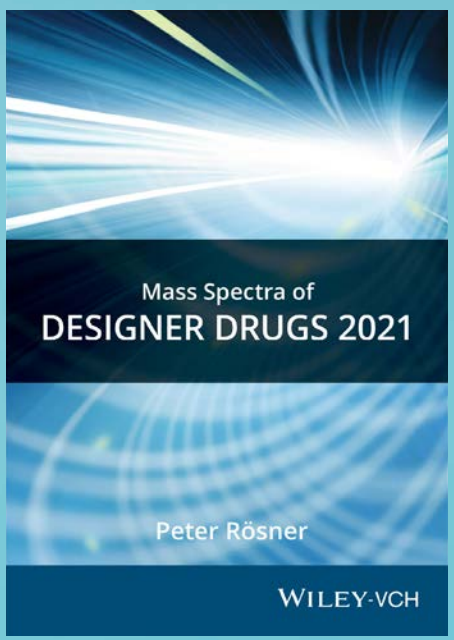
the Analytical Scientist

MASS SPECTROMETRY: SIMPLY THE BEST

As time advances, mass spectrometry applications become more impressive, more innovative, and more impactful. This supplement celebrates such applications, from the determination of carcinogens in plant material to monoclonal antibody characterization. One thing's for sure: the future of this wonderful technique is very bright!

Mass Spectra of Designer Drugs 2021

An Essential & Trusted Forensics Lab Resource



Whether you are researching designer drugs, pharmaceuticals, chemical warfare agent, or any related substances, you can trust this carefully curated data.

Key Features:

- ✓ Over **31,000** mass spectra of **23,879** unique compounds like fentanyls, synthetic cannabinoids, opioids, and many more
- ✓ Data from both legal and underground literature to ensure the most comprehensive and up-to-date mass spec resource
- ✓ Data carefully compiled in cooperation with regional crime labs and other partners worldwide

Wiley Science Solutions - The Leader in Spectral Data

Learn more at
https://sciencesolutions.wiley.com/DD21

WILEY

Contents

DETERMINATION OF PYRROLIZIDINE ALKALOIDS IN PLANT MATERIAL USING SFC-MS/MS

Presenting an SFC workflow for the quantitative analysis of tea samples

By Anja Grüning and Gesa J. Schad, Shimadzu Europa GmbH, Albert-Hahn-Str. 6-10, 47269 Duisburg, Germany, and Jan Stenzler, Shimadzu Deutschland GmbH, Keniastr. 38, 47269 Duisburg, Germany

Introduction

Pyrrolizidine alkaloids (PAs) are potentially carcinogenic plant metabolites. They occur mainly in plants of the Boraginaceae, Asteraceae and Fabaceae families. PAs can exist in two forms: a tertiary amine (free base) and an N-oxide form. Exposure to PAs in food, beverages or phytopharmaceuticals is a possible long-term concern for human health. The European Commission has laid down legislation (1) on the presence of PAs in foodstuffs based on a risk assessment conducted by EFSA (2).

As some analytes are isomers that can't be distinguished by different mass, they have to be separated chromatographically. While LC-MS/MS is the standard method for determination of PAs, separation of these compounds often poses a challenge. Supercritical fluid chromatography (SFC) offers complementary chromatographic selectivity to RP-LC and an advantage for separation of stereoisomers, as shown in the method for determination of 34 PAs including 5 Lycopsamin and 2 Senecionin isomers developed.

Sample Preparation

Tea samples were extracted twice with 0.05 M sulfuric acid by sonication. The pH of the combined extracts was adjusted with ammonium hydroxide before the samples were subjected to a solid-phase extraction (SPE) (3).

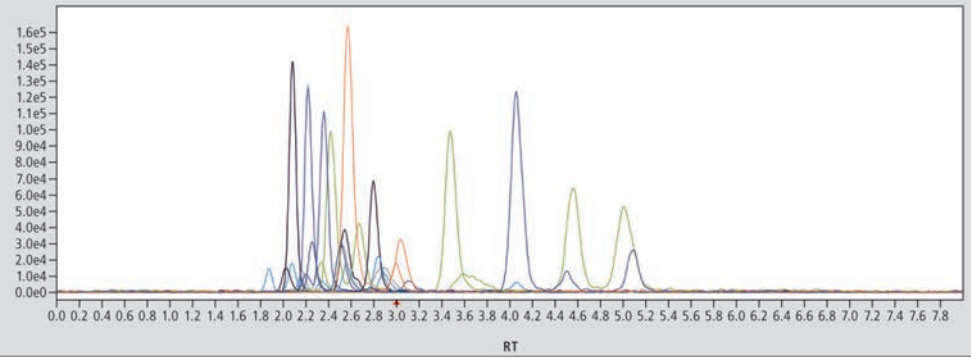


Figure 1: Typical chromatogram of the SFC-MS analysis of 34 pyrrolizidine alkaloids

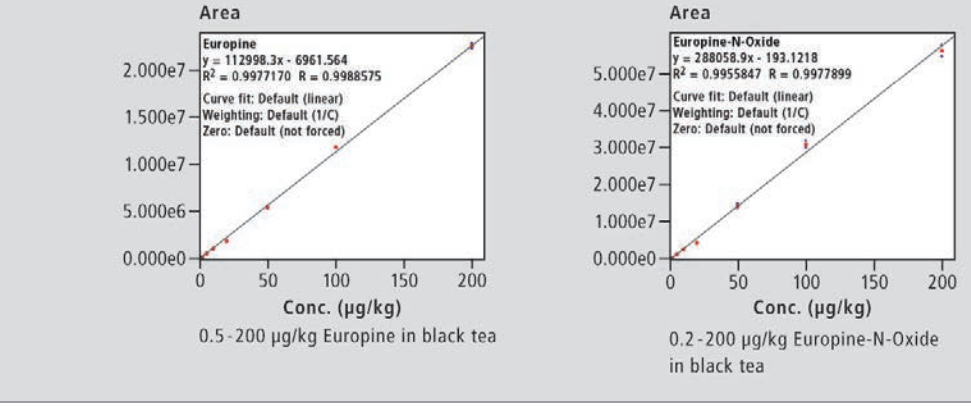


Figure 2: Exemplary calibration curves in black tea matrix

	Black tea LLOQ			Black tea LLOQ	
	ng/mL	µg/kg		ng/mL	µg/kg
1 Echimidine	0.050	0.20	18 Monocrotaline-N-Oxide	0.500	2.00
2 Echimidine-N-Oxide	0.250	1.00	19 Retrosine	0.500	2.00
3 Erucifoline	0.500	2.00	20 Retrosine-N-Oxide	0.500	2.00
4 Erucifoline-N-Oxide	0.125	0.50	21 Senecionine	0.125	0.50
5 Europine (HCl)	0.125	0.50	22 Senecionine-N-Oxide	0.125	0.50
6 Europine-N-Oxide	0.050	0.20	23 Senecyphilline	0.250	1.00
7 Heliotrine	0.050	0.20	24 Senecyphilline-N-Oxide	0.250	1.00
8 Heliotrine-N-Oxide	0.025	0.10	25 Senecivernine	0.250	1.00
9 Intermedine	0.125	0.50	26 Senecivernine-N-Oxide	0.125	0.50
10 Intermedine-N-Oxide	0.125	0.50	27 Senkirkine	0.050	0.20
11 Jacobine	0.250	1.00	28 Trichodesmine	0.125	0.50
12 Jacobine-N-Oxide	0.250	1.00	29 Indicine	0.125	0.50
13 Lasiocarpine	0.025	0.10	30 Indicine N-Oxide	0.125	0.50
14 Lasiocarpine-N-Oxide	0.125	0.50	31 Echinatin	0.125	0.50
15 Lycopsamine	0.125	0.50	32 Echinatin-N-Oxide	0.050	0.20
16 Lycopsamine-N-Oxide	0.125	0.50	33 Rinderin	0.025	0.10
17 Monocrotaline	0.500	2.00	34 Rinderin-N-Oxide	0.050	0.20

Table 1: LLOQs of the pyrrolizidine alkaloids in black tea matrix

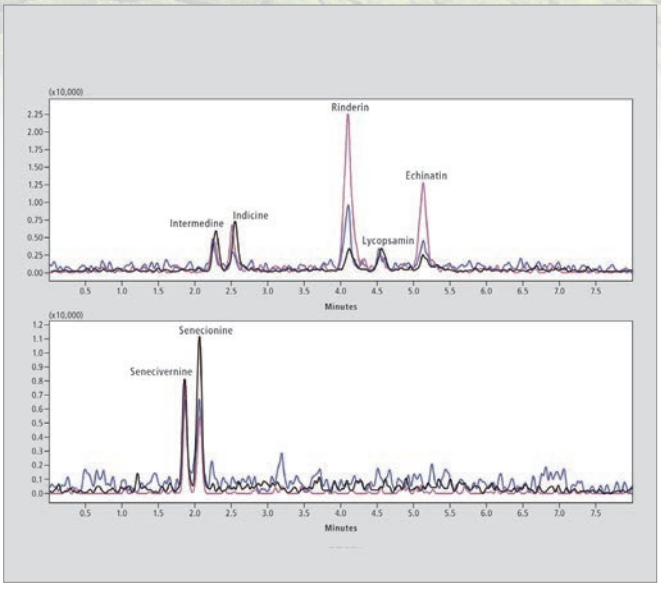


Figure 3: Chromatograms of the separated Lycopsamin and Senecionine isomers

SPE method
SPE cartridge : ISOLUTE® C18 (EC), 200 mg, 3 mL, Biotage

SFC method
Instrument : Nexera UC, Shimadzu
Column : CHIRALPAK®, IG-3, 0.3 x 10 cm, 3 µm SFC, Daicel
See details on mobile phase and time program in the online version of the application note.

MS conditions
Instrument : LCMS-8060, Shimadzu
See details on MS parameters in the online version of the application note.

Results
The main mobile phase used for SFC is supercritical carbon dioxide, to which polar organic solvents (modifiers) are added to control solubility and polarity. Ionic additives in aqueous or organic solution can also be used to adjust selectivity. Method scouting solution (Shimadzu Corp.) was used to determine the best combination of stationary and mobile phases and all parameters were optimized to achieve baseline separation of all isomers in a minimized overall analysis time (Figure 1).

Quantitative Analysis of tea samples
Quantification of 18 PAs and 16 of their related N-Oxides could be achieved. Calibration curves in black tea matrix showed good precision and accuracy. Even in complex matrices, we were able to easily quantify the PAs in the range of at least 2 to 200 µg/kg. For all analytes, weighted regression resulting in $r^2 > 0.99$ was obtained, with S/N > 10 for LLOQ levels.

