative precision and reproducibility. However, for best performance, a spectral library typically generated from additional DDA runs is necessary for targeted data analysis of DIA. This increases the instrument run time and, especially in small experiments, presents a significant overhead.

Biognosys, the leading developer of next-generation commercial proteomics solutions, will now add a new workflow to its DIA analysis software – Spectronaut – that enables reproducible and precise quantification of thousands of proteins in a single sample without the need for DDA-based spectral libraries. This is a simple workflow for label-free proteome quantification that offers significant savings in instrument time, while maintaining high quantitative precision and high reproducibility at the same level as the targeted analysis of DIA data using spectral libraries.

In the technical note, we present an overview of the new spectral library free DIA workflow and its application to the set of six samples derived from healthy and cancerous liver tissue. In this data set, we could quantify over 70,000 peptide precursors and over 5,300 protein groups across samples. The quantitative data completeness was > 90 percent for peptide precursors.

MRM SPECTRUM MODE TO REDUCE FALSE DEFECT REPORTING IN ROUTINE PESTICIDE ANALYSIS

Applying 'MRM Spectrum Mode' and library searching for enhanced reporting confidence in routine pesticide residue analysis.

By Stephane Moreau, David Baker, Neil Loftus and Chris Titman

Multiple reaction monitoring (MRM)-based LC-MS/MS techniques are widely used on triple quadrupole platforms for targeted quantitation as a result of their high selectivity, sensitivity and robustness. In a regulated environment such as food safety, there is a growing need to enhance capability in routine monitoring programs by increasing the number of pesticides searched for in a single analysis, while at the same time delivering the highest confidence in compound identification to reduce false detect reporting. For pesticide analysis in the EU, identification criteria in SANTE/11945/2015 requires the retention time and ion ratio from at least two MRM transitions to be within acceptable tolerance limits. However, even when applying these criteria it is well reported that false positives can occur in certain pesticide/commodity combinations.

To help reduce false negative and false positive reporting, MRM spectrum mode was used to increase the level of confidence in assay specificity by monitoring a higher number of MRM transitions for each target pesticide. With this method, a specific panel of

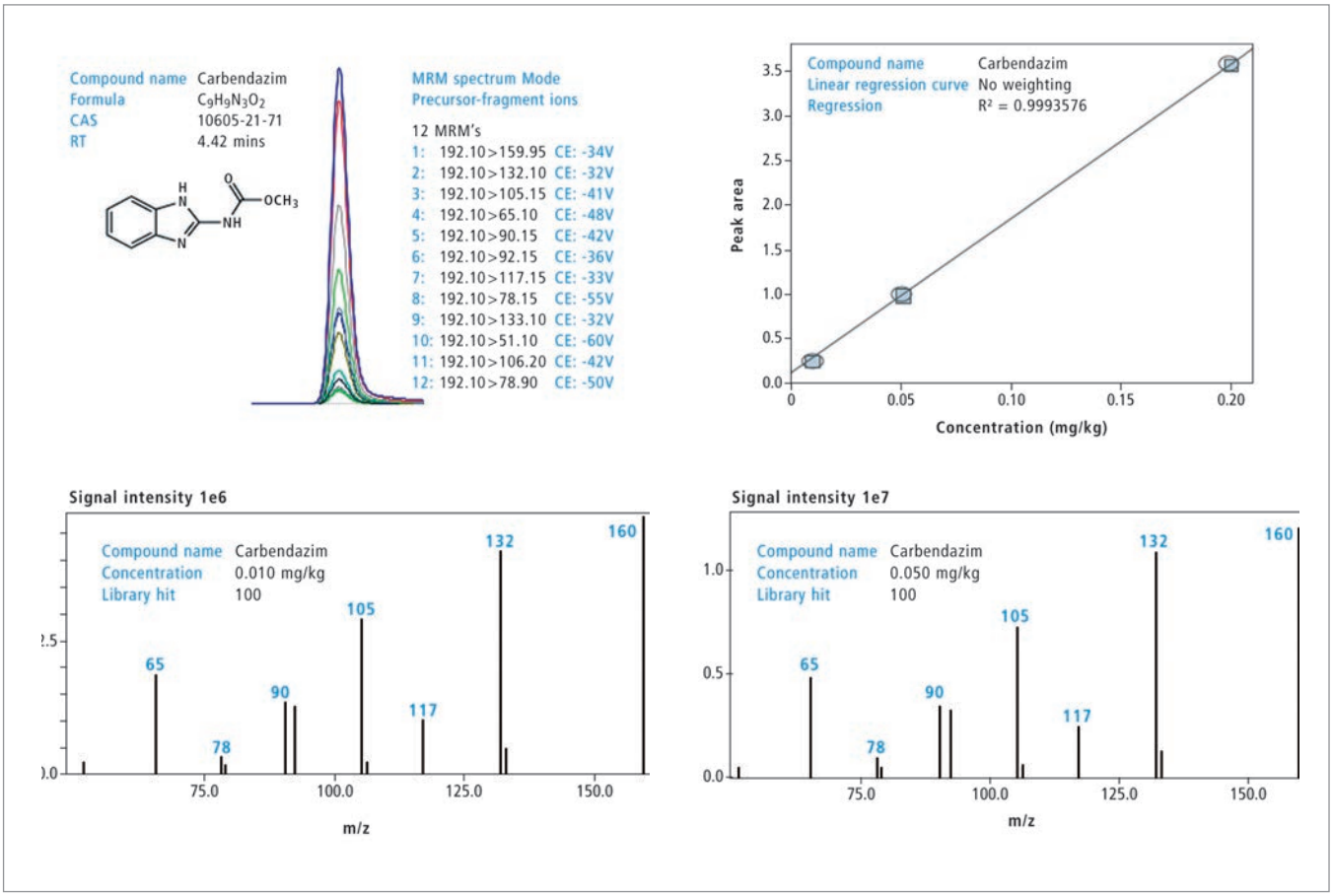


Figure 1. By applying a range of collision energies to carbendazim, 12 precursor-fragment ions are generated. MRM 192.10>159.95 was used in generating sensitive and robust quantitation whilst the product ion spectrum using all 12 fragment ions was used in confirming peak identification.

193 pesticides with 1,291 MRM transitions and a 15-minute cycle time were measured in turmeric, plum, peppermint, parsnip, cherry, lime, pumpkin, tomato and potato matrices. On average, seven MRM transitions were applied to each compound, with more than 10 MRM transitions applied to 34 compounds. As the collision energy is optimized for each precursor-fragment ion transition, the product ion spectrum is highly specific for each pesticide and generates library searchable results, even at very low concentrations and in different food commodities.

For all pesticides in all food commodities tested, MRM Spectrum mode reduced the possibility of false positive and negative results by searching against a reference library of optimized collision energies for each MRM transition. Despite acquiring a higher number of MRM transitions, the library searchable MRM approach (acquiring 1,291 transitions in a single method) results in the same signal intensity, linearity and reproducibility compared to a conventional two-fragment ion MRM method (acquiring 386 MRM transitions in a single method). Using MRM Spectrum mode resulted in a highly confident compound identification with highly accurate and sensitive quantitation in varying food matrices without compromising data quality, and extends the capability of LC-MS/MS in routine pesticide monitoring.

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Download the full application note: tas.txp.to/0517/SHIMADZU

ON-LINE VEHICLE EXHAUST MONITORING

Real-time monitoring of organic emissions in vehicle exhaust using high-resolution PTR-TOF.

By Jens Herbig and Lukas Märk

Automobile exhaust has been identified as a major source of air pollution. Emission testing of combustion engines has therefore been the focus of intense studies. Apart from inorganic emissions, volatile organic compounds (VOCs) and their atmospheric photochemical reaction products have lately drawn much attention due to their negative impact on the atmosphere and their dangers to public health.

IONICON PTR-TOFMS is an analytical technique to quantify such VOCs in real time and with high sensitivity. The ability to monitor hundreds of VOCs simultaneously is particularly useful to investigate the fast-changing conditions in a combustion process and to evaluate the impact of control technologies. In contrast to off-line methods, real-time and on-line analysis enables the measurement of unstable compounds, such as Acrolein, which easily gets lost in off-line sampling procedures.

At the California Air Resources Board (CARB), a PTR-TOF is used to study automotive vehicle exhaust (Figure 1). The high mass resolution of the IONICON PTR-TOF allows the effective separation of isobaric compounds, which is essential in a complex sample. In Matsunaga et al. (1) the researchers report their findings on VOC emissions from gasoline and diesel vehicles. A general observation is that VOC emissions from unburned fuel are extremely high at the beginning of a cold-start drive cycle but vanish as the vehicle warms up.

Another focus of these studies was on gasoline blended with ethanol (E10: 10 percent ethanol in 90 percent gasoline), which is widely used for economic as well as environmental considerations. For such analysis, a real-time instrument like a PTR-TOF is a powerful tool for revealing the relationship between emissions and vehicle parameters (see Figure 2).

Scientists working in environmental research and various other applications choose IONICON systems because of their ability to:

- measure complete mass spectra in < 1s,
- separate isobaric molecules due to high mass resolution,
- identify compounds by their chemical composition, and
- provide market-leading real sub-pptv detection limits for VOCs.

Catering to the needs of the industry as well as science, the IONICON product portfolio offers a range of PTR-TOFMS instruments for robust, automated industrial monitoring to highest performance for cutting edge research.

Reference

1. *A Matsunaga et al., "Motor vehicle exhaust analysis with a proton-transfer-reaction mass spectrometer (PTR-MS) – comparison study with conventional methods for BTEX and other toxic air contaminants". Poster presented at CRC Mobile Source Air Toxics Workshop; February, 2015; Sacramento, CA, USA.*

Please visit www.ionicon.com/automotive for more details.

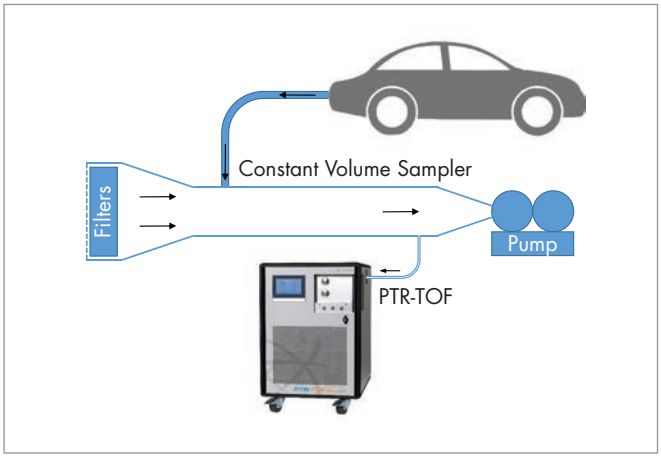

Figure 1. Typical setup for real-time automotive exhaust monitoring with an IONICON PTR-TOF 6000 X2 instrument.

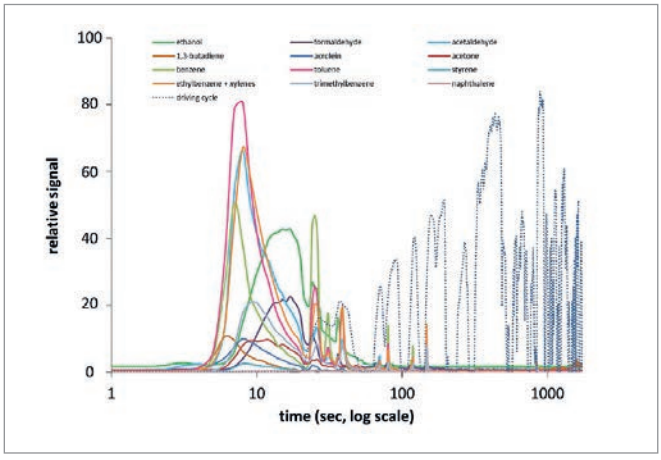

Figure 2. Emissions from gasoline (E10) vehicle exhaust. From Matsunaga et al. (1).

QUANTITATIVE AND QUALITATIVE CHARACTERIZATION USING A NOVEL DATA INDEPENDENT ACQUISITION METHOD

The applicability of SONAR for targeted and discovery proteomics using Aspergillus fumigatus.

By Kirsten Craven

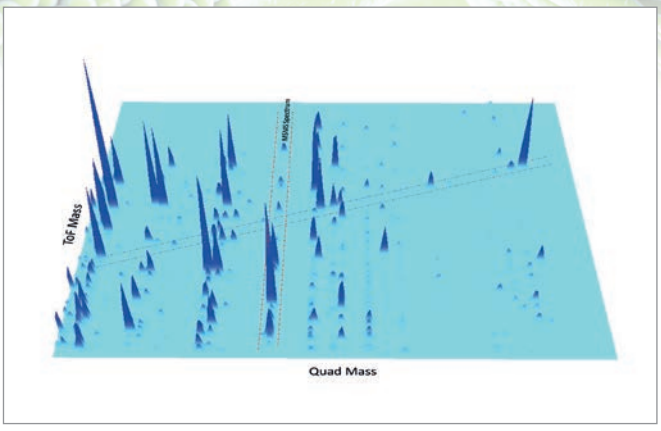

Figure 1. SONAR TOF vs. quadrupole m/z data, showing product ions (vertical bands) from peptides eluting during a 1 min window and the quadrupole sweep (diagonal line).

With accurate mass MS instruments, such as quadrupole time of flight (QTOF), different data acquisition approaches are adopted by analysts depending on the information required about a sample. Typical approaches are either data independent acquisition (DIA) or data dependent acquisition (DDA). Both have different strengths and compromises that must be considered when selecting an analytical method.

The advantage of DIA is that it collects unbiased precursor and fragment ion data on all detectable ions within the sample, without the need for time-consuming method development. This is possible because the MS collects all of the data all of the time, which removes the need for method optimization. With very complex biological samples, traditional DIA can struggle where common fragment ions and chromatographic coelutions are observed. This has driven a requirement for a DIA approach with increased selectivity, to more accurately discriminate analytes.

SONAR delivers this additional selectivity by using the quadrupole in the Xevo G2-XS QTOF as a mass filter, rather than remaining open as would be the case in traditional DIA experiments. Ions are separated prior to fragmentation and data recording. This results in clear MS/MS data for discovery workflows, without the need for time consuming method development. SONAR operates at speeds compatible with fast UPLC chromatographic separation, so there is no compromise in the number of points across a peak and the quality of quantitative results.

Aspergillus fumigates were used to evaluate the benefits of SONAR for biomedical samples. The samples were prepared by extracting proteins from immunoprecipitated Aspergillus fumigates and re-suspended in 0.1 percent RapiGest SF solution prior to reduction, alkylation and overnight digest. For this study, two biological replicates of a control and three independent drug-treated Aspergillus fumigates samples were analyzed. A study pool was created by combining all eight samples during the experiment to act as a QC.

The quadrupole scanning DIA (SONAR) method was applied to characterize the qualitative and quantitative changes in calcineurin complexes as a function of drug treatment; in particular, to examine the differential interactors with calcineurin in the presence of anti-fungal drugs, revealing the mechanism-of-action of calcineurin control over cell wall stress, and identify potential new targets for drug treatment.

The precision of SONAR LC-MS technique was found to be better than 10 percent, affording the detection of even mild protein abundance changes.

Download the application note: www.waters.com/DIAmethod

FULL CHARACTERIZATION OF HETEROGENEOUS ANTIBODY SAMPLES UNDER DENATURING AND NATIVE CONDITIONS

The three different operational modes of the BioPharma Option are described and application data are presented for the major application workflows.

By Kai Scheffler and Eugen Damoc

Mass spectrometry (MS) analysis of antibodies at the protein and peptide levels is critical during the development and production of biopharmaceuticals. The compositions of current-generation therapeutic proteins are often complex due to their heterogeneity, caused by various modifications that are relevant for their efficacy. Intact proteins analyzed by ESI-MS are detected in higher charge states that also provide more complexity in mass spectra.

Analysis of proteins in native or native-like conditions with zero or minimal organic solvents and neutral or weakly acidic pH can allow proteins to preserve non-covalent interactions and retain high degrees of folding. This effect has analytical benefits: greater protein folding leads to reduced charge states, increased mass separation, and increased signal at higher *m/z*. This strategy has been utilized for analysis of antibodies and antibody–drug conjugates present in highly complex mixtures of different antibody/drug combinations (1). Requirements for performing native MS on antibody samples include scanning towards 8,000 *m/z* and increased transmission optimization for large compounds. This feature has so far only been available on the Thermo Scientific™ Exactive™ Plus EMR mass spectrometer. Here, we show results obtained after successful implementation of the High Mass Range (HMR) Mode as part of the BioPharma Option now available on both the Thermo Scientific™ Q Exactive™ Plus and Q Exactive HF™ mass spectrometers, aimed at adding the capability to perform native MS analysis with mass detection up to 8,000 *m/z* without compromising performance of normal operation modes. These enhanced capabilities are necessary for the analysis of antibody samples on the intact level, under native conditions requiring the detection of masses beyond the standard mass range of up to 6,000 *m/z*.

The BioPharma Option adds superior denatured and native MS intact mass analysis and subunit top/middle-down analysis capabilities to the most powerful benchtop peptide mapping instruments available. The BioPharma Option offers distinct operational modes that have been optimized for the top three protein characterization workflows:

- intact mass analysis under native and denaturing conditions with the new HMR Mode,
- subunit and top/middle-down analysis with Protein Mode,
- peptide mapping with Standard Mode.