Exemplary calibration curves are displayed in Figure 2. Figure 3 shows chromatograms of the separation of Lycopsamin and Senecionine isomers. Table 1 states LLOQs for the different PAs. A total of 10 commercially available tea samples were analyzed to demonstrate the benefits of this single-run method.

Conclusions
An SFC-MS/MS method applicable to food sample for high-sensitivity analysis of 34 PAs was developed, which achieves baseline separation of 5 Lycopsamin and 2 Senecionin stereoisomers in 8 min.

References

1. *COMMISSION REGULATION (EU) 2020/2040 of 11 December 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs*
2. *EFSA, Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. EFSA Journal 2017;15(7):4908, 2017: p. pp. 34.*
3. *Method description BfR-PA-Tee-2.0/2014; Determination of pyrrolizidine alkaloids (PA) in plant material using SPE-LC-MS/MS*

See the online app note: https://www.shimadzu.com/an/literature/lcms/ego119034.html

HOW TO IDENTIFY UNKNOWNS FROM LC/MS DATA

Using ACD/MS Structure ID to deconvolute data and assign structures to peaks

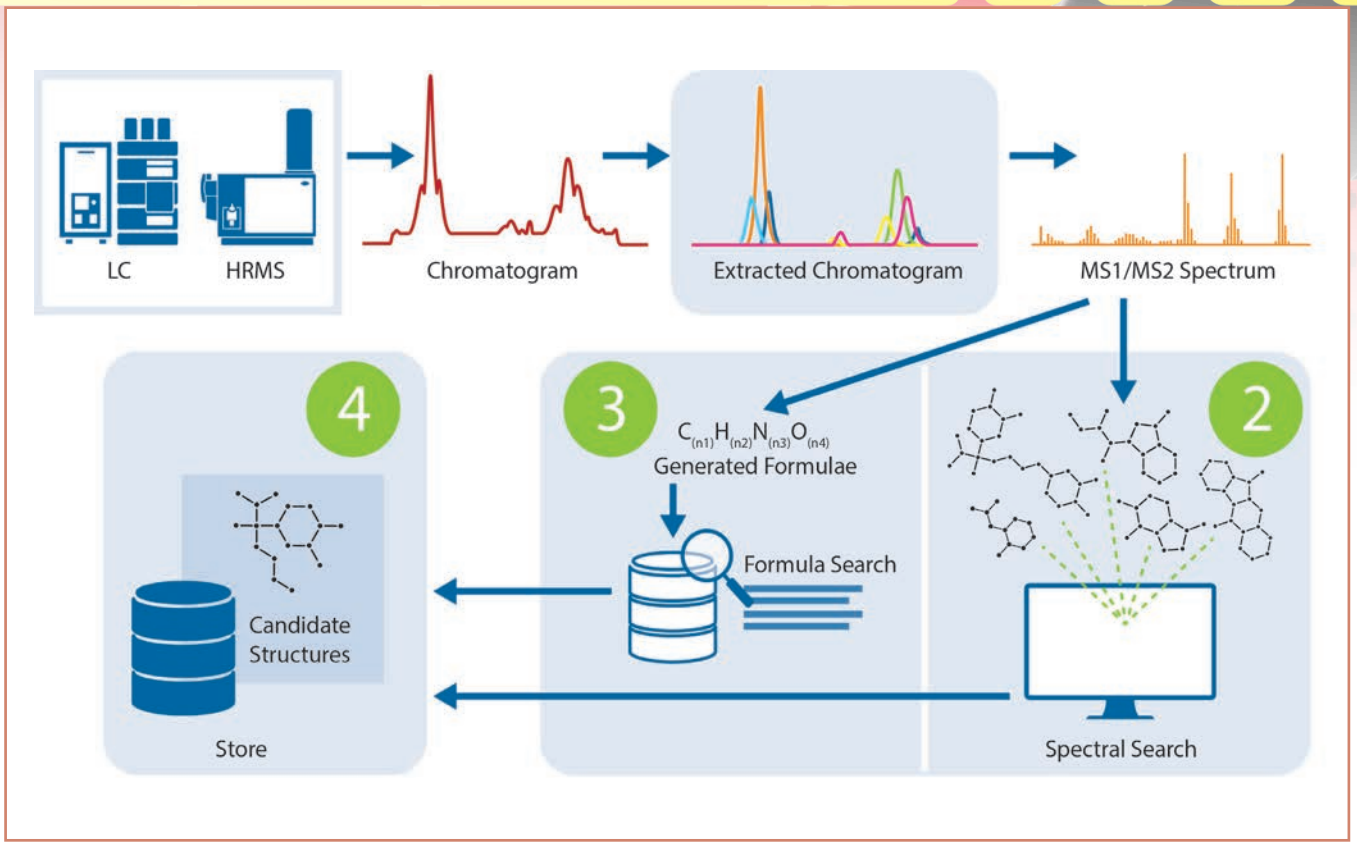
By Richard Lee, Joe DiMartino, and Brent G. Pautler

Combine analytical information from several sources (molecular ion mass, isotopic pattern, predicted fragmentation, and known inclusion/extraction criteria) to identify the most likely structural candidates from databases of several million compounds

Identification of unknowns is one of the biggest challenges in screening assays. High resolution and mass accuracy give greater precision for predicting elemental composition, but lack structural information. Even fragment ion analysis can only highlight certain functional groups. However, chemically intelligent MS software can use diverse analytical information, including predicted elemental formulae, fragment ions, and known fragment inclusion/exclusion criteria, to produce an accumulative filter and refine a mass-based library search down to a manageable number of structure candidates.

To illustrate, data for an unknown pharmaceutical sample analyzed by HPLC-FTMS (Thermo Fisher Scientific), is processed via MS Structure ID. PubChem and ChemSpider databases are used for the mass search.

Following extraction of chromatographic components, a peak with tR = 5.10 minutes and [M+H]+ = 272.2017 m/z is selected for identification. This component has MS2 data associated with its [M+H]+ peak, which includes the 272.2017 m/z parent mass. This is used as the source for elemental



composition determination, and to query the local PubChem and ChemSpider databases (~96 million compounds). Based on the accurate mass and resulting isotope pattern, the Formula Generator suggests the formula $C_{18}H_{25}NO$.

Searching the databases for the selected m/z value and elemental composition, with a tolerance of 2ppm, yields 5752 structures (after removing duplicate stereoisomers). Additional filtering is performed via an include/exclude structure fragment list. The candidate list is reduced to 22 structures once duplicate structures are removed.

MS Workbook Suite can further rank structures based on an "Assignment Score." The AutoAssignment tool generates in silico fragments from the parent structure, which are then scored based on alignment with the MS2 spectrum. After running AutoAssignment, 8 structures possess a Match (dMS) value of 0.75 or higher, with only 2 having values above 0.80. The top structure candidates can be examined more closely by reperforming AutoAssignment/Fragmentation analysis, increasing the number of generated fragments to find the best structural match.

This example demonstrates how to use MS Structure ID to quickly search a wide range of potential structures, curate a reasonable and relevant list of candidates, and identify the most likely structure for a chromatographic peak, all in one workflow.

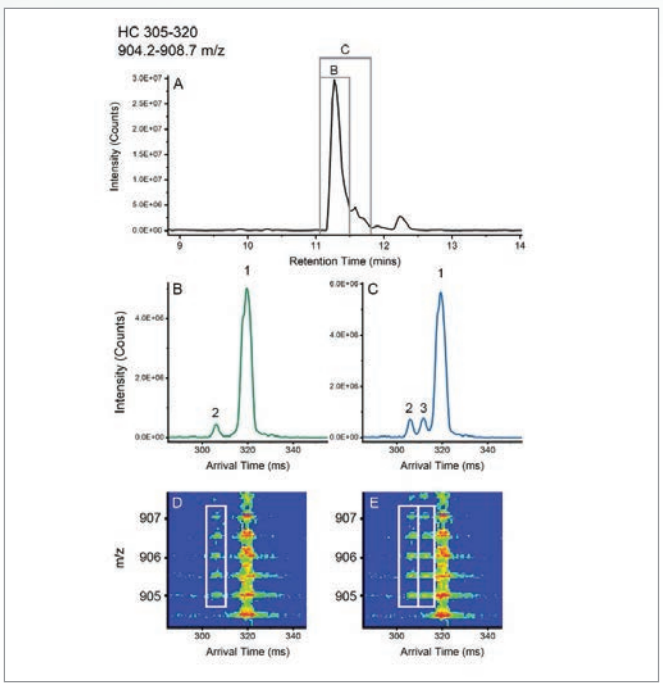
RAPID PEPTIDE MAPPING AND MONITORING OF POST-TRANSLATIONAL MODIFICATIONS

High-Resolution Ion Mobility Mass Spectrometry enables identification of co-eluting isomerization PTMs

By Jim Arndt, Andreas Krupke, Roxana McCloskey, Melissa Sherman

HRIM-MS enables >96.5 percent sequence coverage with identification of PTMs like deamidation and isomerization in under 20 minutes, while providing structural information not provided by LC-MS alone. Identifying mass-neutral PTMs and reducing the risk of under-reported or missing key PTMs.

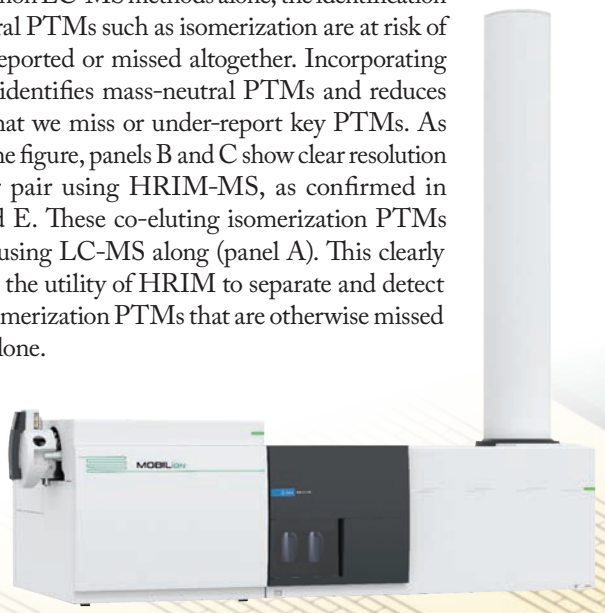
Peptide mapping by liquid chromatography-mass spectrometry (LC-MS) is the only assay that can provide quantitative information of post-translational modifications (PTMs) at the amino acid level. The level of detail comes at the expense of analysis time; peptide mapping using LC-MS can require shallow analytical gradients taking upwards of 90 minutes to resolve near-isobaric PTMs, such as asparagine deamidation, or isobaric PTMs such as aspartate isomerization. Even then, some deamidation and isomerization events can go undetected due to lack of chromatographic resolution.