For the Q Exactive Plus mass spectrometer, the BioPharma Option includes: Standard Mode, Protein Mode, Enhanced Resolution Mode with resolution up to 280,000 @ *m/z* 200, and the HMR Mode with extended mass range up to *m/z* 8,000.

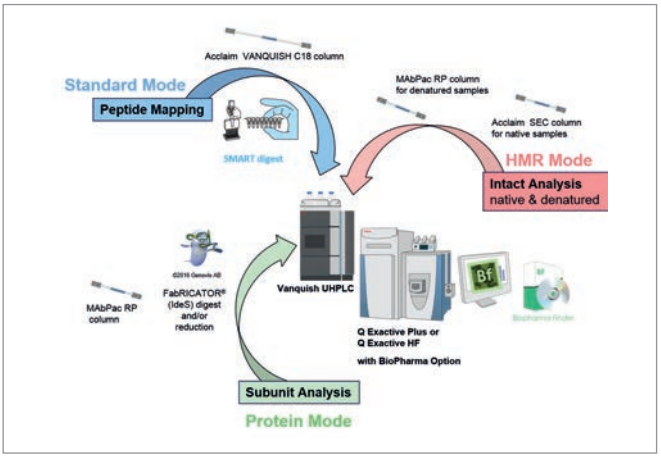

Figure 1. The Q Exactive Plus and Q Exactive HF mass spectrometers equipped with the BioPharma Option provide three different modes to cover the three major workflows in BioPharma: peptide mapping in Standard Mode, subunit analysis in Protein Mode and intact mass analysis under native and denaturing conditions in HMR Mode.

For the Q Exactive HF mass spectrometer, the BioPharma Option includes: Standard Mode, Protein Mode, and HMR Mode with extended mass range up to *m/z* 8,000.

The increase of the upper mass range on the mass spectrometers was achieved by implementing instrument control software changes. The analysis of molecules across the full mass range, including the detection of proteins under native conditions, required the use of optimized parameter settings to ensure efficient desolvation in the front region of the instrument, efficient transfer via multipoles, efficient trapping in the C-trap/HCD region, and sensitive injection and detection in the Orbitrap™ mass analyzer. Critical parameters include the optimization of in-source fragmentation that strongly influences the support of the desolvation process. Also, for transmission efficiency, specific voltages have been evaluated and optimized to ensure robust and sensitive performance in the higher mass range when performing analyses under native conditions, experiments that have not been possible so far on this type of mass spectrometer. Additionally, the standard calibration routine previously used was modified and adapted to ensure high mass accuracy across the full mass range. The addition of the BioPharma Option to the Q Exactive Plus and Q Exactive HF mass spectrometers does not compromise the performance of the instruments in any way, but rather extends it. That is, small molecule applications always run in Standard Mode and can be performed with the same level of sensitivity as on instruments not equipped with the add-on option.

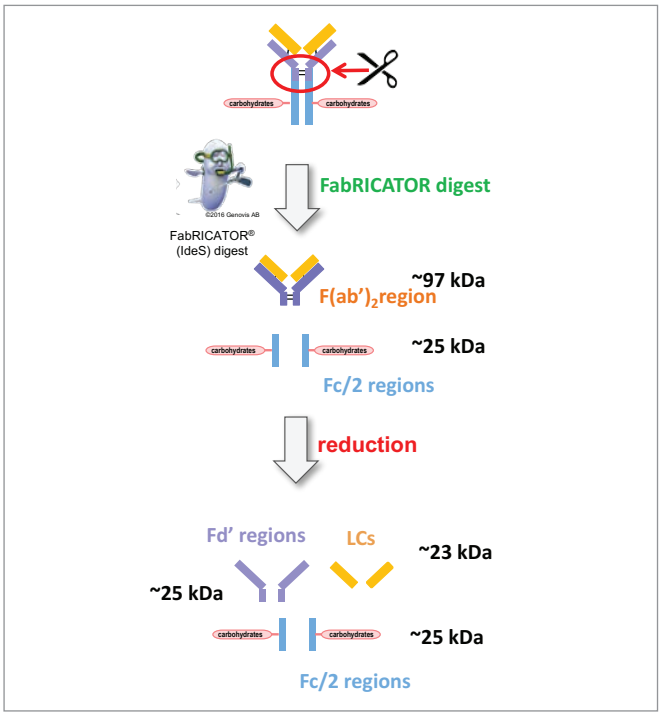

Figure 2. Schematic displaying the subunits obtained after FabRICATOR (IdeS) digest, which are the F(ab')^2 region and the Fc/2 subunits resulting after reduction in the Fd', LC, and the Fc/2 region.

We have analyzed the profiles of three monoclonal antibodies – trastuzumab, bevacizumab, and infliximab – under denaturing and native conditions. The samples were analyzed with three different workflows (Figure 1):

- analysis on the intact level under native and denaturing conditions,
- analysis of subunits following IdeS digestion and or reduction,
- peptide mapping following sample preparation applying the Thermo Scientific™ SMART Digest™ kit.

With the data collected on the different types of samples and presented in this study, we demonstrate successful analysis after implementation of the HMR Mode, successful desolvation, and optimized critical hardware operation settings. This makes the instrument an ideal platform to cover the three major workflows in the BioPharma Option: intact mass analysis under denaturing and under native conditions in HMR Mode, subunit analysis (reduced mAb and/or IdeS digested mAb) on the MS^1 and MS^2 level in Protein Mode, and lastly, peptide mapping in Standard Mode.

Experimental

Sample Preparation

The three commercially available monoclonal antibodies, trastuzumab (tradename Herceptin®), infliximab (tradename Remicade®) and bevacizumab (tradename Avastin®) obtained in the manufacturer's formulation buffer were used for all experiments.

Intact analysis: For native intact mass analysis using size exclusion chromatography (SEC)-LC-MS, the antibodies were injected without any further dilution. For LC-MS analysis under denaturing conditions, antibody samples were diluted in 0.1 percent formic acid. For direct infusion analysis under denaturing conditions, samples were desalted via Bio-Rad® P6 desalting columns and diluted to achieve 50 percent acetonitrile/0.1 percent formic acid in the solvent.

Subunit analysis: For subunit analysis, samples were reduced in 4M guanidine hydrochloride (GdHCl)/50 mM tris(2-carboxyethyl)phosphine (TCEP) and incubated at 57 °C for 45 min. For LC, Fc/2, Fd', F(ab')$_2$ subunit analysis, samples were first digested with the FabRICATOR® (Genovis) enzyme according to the manufacturer's protocol to obtain the Fc/2 and F(ab')$_2$ subunits, and in a subsequent step, reduction was performed using 4M GdHCl/50 mM TCEP and incubated at 57 °C for 45 min. FabRICATOR is also commonly known as IdeS (immunoglobulin-degrading enzyme from *Streptococcus pyogenes*), an engineered recombinant protease overexpressed in *Echerichia coli*. The protease cleaves specifically below the hinge region to yield F(ab')$_2$ and Fc/2 subunits (Figure 2).

Peptide analysis: For peptide analysis, 100 µg total protein per antibody sample was diluted to a volume of 50 µL and combined with 150 µL of SMART Digest buffer. The final 200 µL of sample were added to one vial of the SMART Digest kit (P/N 60109-101) containing immobilized, heat-stabilized trypsin. The proteolytic digestion was carried out at 70 °C and at 1,400 rpm shaking for 60 min. After completed digestion, the sample was separated from the beads and transferred into a fresh vial followed by reduction with 10 mM dithiothreitol (DTT) for 45 min at 57 °C. For disulfide bridge analysis, part of the sample was analyzed non-reduced.

Chromatography

All experiments were performed using Thermo Scientific™ Vanquish™ Horizon or Vanquish Flex Quarternary UHPLC systems.

For intact mass analysis under native conditions, proteins were online desalted using SEC on an Acclaim SEC-300 4.6 x 300 mm column (5 µm particle size, P/N 079723 column and isocratic elution with 50 mM ammonium acetate).

For reversed-phase chromatography of proteins, subunits, and peptides under denaturing conditions, the Thermo Scientific™ MAbPac™ RP 50 mm x 2.1 mm column was used (P/N 088648) with a gradient of solvent A consisting of water/0.1 percent formic acid and solvent B consisting of acetonitrile/0.1 percentformic acid.

Peptide mapping experiments were performed on a Thermo Scientific™ Acclaim™ VANQUISH™ C18 2.1 x 250 mm reversed-phase column with 2.2 µm (120 Å) particles (P/N 074812), run with a gradient of solvent A, consisting of water/0.1

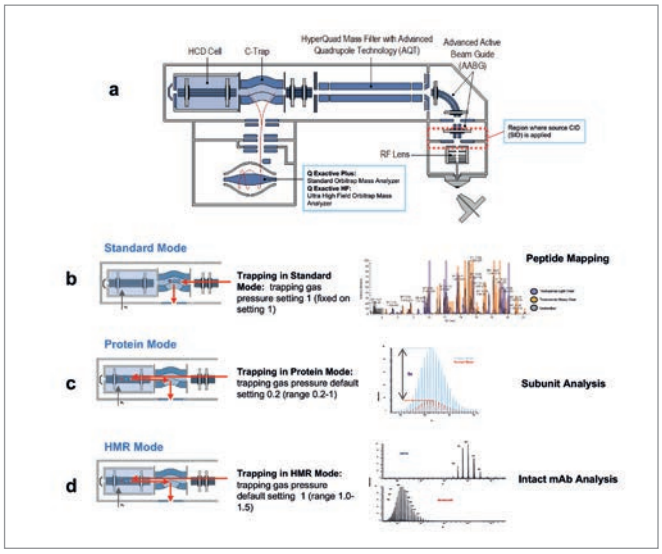

Figure 3. a) Schematic of the Q Exactive Plus and Q Exactive HF mass spectrometers and differences in the trapping path in the three different operating modes available: b) Standard Mode, c) Protein Mode and d) HMR Mode.

percent formic acid and solvent B, consisting of acetonitrile/0.1 percent formic acid.

Mass spectrometry

The mass spectrometers used for all experiments were commercially available Q Exactive Plus and Q Exactive HF mass spectrometers, each equipped with the BioPharma Option and controlled by Exactive Series Tune 2.8 software.

The top-down experiments of trastuzumab light and heavy chains were performed on the Q Exactive HF mass spectrometer with direct nanospray infusion. The mass range was set to 300–3,000 *m/z* and fragment ion spectra were acquired for several light and heavy chain precursor ions at a resolution setting of 240,000 for the heavy chain (HC) and 120,000 for the light chain (LC). The AGC target was 5e6 for the HC and 3e6 for the LC. 500 and 200 ms maximum injection times were set for the HC and LC, respectively, with spectra acquired with 10 µscans and a precursor isolation width of 1.2 Th. Rolling averaging was used for acquisition of the heavy chain top-down fragment ion spectra. Several hundred µscans per precursor were averaged with five different collision energies in the range of 10-18 percent. Fragment ions from all spectra from the different precursors and different collision energies applied were combined and submitted for sequence matching using ProSight Lite software.

IdeS digested and reduced trastuzumab was acquired on a Q Exactive Plus mass spectrometer using a Full MS method with the mass range set to 600–2,400 *m/z*, and a resolution setting of 140,000. Spectra were acquired with 5 µscans and an AGC target value of 3e6 with a maximum injection time of 200 ms. Top-down

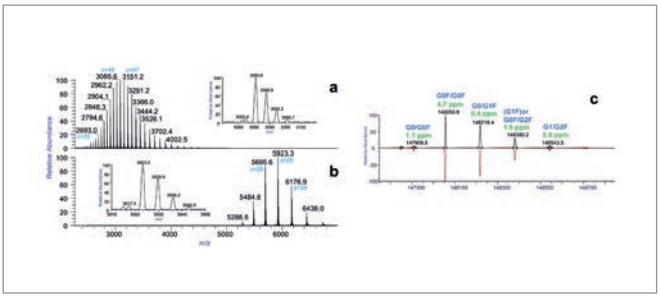

Figure 4. Full MS spectra acquired from intact trastuzumab under denaturing (a) and native conditions (b) and mirror plot of the spectra reflecting the masses of the different glycoforms obtained after deconvolution (c).

fragmentation was performed with a method consisting of MS2 scans using a fixed 200 Th-wide isolation window with a center mass of 950 *m/z*, a resolution setting of 140,000, an AGC target value of 3e6, 10 µscans, a maximum injection time of 500 ms and a fixed first mass of 300 *m/z*. Five different normalized collision energies between 10 and 18 percent were used in separate, duplicate runs. On average, 25 scans with 10 µscans each were acquired for both subunits at each of the five different collision energies in duplicate runs, resulting in a total of 2,500 µscans used for sequence matching.

Data analysis:

Intact protein, FabRICATOR digest, and peptide mapping raw data files were analyzed with Thermo Scientific™ BioPharma Finder™2.0 software. For top-down analysis data, ProSight Lite software was used after spectral deconvolution with the Xtract algorithm.

Results and discussion

Standard Mode, Protein Mode and HMR Mode

There are many factors that play a key role in the analysis of proteins, some of which relate to sample preparation (buffers, solvents, additives) while others relate to the mass spectrometer's source conditions as well as the physical environment inside the instrument (2,3). The Q Exactive Plus and Q Exactive HF mass spectrometers (Figure 3a) have previously been introduced with the Protein Mode option, which was one of many advancements for intact protein analysis on the Orbitrap platform. For these two instruments, an automated HCD gas control was introduced by using an electronically controlled valve for nitrogen gas in the HCD cell. This allowed easier optimization of the experimental conditions required for different types of analyses needing to be run on a single platform.

In Standard Mode, pressure settings are factory-optimized and suitable for most analyses (e.g. any small molecules application as well as peptides) and ions are cooled in the C-trap (Figure 3b).

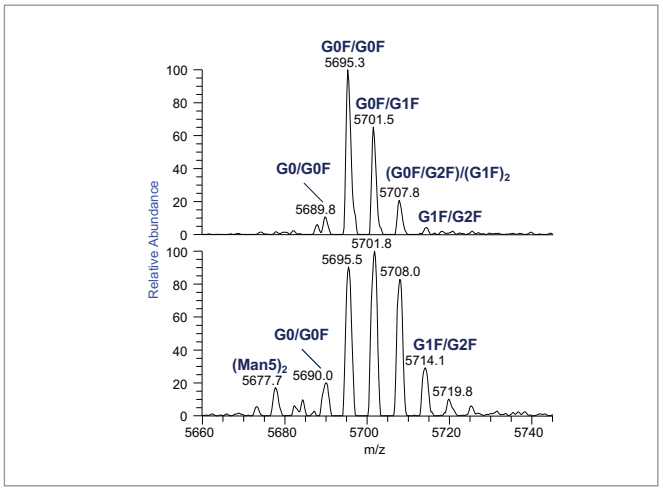

Figure 5. Observed glycoform patterns for two different lots of trastuzumab on the raw data level, showing significantly different relative abundances.

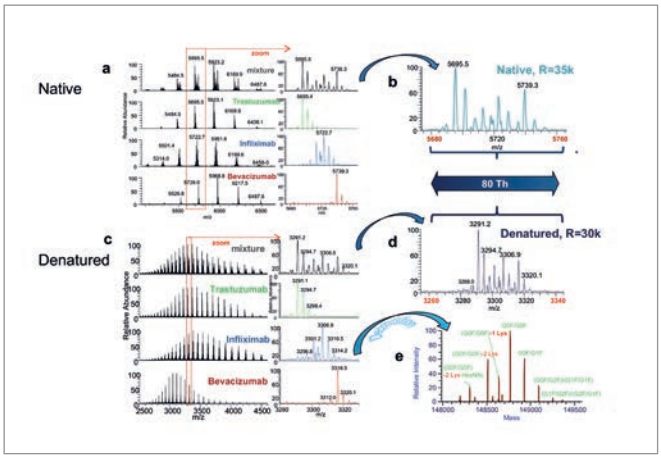

Figure 6. Analysis of the three mAbs trastuzumab, infliximab, and bevacizumab individually, as well as a mixture, under denaturing and native conditions.

The trapping gas pressure setting is 1, which corresponds to a high vacuum pressure delta (ΔHV) of ~3.1 e-5 mbar. The ΔHV is defined as the difference between HV with HCD gas on minus HV with HCD gas off.

In Protein Mode, the default trapping gas pressure setting is 0.2, which corresponds to a ΔHV that is five times lower than in Normal Mode. Additionally, ions are transferred and cooled in the HCD cell and thus have a longer flight path (Figure 3c).

The combination of reduced C-trap and HCD cell gas pressures, and trapping ions in the HCD cell prior to mass analysis, extends the life time of protein ions, resulting in increased signal intensities of isotopically resolved species (Figure 3c).

For higher gas pressures, high charge states of the same protein decay faster than lower charge states. This is because center-of-mass collision energy is proportional to the charge state z and can be expressed in this simple formula:

$$K_{ce}=E^{*}m/(M/z)$$

with *M/z*: the mass-to-charge ratio for a given charge state
m: mass of residual gas, nitrogen
E: ion energy inside the Orbitrap

This explains observations of charge envelope shifts on the *m/z* scale when comparing data acquired in different modes applying different pressure regimes in the HCD cell and C-trap region.