Deamidation of N318 on HC 305-320 as observed by LC-HRIM-MS. A deamidation coelutes with the native peptide (panels B and D). A second deamidation is resolved from the native peptide (panels C and E). The deamidation is evident based on the 1 Da shift (0.5 m/z) shown in panels D and E.

The MOBIE High-Resolution Ion Mobility Mass Spectrometry (HRIM-MS) platform provides an exceptionally long 13-meter ion path providing superior ion mobility resolution and the ability to rapidly and reproducibly separate and identify isobaric PTMs. The key advantage of implementing HRIM-MS in the peptide mapping workflow is that lengthy chromatographic gradients are no longer required to resolve isobaric PTMs; these are resolved in the IM dimension instead, so analytical gradients can be shortened without loss of peak separation. Implementing HRIM into a rapid 20-minute peptide mapping workflow provides an additional dimension of separation, enabling resolution of isobaric species undetected by LC-MS alone in under 20 minutes, while achieving >96 percent sequence coverage.

Using common LC-MS methods alone, the identification of mass-neutral PTMs such as isomerization are at risk of being underreported or missed altogether. Incorporating HRIM-MS identifies mass-neutral PTMs and reduces the chance that we miss or under-report key PTMs. As indicated in the figure, panels B and C show clear resolution of an isomer pair using HRIM-MS, as confirmed in panels D and E. These co-eluting isomerization PTMs were missed using LC-MS along (panel A). This clearly demonstrates the utility of HRIM to separate and detect co-eluting isomerization PTMs that are otherwise missed by LC-MS alone.



Raising the bar with what's under the bench

Introducing the most advanced nitrogen gas generator for your LC-MS

Built upon decades of innovation in gas generation for the lab, Genius XE has been designed with superior performance and user experience in mind. With increasingly sensitive applications and productivity demands, the quality of instrument gas is more important than ever. This is why Genius XE features **Multi-Stage Purification™** and innovative ECO technology, defining **a new benchmark in gas quality and usability.**

www.peakscientific.com/genius

*Your local **gas generation** partner*

ACCURATE LC-MS BIOANALYTICAL QUANTIFICATION OF ITACONIC ACID USING THE AUTOMATED ANDREW+ PIPETTING ROBOT FOR OSTRO SAMPLE PREPARATION

Showcasing the utility of the Andrew+ Pipetting Robot in sample preparation for use in research

By Kathryn Brennan and Mary Trudeau

The following work demonstrates the capabilities of the Andrew+ Pipetting Robot, an automated liquid handling device, for sample preparation and extraction. The Andrew+ was used to autonomously prepare and extract itaconic acid from human plasma using the Ostro Protein Precipitation and Phospholipid Removal 96-well Plate, with subsequent LC-MS/MS analysis and quantification using the ACQUITY PREMIER System and Xevo TQ-S micro Mass Spectrometer.

Benefits

- Reduces user-to-user error with transferrable methods
- Uses "dominoes" that fit specific consumables which can be easily moved around the deck
- Easy-to-use OneLab Software for creating, transferring, and recording methods
- Automated liquid handling increases efficiency, allowing the user to perform other tasks
- Mitigates risk of manual error with liquid handling capabilities
- OneLab software incorporates "user actions" with image and video guidance, which allows users to have control between certain steps in a method, as well as tips for specific applications

Introduction

Sample preparation is a crucial step in an overall LC-MS bioanalysis workflow that is used to extract, purify, and concentrate samples from biomatrices prior to analysis. Common sample preparation techniques include sample dilution, protein precipitation (PPT), liquid-liquid extraction (LLE), and solid-phase extraction (SPE). A sample purification and cleanup step is often required in order to achieve optimal sensitivity, selectivity, and accurate quantification during LC-MS analysis.

PPT is one of the simplest forms of sample cleanup. It involves taking a biological sample, diluting it with a protein disrupting solution (usually comprised of organic solvent), and extracting the analyte of interest from the matrix into the solution. This workflow may seem simple; however, it is common for errors to occur during this step due to the need for manual intervention (1). Such errors can occur when the user is handling a pipette to transfer or dilute samples. The user can inadvertently pipette the wrong volume, dilute the wrong sample, or cause contamination by splashing during sample aspiration. Moreover, the need to reduce manual error is critical for the improvement of sample preparation workflows.

The Andrew+ Pipetting Robot, an automated liquid handling device, can effectively carry out pipetting and extraction steps for sample preparation workflows. Andrew+ runs on the cloud-native OneLab Software to create methods for liquid handling and extraction of biological samples, as well as to initiate flow through a vacuum apparatus, all on one deck. The easy-to-assemble dominoes can be placed in their appropriate spaces and connect via a magnet to ensure that nothing can be moved out of place during any steps in the method. The following work demonstrates how the Andrew+ autonomously prepared a standard curve and QCs, and carried out a simple pass-thru extraction method of itaconic acid from human plasma using the Waters Ostro PPT and Phospholipid Removal Plate for subsequent LC-MS analysis and quantification.

Results and Discussion

The Andrew+ prepared a calibration curve for itaconic acid in human plasma ranging from 0.5 to 100 ng/mL (N = 2), and QC samples at 0.75, 7.5, and 75 ng/mL (N = 4), respectively. More information on standard curve preparation extraction using the Ostro plate and LC-MS analysis can be found in Waters application note 720006683 (2). A recovery experiment

Figure 1. Andrew+ Pipetting Robot deck layout for sample preparation and extraction of itaconic acid using Ostro PPT and Phospholipid Removal 96-well Plates.

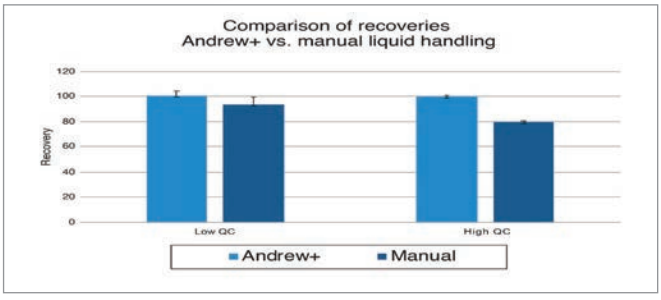


Figure 2. Recovery of itaconic acid from human plasma comparing Andrew+ vs. manual sample preparation and extraction with Ostro PPT and Phospholipid Removal 96-well Plates.

Intra-day accuracy and precision of QC samples (N = 4)			
	Concentration (ng/mL)	% Accuracy	%RSD
LQC	0.75	104.9	3.6
MQC	7.5	112.8	2.8
HQC	75	106.9	1.3

Table 1. Intra-day accuracy and precision of QC samples (N = 4) of itaconic acid in human plasma using Andrew+ sample preparation and extraction with Ostro PPT and Phospholipid Plates and LC-MS analysis.

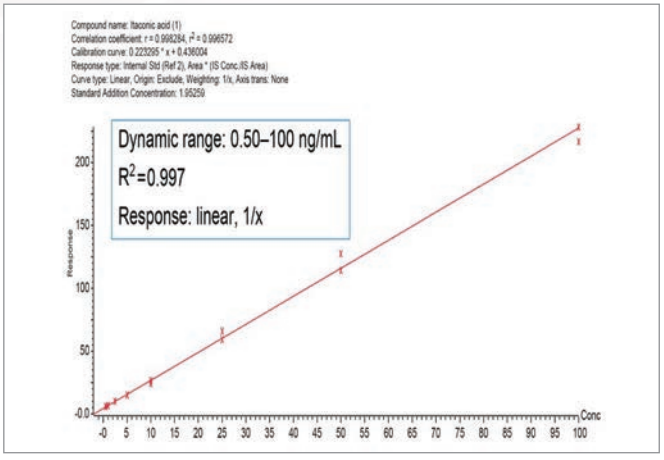


Figure 3. LC-MS quantitative performance of itaconic acid extracted from plasma with Ostro PPT and Phospholipid Removal Plates using the Andrew+ Pipetting Robot.

was conducted for the low and high QC concentrations by the Andrew+ and by hand to compare the accuracy and precision of the fully automated workflow to a manual workflow. The Andrew+ loaded plasma samples and protein disruption solvent onto the Ostro PPT Plate and aspirated to mix. The vacuum was then applied by the Andrew+, with a constant pressure of -5 psi for five minutes. A user action notification in the software instructed that the precipitated samples be manually removed and transferred to a nitrogen evaporator, blown down to dryness, and placed back on the Andrew+ for reconstitution. The full Andrew+ deck layout can be seen in Figure 1.

For analysis, the ACQUITY PREMIER System and Column was used to mitigate analyte metal chelation and ensure recovery of itaconic acid from the column and system. Percent recoveries for low and high QCs were better than manual recoveries at 100 percent and 99 percent, respectively. The comparison of recoveries for the automated workflow performed by the Andrew+ versus the manual workflow can be seen in Figure 2. Intra-day accuracy and precision of quality control samples with an N = 4 per concentration can be seen in Table 1. Accuracies were between 104.9 and 112.8 percent, with RSDs between 1.3 and 3.6 percent. Excellent quantitative performance was achieved with an R2 = 0.997 over a 200-fold concentration range, seen in Figure 3.

Conclusion

This work demonstrates successful automated sample preparation and PPT extraction using the Andrew+ Pipetting Robot with the Waters Ostro 96-well Plate. When compared to a manual workflow, the Andrew+ had better recoveries and improved RSDs, with great intra-day QC accuracy and precision, meeting recommended performance criteria for bioanalytical quantitation assays (3). The Andrew+ method demonstrated the ability to autonomously pipette and extract a full standard curve, and give excellent quantitative performance. Overall, the Andrew+ Pipetting Robot, and broadly applicable bioanalytical workflow, offers an easy-to-use automated liquid handling and extraction solution for Ostro PPT and phospholipid removal.

References

1. *Chapter 5 Automation Tools and Strategies for Bioanalysis, in Progress in Pharmaceutical and Biomedical Analysis, David A. Wells, Editor 2003, Elsevier. p. 135–197.*
2. *Brennan K, et al. Bioanalytical LC-MS Quantification of Itaconic Acid: A Potential Metabolic Biomarker of Inflammation. Waters Application Note, 720006683, 2019, p.1–7.*
3. *Bansal S, DeStefano A. Key Elements of Bioanalytical Method Validation for Small Molecules. The AAPS Journal 2007, 9 (1), E109–E114.*

MOBILE AIR QUALITY VOC MONITORING WITH PTR-TOF QB REAL-TIME ANALYZERS

Tracking air pollution where and when it matters, and contributing to public safety

By Lukas Maerk

IONICON real-time volatile organic compound (VOC) monitors are frequently deployed in mobile laboratories. In this case study: a mobile proton transfer reaction–time of flight–cube design (PTR-TOF QB) analyzer was deployed to map VOCs from a smoke plume caused by a landfill fire in Sweden.