In HMR Mode, the default trapping gas pressure setting is 1, but it can be slightly increased up to 1.5 for improved trapping of certain species such as protein complexes and heterogeneous large proteins (e.g. antibody–drug conjugates). The trapping path in HMR Mode is the same as in Protein Mode, with ion cooling taking place in the HCD cell. Additionally, mass detection is enabled, ranging up to *m/z* 8,000 compared to *m/z* 6,000 in the two other modes.

The trapping gas pressure in all modes is set and saved in the tune files. Since a method allows for segmentation using different tune files, different pressure settings can be used within one LC-MS run. In contrast, the mass range setting is set in the method and the method editor allows for several nodes with different experiment types using different mass ranges within one LC-MS run.

Intact mAb analysis under native and denaturing conditions

The analysis of intact antibodies under native and denaturing conditions requires different chromatographic conditions. Whereas some researchers refer to the native analysis of antibodies as the mass detection of the intact antibodies, but under denaturing conditions, we refer here to native analysis as the analysis of the intact antibody under native conditions: near neutral pH, with no acid or organic solvent involved.

Under denaturing conditions, the protein is exposed to acid and organic solvent and separated over a reversed-phase column resulting in an envelope representing charge states from ~35 to 65, detected in a mass range from ~2,000 to 4,000 *m/z*. Under native conditions, the protein is kept in aqueous solution at near-neutral pH, only containing volatile salts such as ammonium acetate. These conditions preserve the protein's three-dimensional structure, providing a smaller surface to accept protons during the ionization process. This results in an envelope representing fewer and lower charge states, typically ranging from 20 to less than 30, detected in the mass range between 5,000-7,000 *m/z* (Figure 5). The reduced number charge states, representing the ions detected in native conditions, results in improved sensitivity.

For the analysis of mAbs under native conditions, the parameter settings regarding in-source CID, probe heater, and capillary

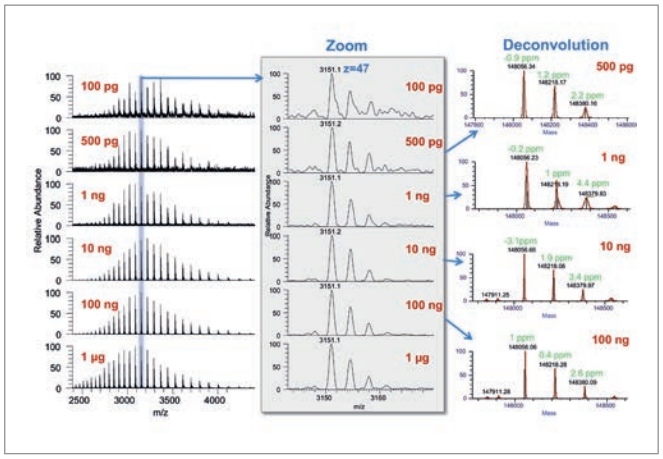

Figure 7. Serial dilution series of trastuzumab ranging from 100 pg to 1 µg total protein loaded on column.

temperatures were found to have a significant impact on the declustering/desolvation efficiency and thus spectral quality (5).

Under native conditions, a higher spatial resolution is obtained due to the detection at higher *m/z* as highlighted in the zoom of the most abundant charge states under both conditions in Figure 4. The glycoform pattern, however, and the masses obtained after deconvolution match well with very good mass accuracies obtained.

The glycoform patterns of antibodies generally, but also of commercially available antibodies, show variations and differences can be detected when different production batches are compared. We have analyzed two different batches of trastuzumab, one that we had obtained several years ago and one that we had obtained in 2016. The observed glycoform patterns are different in the relative abundance and number of glycoforms as detected on the raw data level, as well as after deconvolution (Figure 5). The respective patterns are consistent and reproducible across different instruments and platforms.

The three antibodies trastuzumab, infliximab, and bevacizumab were analyzed under native and denaturing conditions as single samples, as well as in a mixture (Figure 6a, c). The mixed sample provided the most complex pattern (also due to Lys-heterogeneity of infliximab, Figure 6e), which can be well resolved in both conditions (Figure 7b, d). However, under native conditions a higher spatial resolution is obtained due to the detection at higher *m/z* values.

To demonstrate the sensitivity of the instrument for intact mAb analysis under denaturing conditions, we have performed a dilution series of trastuzumab ranging from 100 pg to 1 µg total protein injected on column as shown in Figure 7.

mAb subunit analysis

The three antibodies trastuzumab, infliximab and bevacizumab were analyzed after reduction and after FabRICATOR digest

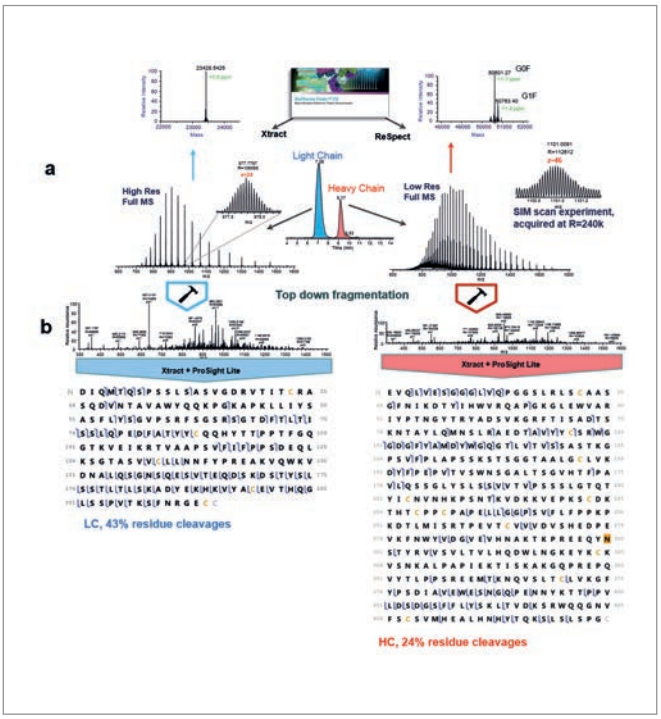

Figure 8. Detection of the separated light (LC) and heavy chains (HC) in Full MS as well as a SIM scan experiment of one charge state of one glycoform of the heavy chain providing a baseline resolved isotope pattern (a). Top-Down subunit analysis of light and heavy chain of trastuzumab and assignment of detected fragment ions to the expected amino acid sequence (b).

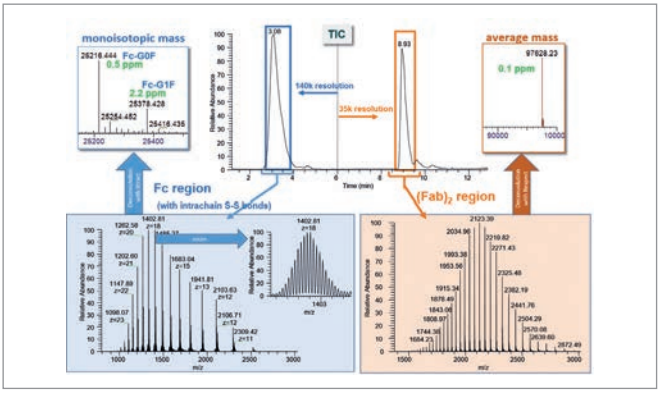

Figure 9. Total ion chromatogram and obtained MS spectra of trastuzumab after FabRICATOR digest without reduction resulting in the Fc/2 and F(ab')$_2$ regions with both subunits bearing intact disulfide bonds, resulting in observed molecular weights of 25kDa for the Fc/2 subunits and 97 kDa for the F(ab')$_2$ subunit.

with and without reduction. Figure 8 shows reduced trastuzumab as a representative for UHPLC separation of light and heavy chains typically obtained on the MAbPac RP column. The method setup comprised the full scan acquisition at high (light chain) and low (heavy chain) resolution settings, to achieve intact molecular weight information in the first instance. In a separate direct infusion experiment acquiring SIM scan data of a single charge state of a single glycoform, isotopic baseline-resolved peaks were also obtained for the heavy chain. In a third experiment

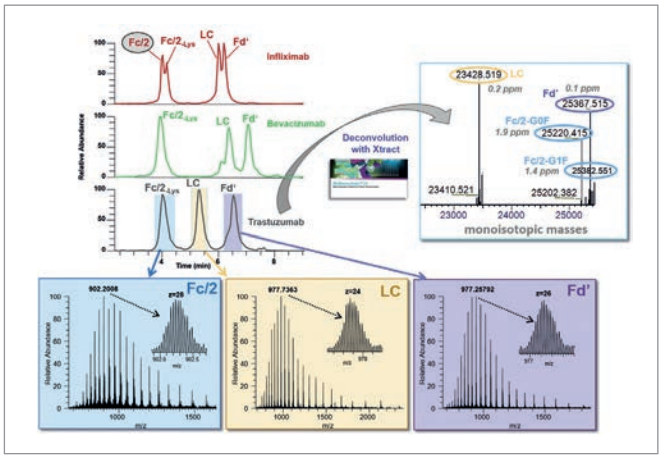

Figure 10. Total ion chromatograms of separated subunits obtained after FabRICATOR digest and reduction from trastuzumab, bevacizumab, and infliximab. Obtained Full MS mass spectra and baseline-resolved isotope patterns of the individual charge states, as well as the result after deconvolution, is showcased for trastuzumab.

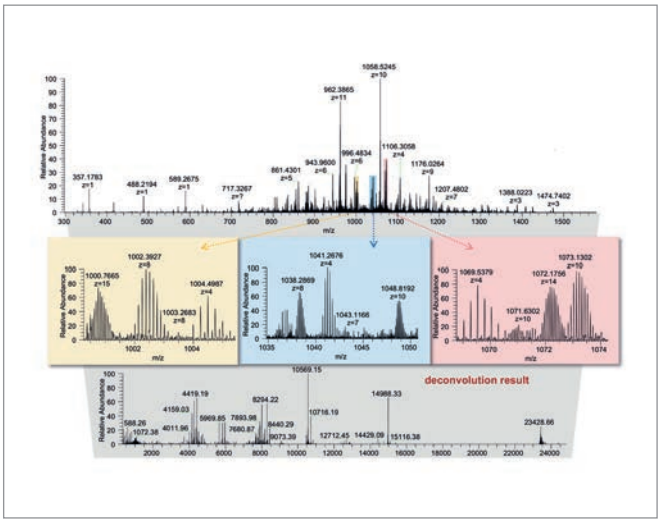

Figure 11. Top-down spectrum of the light chain of trastuzumab (top), zoom into three regions to highlight well resolved isotope patterns of fragment ions with different charge states, and mass spectrum obtained after deconvolution (bottom).

performing top-down analysis, MS/MS spectra for the light and the heavy chain were acquired and applied for sequence confirmation based on the fragment ions detected matched to the expected subunit sequences (6).

Another option to dissect an antibody in subunits is to perform a FabRICATOR digest using the immunoglobulin-degrading enzyme (IdeS) from *Streptococcus pyogenes*, an engineered recombinant protease overexpressed in *Echerichia coli*. The protease cleaves specifically below the hinge region to yield $F(ab')_2$ and Fc fragments (see Figure 2). Since the molecular weight of the $F(ab)_2$ subunit is too large (~90 kDa) to obtain isotopic resolution, and the Fc subunit with MW of about 25 kDa can easily be isotopically resolved, we have applied a method similar as for the analysis of the reduced mAb. In this method, the resolution is switched from high resolution (140k) for the detection of the Fc/2 subunit to lower resolution (35k) for the detection of the $F(ab)_2$ subunit (Figure 9). Due to the different resolution settings applied, the isotopically resolved spectra are deconvoluted with Xtract, whereas the unresolved spectra are deconvoluted with Respect. Both algorithms are implemented in the BioPharma Finder software. Both subunits are detected with very high mass accuracy between 0.1 and 2.2 ppm.

The $F(ab)_2$ subunit can be further separated into the light chain (LC) and Fd' region upon reduction. Figure 10 demonstrates the chromatographic separation of the three subunits Fc/2, LC, and Fd' obtained from trastuzumab, bevacizumab, and infliximab after FabRICATOR digest and reduction, nicely achieved in only 9 minutes based on the superb separation capabilities of the MAbPac RP column. Infliximab shows a significantly lower degree of Lys-truncation than the other two mAbs, resulting in a doublet chromatographic peak (Fc/2 and Fc/2-Lys) and relates to the glycoform pattern obtained on the intact level (Figure 6).

Mass spectra were acquired at high resolution to obtain monoisotopic masses after deconvolution with Xtract. Mass accuracies for all species are below 2 ppm, obtained with external calibration as depicted for trastuzumab.

In a separate experiment, a top-down analysis of the subunits as shown in Figure 10 was performed. Experiments were performed in Intact Protein Mode with a resolution setting of 140,000, to ensure resolving and detection of isotope patterns from highly charged and overlapping species. Figure 11 shows one example of an HCD spectrum obtained from the trastuzumab light chain. This MS/MS spectrum nicely demonstrates the peak density across the mass spectrum and the well-resolved and nicely shaped isotope patterns of detected fragment ions in different charge states, resulting in very good sequence coverage. Top-down spectra from the three subunits resulted in 39 percent bond coverage for the Fc/2 subunit, 38 percent bond coverage for the Fd' subunit, and 49 percent bond coverage for the light chain (6).

Peptide mapping

The three antibodies trastuzumab, infliximab and bevacizumab were analyzed on the peptide level after performing a SMART digest. Obtained base peak chromatograms are very similar but show distinct differences (Figure 12). All antibodies

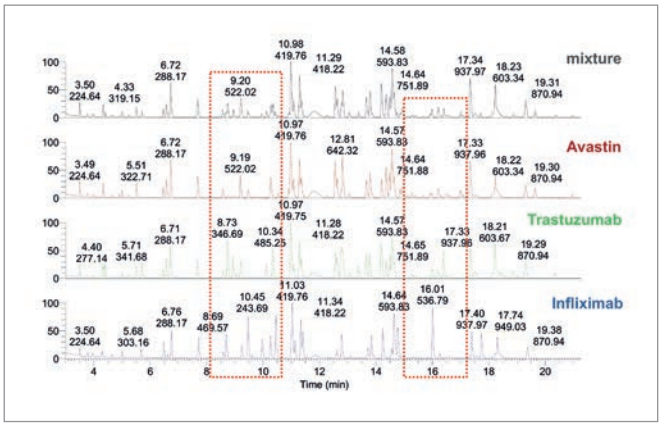

Figure 12. Base peak chromatograms obtained from individual digests of trastuzumab, bavacizumab, and infliximab as well as a mixture of all three. The basic peak pattern is very similar due to the high similarity of the amino acid sequences amongst the three mAb. Areas reflecting distinct differences are highlighted with the red boxes.

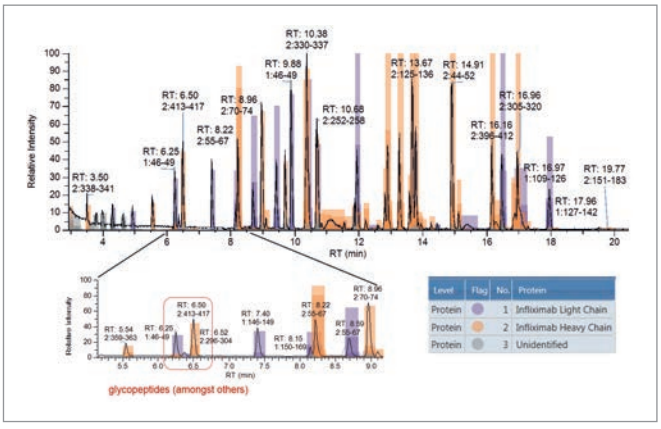

Figure 13. Peak shading provided by BioPharma Finder software based on peptides assigned to the light chain or heavy chain of infliximab. Some peaks remain unidentified, most commonly towards the onset or the end of the chromatogram.

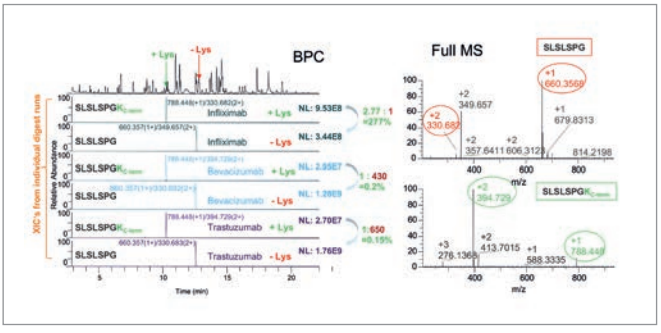

Figure 14. Base peak chromatogram of infliximab and XICs for the C-terminal peptides of the heavy chain with and without lysine truncation for infliximab, bevacizumab and trastuzumab. XICs were taken from the individual digests of the three mAb analyses.

were identified with 100 percent sequence coverage when analyzed separately as well as in a mixture. Glycopeptides, as well as common modifications such as low level oxidation and deamidation, are confidently identified in many variations based on MS/MS spectra.

Figure 13 details the chromatogram of infliximab and the chromatographic peak shading provided by BioPharma Finder software after performing a peptide mapping data analysis based on the known amino acid sequence. This feature facilitates the optical evaluation of results obtained, in particular if the desired 100 percent sequence coverage is not obtained. A quick evaluation will provide missed peaks, either having a too-broad elution profile requiring parameter optimization, or due to deviations or errors in the provided amino acid sequence.