Landfill fires occur frequently and toxic emissions from such fires can constitute a major public health concern. In the outskirts of Stockholm, a 100,000-ton landfill had been on fire since Christmas 2020 for a couple of weeks, with dense smoke spreading to nearby residential areas and more distant urban settlements. Stockholm's Environment and Health Administration observed high particle levels in the most severely impacted communities, but toxic organic gases are more difficult to measure, especially when the source is near and concentrations change rapidly within the moving plume.

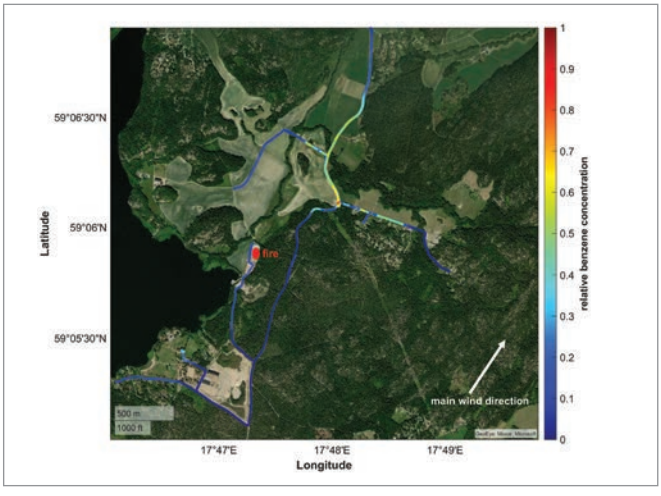
When being contacted by their Swedish colleagues, researchers from the University of Oslo (UiO) in Norway offered rapid help, overcoming closed borders due to COVID-19, and deploying their new mobile PTR-MS laboratory to Sweden. On two consecutive days, the IONICON PTR-TOF QB real-time VOC analyzer aboard the hybrid SUV mapped air pollution levels in the immediate vicinity of the fire and in the nearby villages, thereby determining what type of toxic organic gases are predominantly released from the landfill fire and what concentration levels the fire response team and the population are exposed to.

Results of PTR-TOF QB mobile lab smoke plume monitoring

The measurements revealed high concentrations of benzene and styrene, as well as of other substituted aromatic and polyaromatic compounds. Other hazardous air pollutants detected in the landfill fire plume include formaldehyde and organic cyanides. The mobile PTR-MS laboratory, supported by IONICON Analytik, Austria, turned out to be a key element in the response to a major environmental emergency in Sweden.

IONICON provided a small and lightweight version of the PTR-TOF 1000 real-time trace VOC monitor for this important, time-critical measurement campaign. The PTR-TOF QB allows for a straightforward integration for urgent deployment into makeshift mobile labs such as regular passenger cars. Of course, the analyzer can also be integrated in existing 19" racks of professional air quality monitoring vans. The PTR-TOF QB VOC monitor has an overall weight of <90 kg and can be collapsed into two parts, where each cube weighs <50 kg. These aspects enable the monitor to be easily transported and installed in almost any place offering huge flexibility with respect to the height profile or footprint of the system.

University of Oslo's mobile PTR-TOF lab chasing toxic organic gases emitted by a landfill fire near Stockholm. Image credit: M. Norman, SLB-analys.

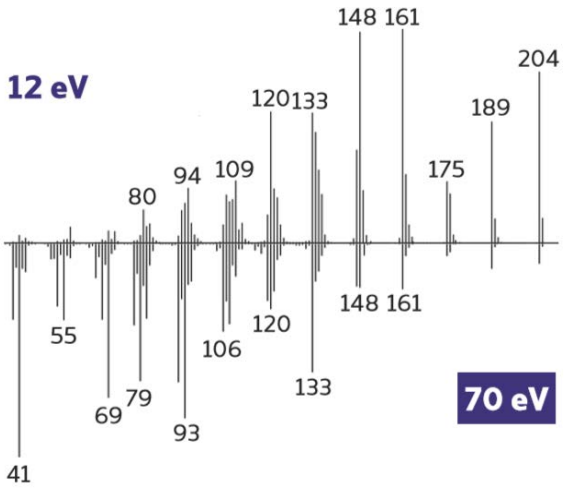


Real-time PTR-TOF data of increased benzene concentrations, downwind of the burning garbage site. Image credit: University of Oslo.

SepSolve Analytical

NEW

TOF MS for GC and GC×GC



BenchTOF2™ **with Tandem Ionisation®**

Powered with ChromSpace® software

ChromSpace

- Enhanced sensitivity
- Superior spectral quality
- Improved selectivity
- Extended dynamic range
- Hydrogen carrier capability
- Both hard- and soft- EI

Discover more

chem.sepsolve.com/TAS/BenchTOF2

MONOCLONAL ANTIBODY CHARACTERIZATION BY SEMI PREPARATIVE FCR-BASED AFFINITY CHROMATOGRAPHY AND HILIC-MS

The TSKgel® FcR-IIIA columns separate monoclonal antibodies into 3 subsets of affinity to the FcγRIIIA ligand: low, medium and high affinity. These correlate with different mAb glycoforms and their ADCC activity. Presented in this note is a workflow consisting of the combination of a TSKgel FcR-IIIA-5PW semi-preparative column and HILIC-MS, allowing for the rapid screening and in-depth characterization of upstream and downstream mAb products.

Monoclonal antibodies (mAbs) are an important class of therapeutics with the immense capacity to treat multiple diseases. Due to the complex nature and glycan heterogeneity of these products, characterization and strict control of their critical quality attributes is necessary to maintain product quality and efficacy. The mAb glycans linked to the Asn-297 glycosylation site on the Fc region impact biologic activities such as antibody-dependent cellular cytotoxicity (ADCC) and stability.

The TSKgel FcR-IIIA columns separate monoclonal antibodies into 3 subsets of affinity to the FcγRIIIA ligand: low, medium and high affinity. These correlate with different mAb glycoforms and their ADCC activity. To quantitate and elucidate the glycan profile of the different glycoforms separated by FcR-IIIA affinity, fractions can be analyzed by releasing and labeling the glycans before analysis on hydrophilic liquid interaction chromatography (HILIC) followed by mass spectrometry (MS).

TSKgel FcR-IIIA-5PW is a semi-preparative affinity column which immobilizes the recombinant FcγRIIIA ligand bonded to porous 10 µm polymethacrylate particles which can load up to 5 mg of mAb. It differs from the analytical column (TSKgel FcR-IIIA-NPR), which is based on non-porous material and is typically loaded with ≤50 µg of mAb. Therefore, the presented workflow benefits from the use of the semi-preparative TSKgel FcR-IIIA-5PW column as more sample can be collected at once (Figure 1).

The added utility of this semi-preparative column allows for material collection in sufficient quantity for in-depth analysis of mAb glycoforms via enzymatic glycan release followed by HILIC-MS.

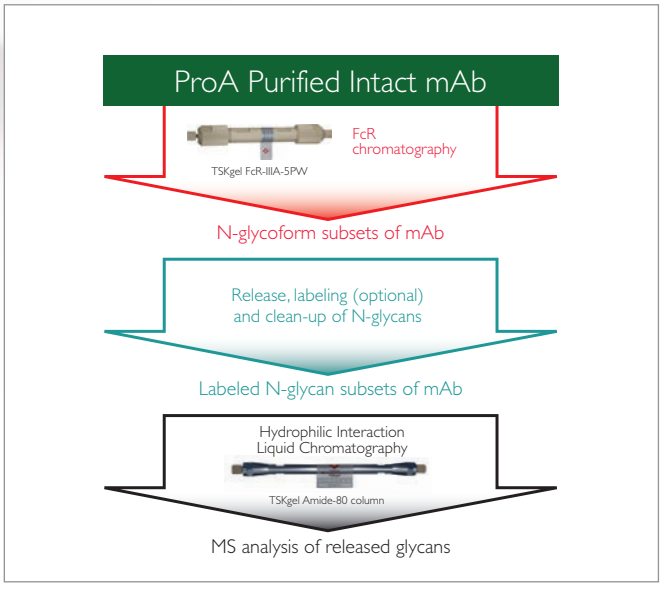


Figure 1. Novel workflow for analysis of released glycans

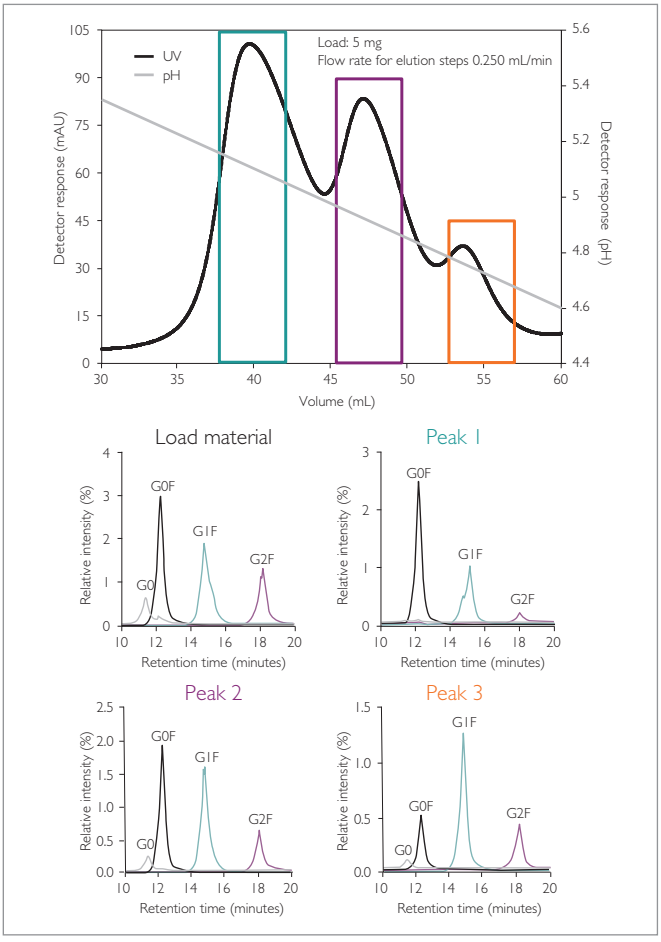


Figure 2. Elution profile of Herceptin biosimilar (upper figure) on TSKgel FcR-IIIA-5PW and relative intensities from HILIC-MS analysis of released glycans from FcR fractions (lower figures)

Materials and Methods

TSKgel FcR-IIIA Conditions

Column:	TSKgel FcR-IIIA-5PW, 10 µm, 7.8 mm ID × 7.5 cm
Mobile phase:	A: 50 mmol/L citrate/NaOH, pH 6.0
	B: 50 mmol/L citrate/NaOH, pH 4.0
Method:	Equilibrate: 5 CV MP A
	Wash: 4 CV 25% MP B
	Elution: linear gradient 25-90% B over 14 CV
	Hold 4 CV at 90% B and 100% B
Flow rate:	Equilibration, load, and wash steps: 0.5 mL/min
	Elution and hold steps: 0.25 mL/min
Instrument:	ÄKTA™ avant 25 FPLC
Detection:	UV @ 280 nm
Temperature:	ambient
Sample:	5 mg protein A-purified trastuzumab (Herceptin® biosimilar)

HILIC-MS Conditions

Column:	TSKgel Amide-80, 2 µm, 2.1 mm ID × 15 cm
Mobile phase:	A: 50 mmol/L ammonium formate, pH 4.4
	B: 100% acetonitrile
Gradient:	From 65-58% B in 35 min
Flow rate:	0.2 mL/min
Instrument:	Shimadzu Nexera® XR UHPLC
Detection:	Fluorescence: Ex 265 nm, Em 425 nm
	MS: SCIEX X500B Q-TOF, ESI positive, m/z 200-3500
Temperature:	50 °C
Sample:	5 µL for load sample and 10 µL from collected FcR-column elution peaks

MS Conditions:

Source gas 1	60 psi	Spray voltage	5000 V
Source gas 2	60 psi	Declustering potential	20 + 0V
Curtain gas	45 psi	Collision energy	7 + 0V
CAD gas	7 psi	Source temperature	450 °C
Accumulation time	0.5 sec	Time bins to sum	4

Results

Figure 2 illustrates protein A-purified trastuzumab analyzed on the TSKgel FcR-IIIA-5PW semi-preparative column. This peak profile is comparable to the analytical TSKgel FcR-IIIA-NPR (not shown), showing low affinity first, then mid and high affinity as pH decreases. Glycans were released and labeled from the collected peaks 1, 2, and 3 and injected onto a TSKgel Amide-80 HILIC column connected to MS for quantitative glycan analysis.

As demonstrated in Figure 3, use of the TSKgel Amide-80 column with mass spectrometry confirms that mAb glycoforms with the highest affinity to FcγRIIIA-ligand (peak 3) also contain the highest amount of galactose in their N-glycan structure (G1F and G2F glycan notations). Peak 2 shows a higher level of G1F relative to peak 1, and peak 1 contains a greater abundance of fucosylated glycans without terminal galactose (GOF).

Glycan	Structure	Load Material (%)
G2		0.00 ± 0.00
G2F		2.06 ± 0.01
G1		0.16 ± 0.01
G1F		26.76 ± 0.29
G0		1.15 ± 0.03
G0F		69.87 ± 0.34

N-acetylglucosamine (GlcNAc)
Fucose
Mannose
Galactose

Glycan	Peak 1 (%)
G2	0.00 ± 0.00
G2F	0.00 ± 0.00
G1	0.00 ± 0.00
G1F	7.60 ± 1.12
G0	0.00 ± 0.00
G0F	92.40 ± 1.12

Glycan	Peak 2 (%)
G2	0.00 ± 0.00
G2F	2.89 ± 0.11
G1	0.00 ± 0.00
G1F	40.56 ± 0.37
G0	2.05 ± 0.48
G0F	54.51 ± 0.27

Glycan	Peak 3 (%)
G2	0.00 ± 0.00
G2F	5.47 ± 1.10
G1	0.00 ± 0.00
G1F	71.69 ± 1.16
G0	2.29 ± 0.40
G0F	20.55 ± 0.44

Figure 3. HILIC-MS: Relative abundance of 6 different N-glycans within the 3 peaks from fractions collected by TSKgel FcR-IIIA-5PW

Conclusions

This two-step workflow, consisting of the combination of semi-preparative TSKgel FcR-IIIA affinity chromatography and HILIC separation, allows for the rapid screening of upstream and downstream mAb products. Utilizing HILIC-MS to confirm the presence and relative quantity of N-glycans in different mAb glycoforms permits in-depth characterization of mAbs. This type of analysis can be conducted on almost any mass spectrometer instrument, therefore bypassing the need for high-resolution equipment. The added utility to use the same sample material for orthogonal chromatography methods is a novel benefit for drug development and quality control. Additional advantages to this workflow include the ability to monitor FcγRIIIA affinity and relative ADCC activity without the need for a costly, labor-intensive and time-consuming bioassay.

HIGH RESOLUTION MULTI-REFLECTING TIME-OF-FLIGHT MASS ANALYZER WITH FOLDED FLIGHT PATH®

Celebrating ten years of high resolution

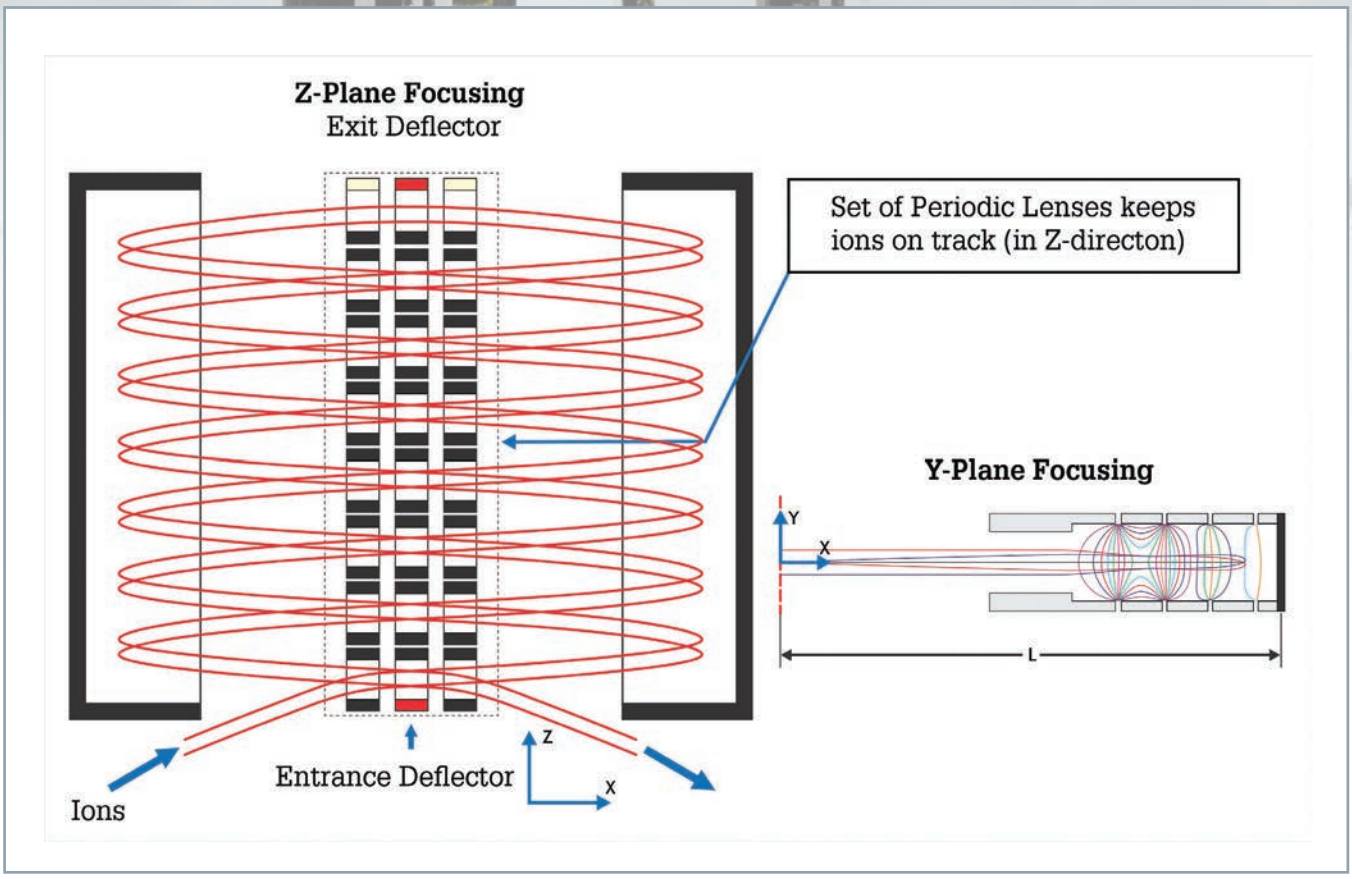
By Viatcheslav Artaev

LECO's Folded Flight Path® (FFP®) technology revolutionized high-resolution Time-of-Flight Mass Spectrometry. Using planar gridless electrostatic mirrors and an array of Einzel focusing electrostatic lenses, the FFP can create a flight path of 40m in a sensible footprint, enabling resolving powers beyond 50,000.

The first Time-of-Flight (ToF) mass spectrometer was proposed in 1946, and the design has been refined and iterated upon ever since. With an unlimited mass range visible in each spectrum and a high spectral generation speed, the advantages of ToF MS were immediately clear. Though the instruments improved, they eventually hit physical limitations that restricted the resolving power.

In 1973, ToF analyzers took a huge step forward with the introduction of the reflectron, an electrostatic ion mirror. Reflectrons provided time-energy focusing and improved the resolving power from several hundred to several thousand or more. To increase resolving power beyond 15,000, the height of the analyzer's flight tubes had to increase significantly.

The next major iteration in ToF resolution came with multi-reflecting reflectrons. Ion mirrors can bounce the ions multiple times through grids defining electrical fields before they arrive at the detector, increasing the flight time significantly. However, these grids also cause significant ion losses, limiting their usefulness.



In 1989, a planar gridless electrostatic mirror was proposed. Without the grids, ion loss from the reflections was minimized. Unfortunately, this design could not mitigate the ion trajectories' divergence in the Z-direction, and there were still significant losses, resulting in a lack of practical improvement.

In 2011, LECO engineers took that idea and worked in an array of Einzel focusing electrostatic lenses. This allowed for both Y-direction and Z-direction focusing, minimizing ion losses. With this Folded Flight Path (FFP®) design, resolving power took a huge leap forward, easily reaching 25,000 within a sensible footprint. An additional reflection within the *FFP* sent the ions through the array in another pass, doubling resolving power to 50,000.

This LECO technology is unique in being capable of generating full mass range, high-resolution mass spectra with fast acquisition speeds that enable true GCxGC separations to be performed. This high level of GCxGC-HR-TOF-MS performance is vital for successful non-target-screening (NTS) applications.

With each new development of ToF MS, the advantages of this technology continue to grow. The *FFP* was the next step forward, bringing even more clarity and resolution to seeing what is in your samples.