Figure 14 highlights, on the peptide level, the low degree of Lys-truncation of infliximab compared to trastuzumab and bevacizumab, confirming the results obtained on the intact and subunit levels. XICs were created for both versions of the peptide, with and without lysine truncation. For XICs, the singly and double charged precursor masses were considered with a ± 5 ppm mass window. The relative intensities for the charge states of the peptide with and without C-terminal lysine are different. Since lysine is a basic amino acid strongly capturing a proton, the peptide containing the C-terminal lysine shows a more abundant doubly charged than singly charged ion. Comparing the relative intensities obtained for the two versions of the peptides shows, for trastuzumab and bevacizumab, only low levels of the lysine-containing peptides (0.15 percent and 0.2 percent) reflecting a high degree of lysine clipping. For infliximab, only low levels of lysine clipping are observed, resulting in a ratio of ~1:3 clipped vs. unclipped.

Disulfide bond mapping

Figure 15 highlights results from disulfide bond experiments on trastuzumab comparing a reduced versus non-reduced sample. Differences in the base peak chromatograms are obvious by visual inspection, and using BioPharma Finder software they are identified as either free Cys-containing peptides (reduced sample) or as part of disulfide linked peptides (non-reduced sample) and provided as shaded peaks in the chromatogram. All expected intra- and interchain disulfide bonds were detected and confirmed with very good mass accuracies.

Figure 16 showcases one example of a light chain's intrachain disulfide bond, highlighted in the sequence window. The XIC of the precursor mass of the linked peptide shows the

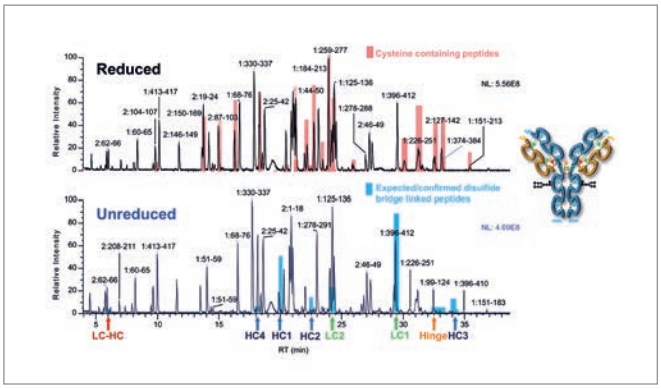

Figure 15. Comparison of chromatograms obtained from a digested and reduced sample of trastuzumab vs a digested and unreduced sample. Color shading highlights cysteine-containing peptides.

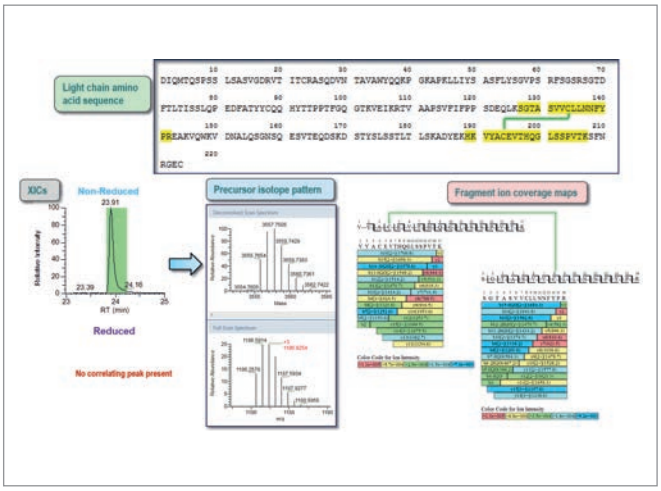

Figure 16. Example of an identified and confirmed intrachain disulfide linked peptide represented in the amino acid sequence. The XIC based on the linked peptide mass shows the absence upon reduction of the sample. The detected isotope pattern in the non-reduced sample matches very well with the theoretical isotope pattern. The fragment ion coverage map provides fragment ion assignment based on fragment ions of the linked peptide detected in the MS/MS spectrum.

absence of this mass in the reduced sample. The precursor ion's isotope pattern detected in the non-reduced samples matches very well with the theoretical, simulated one. And lastly the fragment ion coverage map represents fragment ion assignment to both peptides involved, based on the MS/MS spectrum obtained from the linked peptide and providing a high level of confidence in the correct identification.

Conclusions

- Here we cover the three major workflows for characterization of biopharmaceuticals on one single instrument LC-MS platform, using single and mixed antibody samples: 1) intact mass analysis, 2) subunit analysis, and 3) peptide mapping – aiming at confirming antibody sequences and disulfide bonds, elucidating modifications, and probing for scrambled disulfide bonds.
- The example provided showcases the use of the three different modes included in the BioPharma Option: Standard Mode for peptide mapping analyses, Protein Mode for subunit and top-down analysis, and HMR Mode for intact mass analysis under denaturing and native conditions.
- The new HMR Mode now also allows for analysis of antibodies and antibody–drug conjugates under native conditions requiring a higher mass range up to *m/z* 8,000.
- Excellent mass accuracy, resolution, and sequence coverage are obtained for results at all stages of the workflows: intact molecular masses, masses of subunits, top-down fragments of subunits as well as peptides, providing very high confidence results.
- The SMART Digest kit provided an easy to use process with efficient and reproducible digestion of antibody samples in only 60 minutes.
- For infliximab, unusually low lysine truncation was observed and confirmed at all molecular levels: in the fully intact mAb, in the Fc/2 subunit, and at the peptide level.

Acknowledgements

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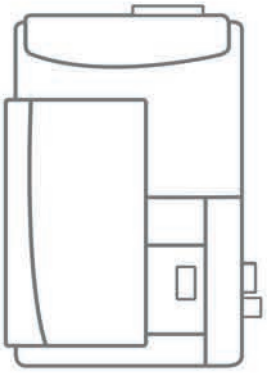

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MAINTAINING SENSITIVE, ROBUST AND RELIABLE PESTICIDE ANALYSIS WITH JETCLEAN SELF-CLEANING IONSOURCE

A more robust GC/MS IonSource for pesticide analysis.

Introduction

With over a thousand agrochemicals in use to ensure production of affordable and reliable food supply, pesticide surveillance is mandatory in almost every country. The ever-decreasing detection limits and need for faster, lower-priced analysis have meant preparation techniques such as QuEChERS are used routinely. These rapid and simple techniques can often leave significant matrix components in the sample. MS/MS techniques mean this doesn't limit the sensitivity. However, the robustness of a GC/MS IonSource can be impacted by the larger injection volumes and complex extracts.

Residue analysts have become more accustomed than most to a drop off in data quality and the routine IonSource cleaning or exchange symptomatic of these developments.

To address the cause of the problem rather than the symptom, Agilent Technologies developed the JetClean Self-Cleaning-IonSource. JetClean allows an accurately controlled microflow of hydrogen to be added to the source either during analysis, or post analysis. This hydrogen is ionized and the generated acidic protons act as a cleaning agent in the source, preventing the accumulation of non-volatile material on the surfaces. This has a huge benefit to mobility of ions generated, improving long-term response and peak shapes. When combined with column backflush, it can extend the period between source cleans by as much as a factor of 4, meaning that source cleaning becomes an annual preventative measure.

Experimental

An analysis of 1 µl organic honey extracts for 170 pesticides, prepared by QuEChERS, was performed on a 7010 GC/MS/MS using a midpoint column backflush (total column dimension 30 m x 0.25 mm id × 2.5 pg HP-5MS UI) operated in Online-Clean mode with hydrogen flowing constantly during analysis.

The chromatograms in Figure 1 show analytes at 2.5 pg concentrations across the retention range. The benefits of JetClean in response and peak shape are more obvious in the later, high boiling components.

JetClean reduced the average MDL from 0.0151 to 0.008 pg/µl when determined using the 1.25 pg/µl level sample. However, at higher concentrations the effect was not so dramatic.