ESTABLISHMENT OF A ROBUST MONOLCONAL ANTIBODY SUBUNIT PRODUCT QUALITY ATTRIBUTE MONITORING METHOD SUITABLE FOR DEVELOPMENT, PROCESS MONITORING, AND QC RELEASE

By Samantha Ippoliti, Ying Qing Yu, Nilini Ranbaduge, Weibin Chen

This application note presents a subunit multi-attribute method (MAM) method as a complement to peptide MAM approaches in biopharmaceutical development. Typical peptide mapping MAM methods provide a more targeted capability for attribute-based analysis but are challenged by laborious sample preparation, longer run times (lower throughput), and more complex data analysis. A subunit-based MAM approach addresses each of these challenges, while providing an opportunity to monitor a wide range of biotherapeutic product attributes.

Biopharmaceutical developers of both innovators and biosimilars face increased competitive pressures to be faster to the market, lower their costs, and keep their reputations for quality high. It is important to characterize and monitor critical quality attributes (CQAs) throughout the drug development process, and increasingly extend these assays for process monitoring and lot release. Therefore, the analytical methods used for CQA monitoring must be as robust, sensitive, and as fast as possible.

LC-MS peptide mapping MAM gained popularity due to the wealth of information that it provides compared to traditional optical-only detection methods. However, many labs using this approach find that sample preparation can introduce method-induced artifacts and suffer from irreproducibility. In addition, the data acquisition is usually lengthy, limiting throughput, resulting in data sets that can be quite complex. In recent years, biotherapeutic developers turned to mAb subunit MAM analysis to produce critical information more quickly and robustly. For example, Dong et. al established a method for automated purification, subunit digestion, and LC-MS analysis of mAbs for cell culture process monitoring (1). Through this workflow, they were able to monitor glycosylation profiles and nonezymatic lysine glycation in near real time and make adjustments to the cell culture process. A similar method was used by Sokolowska et al, in which Fc subunit methionine oxidation was found to be a critical product attribute and was monitored following photo and chemical stress studies (2). This GMP compliant Xevo QTof LC-MS method has been validated for use in QC for commercial product release and stability studies (3).

In this study, we demonstrate the implementation of a subunit MAM method on two additional ToF-based MS systems (BioAccord and Vion) to monitor antibody glycosylation, glycation, oxidation, and sequence variants. This method demonstrates that subunit based analysis is a core capability of ToF-based LC-MS platforms and when deployed on a compliant-ready informatics platform, such as UNIFI/waters_connect, can be utilized to support mAb development, in manufacturing, and quality functions within a biopharmaceutical organization.

Experimental

Sample Description

50 µg antibody sample was incubated with 50 units of Fabricator (IdeS) enzyme (Genovis) in digestion buffer (25 mM NaCl, 25 mM Tris, 1 mM EDTA, pH 8.0), at a final concentration of 1 mg/mL, for 1 hour at 37 °C. DTT (dithiothreitol) was then added to a final mAb concentration of 5 mM for a partial reduction of inter-chain disulfides by incubation for 30 min at 37 °C. For the deglycosylated samples, 50 µg antibody sample was incubated with PNGaseF (for 50 µg sample, 4 µL PNGaseF from the RapiFluor-MS Kit was used and scaled up as needed [4] - all samples were diluted to 0.1 mg/mL with 0.1% formic acid in water prior to analysis) at 37 °C, at final mAb concentration of 1 mg/mL, prior to the IdeS digestion and reduction steps.

Method Conditions

LC Conditions

LC system:	ACQUITY UPLC I-Class
Detection:	ACQUITY UPLC TUV
Vials:	QuanRecovery with MaxPeak HPS 12 x 32 mm Screw Neck Vial, 300 µL (P/N: 186009186)
Column(s):	Waters ACQUITY BEH C4 300 Å, 1.7 µm, 2.1 x 50 mm (P/N:186004495)
Column temp.:	80 °C
Sample temp.:	6 °C
Injection:	0.5 µg IdeS-digested mAb (5 µL injection of 0.1 mg/mL sample)
Flow rate:	0.25 mL/min
Mobile phase A:	0.1% Formic acid in water
Mobile phase B:	0.1% Formic acid in acetonitrile

Gradient

Time (min)	Flow (mL/min)	%A	%B	Curve
0.0	0.25	80	20	6
10.0	0.25	60	40	6
10.3	0.25	20	80	6
11.3	0.25	20	80	6
11.6	0.25	80	20	6
15.0	0.25	80	20	6

MS Conditions

MS System	ACQUITY RDa	Vion IMS QTof
Ionization mode	Positive	Positive
Acquisition range	400-7000 *m/z* (High Mass)	500-4000 *m/z*
Capillary voltage	1.5 kV	2.75 kV
Cone voltage	50 V	70 V
Desolvation temp.	550 °C	600 °C
Source temp.	N/A	125 °C

Data Management

Data was acquired, processed, and reports generated using the UNIFI v1.9.4 Intact Protein Analysis workflow.

Results and Discussion

MAMs show great promise to provide direct and selective product attribute analysis for biopharmaceutical drug candidates. Here we demonstrate a fast and efficient subunit MAM method, using IdeS enzyme to cleave the mAb at the hinge region followed by reduction of inter-chain disulfide bonds to generate free light chain (LC), Fd, and Fc subunits (see Figure 1) of ~25 kD mass. The free subunits were subjected to reverse phase (RP) chromatography in a 15-minute LC method and the resulting resolved peaks were analyzed via MS for qualitative and relative quantitative analysis.

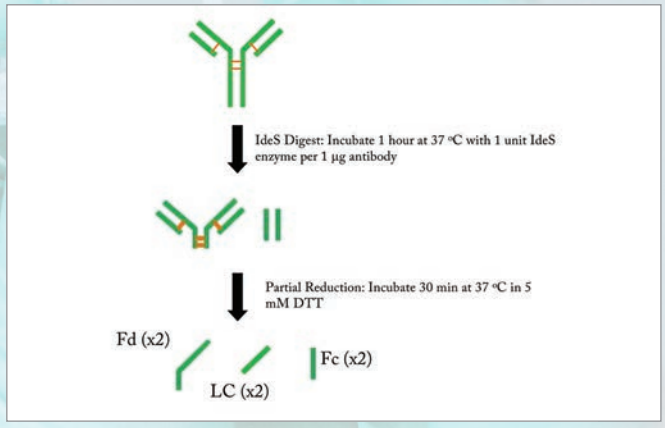


Figure 1. Sample preparation for mAb Subunit MAM, including IdeS digestion and partial reduction.

MAM methods destined for process monitoring, QC release testing, and other studies conducted within regulated labs must be robust and reproducible (5). To assess the robustness of subunit attribute-based analysis on the BioAccord System, triplicate injections of an IdeS-digested trastuzumab sample were analyzed on two systems with the same set of three columns evaluated on each system. Acquired mass spectra for each of the three subunit peaks (Fd, Fc, and LC) were automatically deconvoluted using MaxEnt1 and the resulting masses were matched to trastuzumab species during automated UNIFI data processing (using a 10 ppm mass accuracy threshold). Relative percentages of glycosylated and glycated species were calculated via integrated MS response of the deconvoluted mass peak. LC and Fd glycation were measured at 1.6% and 1.2%, respectively, with less than 8% RSD evident over all injections (n=18). Fc N-glycosylated species ranged from 0.1 to 40% abundance (as shown in Figure 2) and all Fc glycosylation species over 0.5% relative abundance had an abundance variation less than 5% RSD across all injections. This assessment is well within typical expectations for assays supporting CQA monitoring during bioprocessing or product release. After demonstrating the primary robustness of this methodology, it was applied to three case studies similar to analyses typically performed to support developability, clone selection, and formulations.

The first study relates most closely to questions of developability that might be asked during clone screening and those questions typical for cell culture process monitoring. We applied this subunit method to glycoprofiling of cetuximab, an antibody with an N-glycosylation site in the Fd region of the heavy chain, in addition to the typical IgG1 Fc N-glycosylation sites on the heavy chains (Figure 3). Traditionally, N-glycans are profiled by released glycan assays such as 2AB labelling using HILIC-FLR analytical system or HILIC-FLR-MS using MS enhancing tag, such as RapiFluor-MS (4). However, if a released glycan assay is

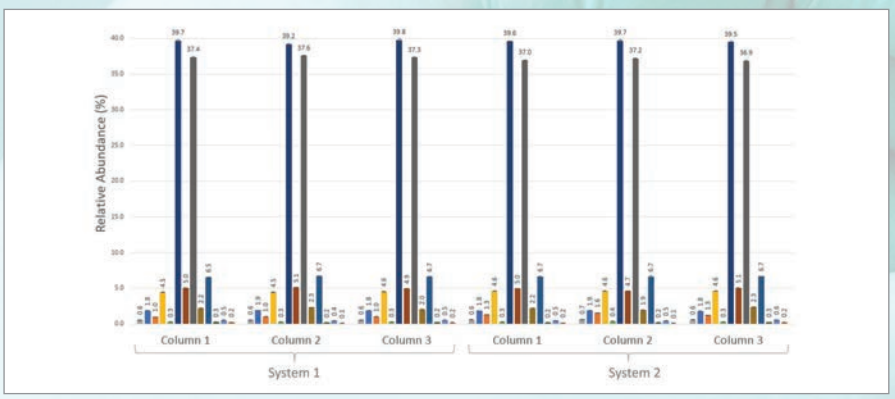


Figure 2. Trastuzumab Fc N-glycosylation species analyzed via subunit MAM method on two BioAccord systems, three columns each, triplicate injections. For all species >0.5% relative abundance, %RSD is <5%.

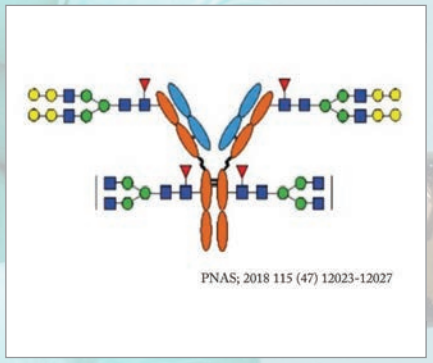


Figure 3. Depiction of cetuximab showing the N-glycosylation sites in the Fd in addition to the typical sites in Fc.

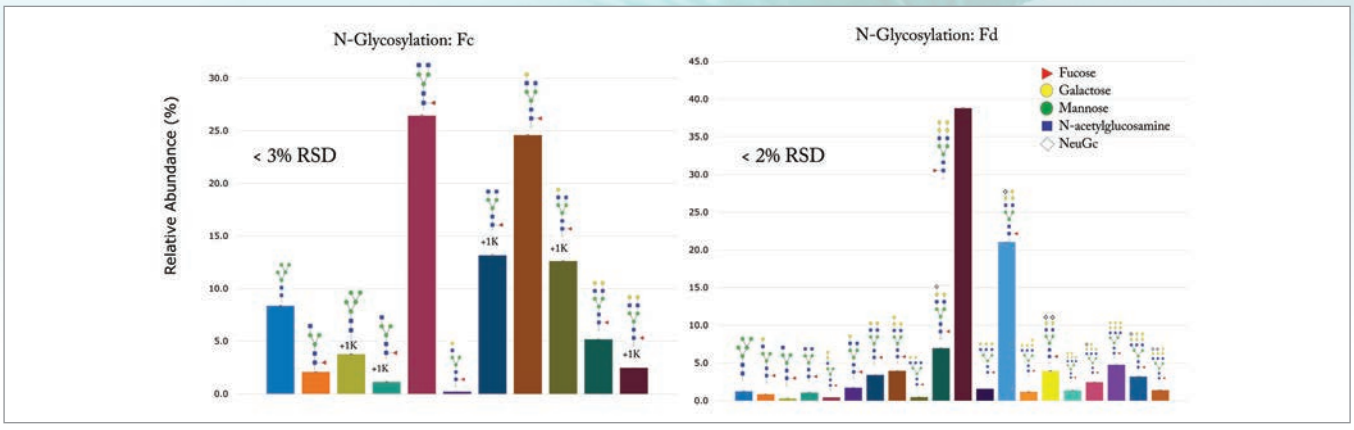


Figure 4. Cetuximab Fc and Fd N-glycosylation species.

used in the case of cetuximab, one would obtain the global picture of all N-glycans present but not specific to which site they occupy. This information is important because Fd and Fc glycosylation have differing effects on antigen recognition, immunogenicity, and serum half-life (6). The IdeS-digested cetuximab sample was analyzed in triplicate and the results for Fc and Fd are displayed in Figure 4. The observed Fd N-glycosylated species (right) are more complex branched structures than the typical Fc N-glycosylation profiles (left). The calculated %RSD for relative abundance of all Fc and Fd glycosylated species was less than 3% in these analyses. These observations are consistent with previously published findings, in which an IdeS subunit digest of cetuximab was separated, fraction collected, and a released N-glycan assay was performed for isolated Fc and Fd separately (7). With the use of a subunit MAM fractionation of the Fd and Fc is not necessary to localize the glycoforms associated with each domain. An additional advantage of using this subunit MAM method is that the Fc C-terminal lysine variant and other CQAs can be monitored simultaneously within the same analysis.

The second case study demonstrates how a subunit MAM approach could be applied to clone selection or process development to monitor product sequence variants. These can occur during production as a result of misincorporation of amino acids due to sequence mutation or suboptimal cell culture conditions (8). To mimic a typical analysis, we used a sample of trastuzumab containing 3 known point mutations – one in the light chain (V104L), resulting in +14 Da mass shift and two in the Fc (E359D and M361L), resulting in a combined -32 Da mass shift. This sample was spiked into originator trastuzumab at levels between 0.5%–50% and analyzed for accuracy in quantitation and linearity. For this study, the samples were deglycosylated prior to IdeS digestion to simplify data analysis of the spiked Fc sequence variants on a BioAccord System. Figure 5A shows a representative component plot with masses for LC, Fd, and Fc subunits conformed within the 10 ppm tolerance and expected lower-level sequence variants for LC and Fc detected with the same criteria. Relative percentages agreed with the expected spiked value and results were linear (R2 = 0.9994) over the 1–50% range (Figure 5B).

The final example is a forced oxidation experiment that mimics efforts common to formulations and product stability studies. Oxidation that occurs during storage of a final drug product can affect its efficacy and therefore impact its shelf life. A control sample of the NIST Reference mAb was stressed with 0.003% or 0.01% hydrogen peroxide (H2O2) for 24 hours at room temperature prior to IdeS digestion, reduction, and subunit MAM analysis on a Vion IMS System. The LC and Fd subunits proved resistant to the stress conditions but a significant increase in oxidation was observed for the Fc subunit (Figure 6). The 0.003% H_2O_2 treatment level

SELECT SERIES

Waters Advanced Mass Spectrometry

Unlocking the Potential of Your Mass Spectrometry Research

SELECT SERIES™ Cyclic™ IMS

Endless possibilities. Limitless research.

Revolutionary **Cyclic Ion Mobility** combined with Time-of-Flight Mass Spectrometry.

waters.com/CyclicIMS

SELECT SERIES MRT

All the benefits of Time-of-Flight. Redefined performance.

Reflect on the possibilities of **Multi Reflecting Time-of-Flight Mass Spectrometry**.

waters.com/MRT

Waters

THE SCIENCE OF WHAT'S POSSIBLE.™

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
waters.com

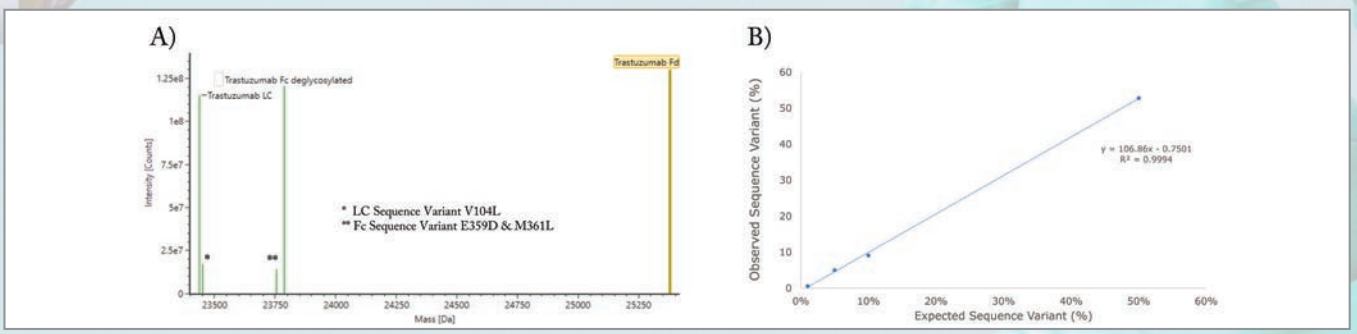


Figure 5. Trastuzumab sequence variant analysis. (A) Component plot for 10% sequence variant spiked sample with automatically labelled LC, Fd, Fc, and the two sequence variant species. LC sequence variant is labelled with (*) and Fc sequence variant species is labelled with (**). (B) % observed sequence variant vs % expected sequence variants, linear from 1–50%.

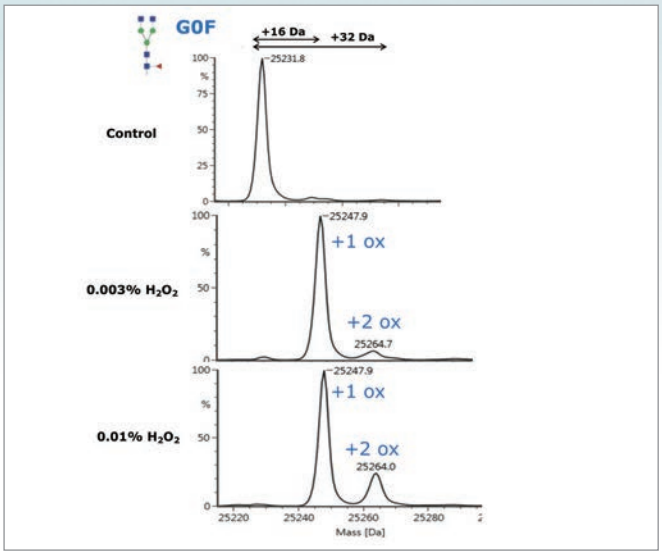


Figure 6. NIST mAb forced oxidation study, Fc species. MaxEnt1 deconvolution (zoom of G0F species), showing +1, +2 oxidation following incubation in 0.003% and 0.01% hydrogen peroxide (H_2O_2).

converted almost all of the Fc species to an oxidized (single and double oxidation) form with a further shift towards doubly oxidized species for the 0.01% H_2O_2-stressed sample.

While subunit MAM methods offer a potentially faster and more efficient way to analyze for mAb CQAs, there are two practical limitations to consider. First, the monitored CQAs must be mass resolved by protein MS. For example, isobaric and near-isobaric species such as isomerization and deamidation will not be resolved using this method and resolution of smaller modifications, such as Met oxidations (+16 Da), are more easily quantified on an 25kD IdeS subunit than a 50kD reduced heavy chain. Second, subunit MAM can only localize the modifications to the LC, Fd, or Fc subunit and the presence of multiple instances of a modification on a given subunit may confound direct interpretation of the results. For these situations, site-specific peptide mapping approaches may be required.

Conclusion

In this work we demonstrated subunit based monitoring of mAb quality attributes across multiple ToF platforms. Subunit MAM-based analysis is more amenable to higher sample throughput and creates less complex data than peptide based MAM methods. These benefits come with potential limitations on the selectivity for specific attributes residing within the same subunit and an inability to monitor deamidation and isomerization based attributes. However, common CQAs such as glycosylation profile, glycation, oxidation, and product sequence variants can be monitored using this simpler approach. The BioAccord and Vion systems, operating under the compliance-ready waters_connect/UNIFI informatics platform, show excellent reproducibility and repeatability for this type of analysis consistent with previous extensive studies with the Xevo QTof platform.