The full application note can be found here: http://bit.ly/2r3IsoP

For more information on JetClean: www.agilent.com/chem/JetClean

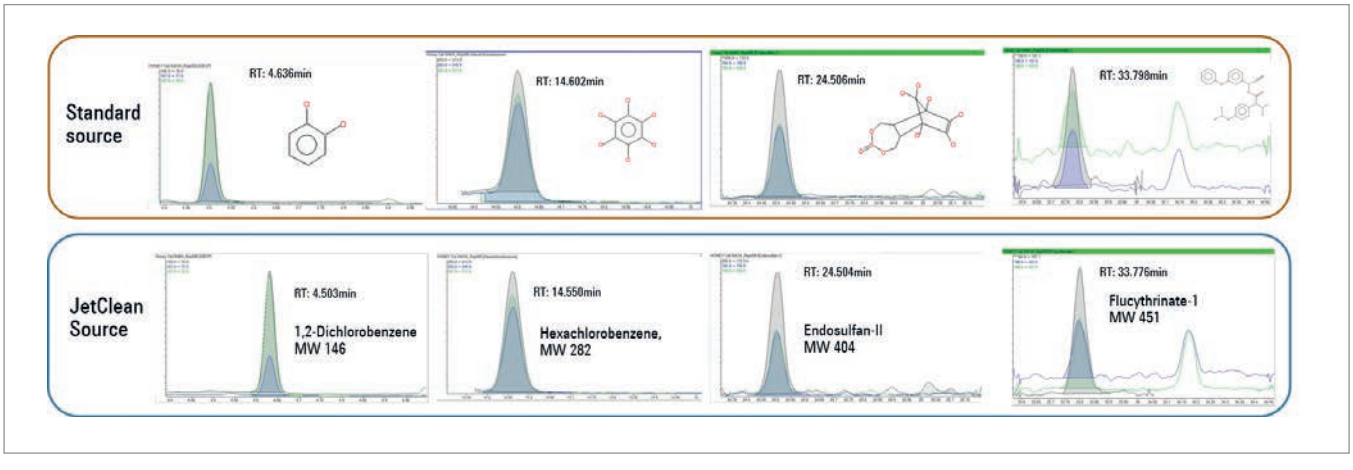

Figure 1. Chromatograms showing analytes at 2.5 pg concentrations across the retention range.

ACCELERATE AND ENHANCE BOTTOM-UP PROTEOMIC WORKFLOWS WITH PROTEUSQC™

Improve process control and peptide/protein quantification in biosamples with the ProteusQC™ standard mixture.

By Andrew J. Percy and Krista Backiel

Abstract

Much research within the field of proteomics has focused on the identification and quantification of candidate protein biomarkers using bottom-up LC-MS methodologies. Although significant progress has been made, bioanalytical development is often slowed by the absence of stable isotope-labeled standards for workflow suitability assessment and quantification data normalization. We describe herein the implementation and merits of a novel standard mixture (referred to as ProteusQC™) for bottom-up proteomic applications.

Scope and Application

To improve assay development time and data quality of qualitative and quantitative analysis in bottom-up (and perhaps middle-down) proteomics, a highly characterized mixture of differently labeled protein/peptides has been prepared and evaluated extensively (Figure 1A).

This stable isotope-labeled mix consists of a set of proteolytically stable, apolipoprotein A-1 (ApoA-1) standards whose combination affords unique analytical benefits. For straightforward use, an aliquot of the ProteusQC mixture (diluted as necessary) is spiked into a neat biosample(s) before bottom-up sample preparation and downstream LC-MS/MS sample processing. Data analysis is readily accomplished with vendor-neutral Skyline software (1), which is further supported by a number of adaptable external tools, such as SProCoP (2) (for QC performance evaluation) and QuaSAR (for curve-based quantification).

In principle, upon tryptic digestion and LC-MS/MS, ProteusQC produces 14 ApoA-1 peptides that are broadly dispersed over a wide chromatographic space and are well suited to serve as reference (or internal) standards for retention time indexing, digestion condition evaluation (Figure 1B), method and system suitability, as well as absolute/relative protein

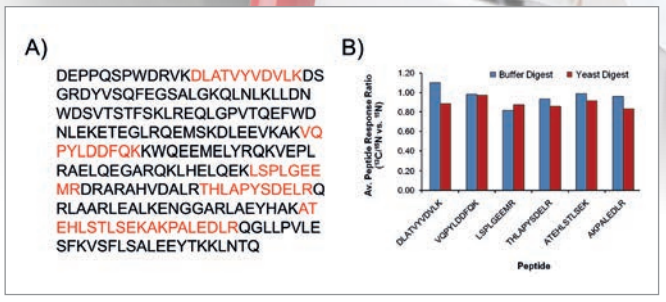

Figure 1. ProteusQC™ and an example of its product utility. A) The mixture consists of ^{15}N-labeled ApoA-1 and 6 ^{13}C/^{15}N-labeled ApoA-1 peptides (highlighted in red). B) Digestion efficiency check of a matrix-free and matrix-containing sample measured by bottom-up LC-PRM/MS.

quantification. Manuscripts are in preparation by MacCoss and colleagues (on development and utility) and Pennington and colleagues (on application to PsA and RA sera patient samples). Independent from this, quantification has been demonstrated with a variety of biosamples processed via targeted LC-MS/MS. In one example, ^{15}N-labeled ApoA-1 peptides from a ProteusQC digest enabled precise and accurate quantification of endogenous ApoA-1, as well as other target proteins, in high-density lipoprotein and mouse sera samples through a global peptide normalization strategy (see CIL application note #46). The use of a single protein as a global standard offers a number of practical (e.g., increases multiplexing) and economic (e.g., decreases cost) advantages, as demonstrated initially by Hoofnagle et al. (3), that are valuable for assay development and performance. In summary, the diverse merits of ProteusQC should help advance the application of bottom-up proteomics in basic research and clinical translation studies.

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DETECTION OF RESIDUAL PESTICIDES ON FRUITS AND VEGETABLES USING PORTABILITY™ MINIATURE MASS SPECTROMETER

Mass spectrometry can be now deployed for onsite pesticide screening in real time

By John MacAskill, Jean Yu and Lynn Chandler

Pesticides are used extensively in food production, ranging from pest mitigation in the field to inhibiting mold during storage and transportation. The growing reliance on pesticides and fungicides in turn requires monitoring and study to assess their environmental and toxicological impacts.

Mass spectrometry in conjunction with various separation techniques is the standard method for performing food and drug analysis. Techniques such as gas chromatography-mass spectrometry (GC-MS) or high performance liquid chromatography-mass spectrometry (HPLC-MS) provide unparalleled specificity and sensitivity, but require large, costly instruments with time-consuming sample preparation and controlled laboratory environments to function properly. Presented here is an application of thermal desorption electrospray ionization (TD-ESI) coupled to a portable mass spectrometer for pesticide screening.

TD-ESI is a mass spectrometry technique from the family of ambient ionization methods that utilizes very simple probing of different surfaces or liquids with no need for sample pretreatment. This feature, combined with the high selectivity of electrospray ionization, offers real-time, highly-sensitive measurements of pesticides with no pretreatment.

In TD-ESI a solid surface or a liquid sample is touched by a simple metallic probe. The probe and any adsorbed analyte is inserted into a custom sampling assembly. The probe is heated to thermally desorb analyte species that become entrained in a high temperature gas flow. This gas flows into the ionization region where desorbed analyte is ionized through charge transfer with ions generated from the electrospray.

Bayspec's Portability™ mass spectrometer is less than 10kg, highly-portable and battery operated. It features a linear ion trap with atmospheric pressure inlet (API). When TD-ESI is combined with Portability™, it is possible to perform fast, in situ detection of a wide variety of chemicals – including pesticides. The simple and fast sampling capability of the TD-ESI allows Portability™ to immediately analyze the surfaces of fresh produce without any sample pretreatment. Fast pesticide screening by Portability™ mass spectrometer can immediately disclose fake organic food products as well as discover elevated levels of residual pesticides.

TD-ESI-MS can screen for residual pesticides on the surfaces of different fruits and vegetables with short analysis times (1– 5 s). MS (as well as MS^n) analyses can be performed on surface-residual pesticides at ppm and sub-ppm levels. The miniaturized TD-ESI ion source is also capable of operating in positive and negative ion mode. Overall, BaySpec's miniaturized Portability™ combined with TD-ESI can find its wide applications for onsite detection of trace toxic chemicals.

Download the full application note: tas.txp.to/0517/BAYSPEC

Figure 1. Portability™ mass spectrometer (left) and TD-ESI sampling device (right).

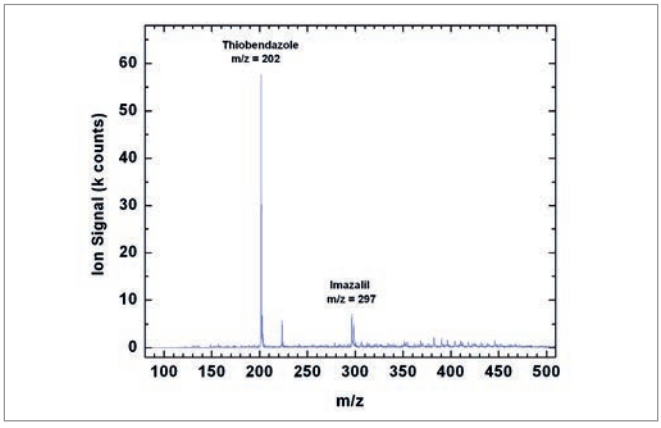

Figure 2. Full scan mass spectrum of an orange sampled using TD-ESI-MS. The fungicides thiobendazole and imazalil are clearly identified from the molecular ions at m/z = 202 and 297 respectively.

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