References

1. *Dong J, Migliore N, Mehrman S, Cunningham J, Lewis M, Hu P. High-Throughput, Automated Protein A Purification Platform with Multiattribute LC-MS Analysis for Advanced Cell Culture Process Monitoring. Anal Chem. 2016. 88. 8673–8679.*
2. *Sokolowska I, Mo J, Dong J, Lewis M, Hu P. Subunit mass analysis for monitoring antibody oxidation. mAbs. 2017; 9:3, 498–505.*
3. *Sokolowska I, Mo J, Pirkolachahi F, McVean C, Meijer L, Switzar L, Balog C, Lewis M, Hu P. Implementation of a High-Resolution Liquid Chromatography-Mass Spectrometry Method in Quality Control Laboratories for Release and Stability Testing of a Commercial Antibody Product. Anal Chem. 2020; 92, 2369–2373.*
4. *Glycoworks RapiFluor MS N-glycan kit Care and Use Manual. 715004903.*
5. *Shion H, Yu YQ, Chen W. Enabling Routine and Reproducible Intact Mass Analysis When Data Integrity Matters. Waters Application Note 720006472en. 2019.*
6. *Giddens J, Lomino J, DiLillo D, Ravetch J, Wang X. Site-selective Chemoenzymatic Glycoengineering of Fab and Fc Glycans of a Therapeutic Antibody. PNAS. 2018; 115 (47) 12023–12027.*
7. *Ayoub D, Jabs W, Resemann A, Evers W, Evans C, Main L, Baessmann C, Wagner-Rousett E, Suckau D, Beck A. Correct Primary Structure Assessment and Extensive Glyco-Profiling of Cetuximab by a Combination of Intact, Middle-up, Middle-down and Bottom-up ESI and MALDI Mass Spectrometry Techniques. mAbs. 2013; 5(5): 699–710.*
8. *Lin T, Beal K, Brown P, DeGruttola H, Ly M, Wang W, Chu C, Dufield R, Casperson G, Carroll J, Friese O, Figueroa Jr. B, Marzilli L, Anderson K, Rouse J. Evolution of a Comprehensive, Orthogonal Approach to Sequence Variant Analysis for Biotherapeutics. mAbs. 2019. 11:1, 1–12.*

AccuTOF™ GC-Alpha JMS-T2000GC

High Performance Gas Chromatograph Time-of-Flight Mass Spectrometer

Key Technology 1: New High-Performance Hardware

- Resolving Power: > 30,000
- Mass Accuracy: < 1ppm
- Optional Soft Ionization: CI, PI, FI
- Combination Ion Sources: EI/FI/FD and EI/PI

Key Technology 2: Next Generation Analysis Software for Simple, Speedy Operation

- Combines EI and soft ionization data for automatic qualitative analysis
- Chromatographic peak deconvolution
- Group analysis for extracting compounds with common substructures
- Differential analysis for directly comparing 2 samples
- Also supports the analysis of EI data alone

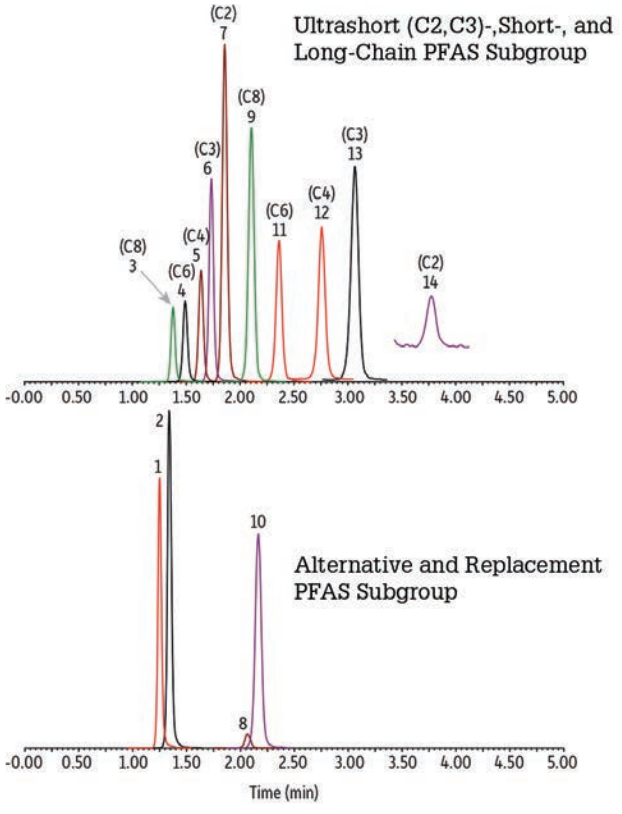
JEOL
Solutions for Innovation

go.jeolusa.com/JMST2000_TAS_Supplemental
salesinfo@jeol.com
978-535-5900

COMPREHENSIVE ANALYSIS OF C2-C8 PFAS USING A NOVEL LC COLUMN

Overcoming traditional limitations in PFAS analysis with innovative column technology

By Shun-Hsin Liang, Ph.D



Mobile Phase	
A:	Water, 10 mM ammonium formate, 0.05% formic acid
Bs	60:40 Acetonitrile:methanol, 0.05% formic acid

Time (min)	Flow (mL/min)	% A	% B
0.00	0.5	15	85
8.00	0.5	15	85

Figure 1: Chromatogram of a 400 ng/L standard.

	Average % Accuracy (%RSD)								
Matrics	Tap Water		River Water		Groundwater**		POTW Water		Deionized Water
1Conc. (ng/L)	40	160	40	160	40	160	40	160	10* (LLOQ)
TFA	106 (16.9)	97.9 (7.10)	97.4 (10.8)	97.6 (6.12)	97.5 (14.5)	103 (8.87)	102 (17.1)	96.4 (7.33)	107 (3.55)
PFPrA	95.1 (4.08)	105 (3.48)	94.5 (6.85)	104 (2.36)	103 (9.37)	105 (8.34)	91.8 (4.90)	104 (7.09)	109 (1.61)
PFBA	106 (6.80)	117 (3.18)	105 (7.40)	114 (4.91)	111 (2.48)	120 (3.27)	106 (6.58)	114 (4.85)	104 (4.91)
PFHxA	93.3 (7.41)	111 (2.61)	91.8 (11.34)	103 (4.55)	102 (6.62)	109 (7.11)	103 (8.37)	108 (3.13)	115 (1.64)
PFOA	100 (4.24)	107 (3.14)	103 (6.71)	105 (2.64)	92.6 (3.85)	107 (3.09)	102 (4.57)	109 (3.64)	106 (3.28)
HFPO-DA	95.7 (11.9)	108 (9.05)	86.6 (8.97)	104 (5.45)	94.1 (18.6)	105 (9.35)	95.2 (8.49)	106 (9.23)	102 (16.8)
ADONA	106 (3.75)	116 (2.38)	100 (6.86)	100 (4.59)	104 (4.91)	113 (5.23)	111 (5.26)	115 (2.65)	105 (4.76)
PFEtS	94.8 (9.68)	110 (5.39)	89.4 (7.43)	102 (9.76)	96.5 (4.09)	108 (6.11)	104 (8.18)	109 (5.23)	99.8 (9.85)
PFPrS	104 (4.97)	115 (4.19)	95.0 (3.87)	107 (4.26)	106 (10.6)	114 (3.36)	111 (4.88)	114 (2.96)	108 (3.28)
PFPS	97.4 (10.1)	113 (3.97)	93.6 (5.24)	104 (4.19)	97.8 (4.47)	107 (4.23)	94.1 (10.7)	108 (4.48)	100 (11.0)
PFHxS	99.4 (15.7)	114 (3.56)	94.3 (9.79)	104 (5.28)	95.2 (5.63)	112 (3.20)	104 (8.19)	111 (4.07)	107 (11.7)
PFOS	104 (7.54)	107 (7.69)	103 (8.43)	105 (7.23)	97.3 (14.9)	110 (4.84)	109 (7.47)	108 (7.53)	102 (4.20)
P9cL-PF3ONS	98.7 (3.52)	105 (8.35)	91.8 (7.66)	103 (5.68)	94.7 (9.83)	105 (8.90)	105 (6.76)	107 (8.27)	107 (4.31)
11Cl-PF3OUdS	106 (10.1)	113 (3.54)	95.0 (3.52)	113 (8.15)	107 (6.61)	112 (4.54)	119 (4.25)	120 (9.10)	98.2 (11.3)

*20ng/L LLOQ for TFA
**Groundwater was diluted fivefold for TFA only

Table 1: Method accuracy and precision. These results demonstrate that switching to a mixed-mode LC column provides the capability to analyze currently monitored and emerging PFAS contaminants in a single, short, isocratic run, preparing labs for the future of PFAS testing.

Accommodating a growing interest in monitoring ultrashort-chain PFAS is not possible using a traditional reversed-phase LC column chemistry. Instead of developing separate analyses, Restek's novel Polar X LC column employs mixed-mode retention mechanisms that make the analysis of ultrashort-chain, legacy, and even alternative PFAS compounds possible in a single method.

While not currently regulated, ultrashort-chain (C2-C3) per- and polyfluoroalkyl substances (PFAS) are of great interest. Current testing methodologies using reversed-phase LC columns cannot be used because of a lack of retention, so either a separate method or a different column is required.

A unique, hybrid ion-exchange/HILIC column (Raptor Polar X) was used to develop a comprehensive LC-MS/MS method for the analysis of ultrashort-chain through long-chain (C8 and up) and alternative PFAS in water sources (tap, river, groundwater, and sewage effluent). The Raptor Polar X's multimode retention mechanisms allow for retention with a single isocratic run. All analytes eluted in 4 minutes with good peak shapes (Figure 1). The overall analytical cycle time was 8 minutes to ensure that there were no matrix-related interferences.

Method linearity from 20-800 ppt for trifluoroacetic acid (TFA) and 10-800 ppt for all other analytes provided r^2 values >0.996 and deviations <20% using a 1/x weighted quadratic regression. Samples were fortified at the low and high concentrations of their calibration ranges and ran in duplicate for each analytical batch. A total of three batches were measured on different days. Concentrations of fortified samples were adjusted to account for any observed background contamination in sample blanks. Results are presented in Table 1.

QUALITATIVE FLEXIBILITY COMBINED WITH QUANTITATIVE POWER

Using the ZenoTOF 7600 system, powered by SCIEX OS Software

The key to achieving robust analytical results lies in the combination of sensitivity, selectivity, and specificity. Sensitivity ensures there is plenty of signal to identify and quantify analytes of interest. Selectivity differentiates analyte signal from noise and interferences. Specificity ensures compound identifications are accurate and confident. The technological advancements in the ZenoTOF 7600 system combine qualitative flexibility and quantitative power for the most demanding sample types and workflows.

A hybrid collision cell is at the heart of the technological advancements in the ZenoTOF 7600 system. Previously, QTOF mass spectrometers have suffered from duty cycle losses as a result of mating time-of-flight (TOF) analysis, a pulsed measurement technique, with the continuous beam coming from the quadrupole ion path. A series of ion-staging events and reverse-mass sequential ion release, with high-capacity ion traps, allow for duty cycle losses to be mitigated and for MS/MS sensitivity gains of 4 to 20 times (1). The cell also has the ability to perform both collision-induced dissociation (CID) and electron-activated dissociation (EAD) experiments for high-resolution MS/MS flexibility. Electron kinetic energies can be precisely tuned from 0-25 eV without the use of chemical transfer reagents. This tunability means EAD can be performed on a wide array of analytes, from multiply-charged peptides to singly-charged small molecules (2). The ability of the EAD cell to contain a high density of electrons allows for rapid reaction rates that keep up with fast chromatographic separations.

Key innovations in the SCIEX ZenoTOF 7600 system

- Zeno trap provides an increase to ≥90% duty cycle across the entire mass range for MS/MS acquisition modes (Zeno IDA and Zeno MRMHR)
- MS/MS sensitivity improvements from 4 to 20 times
- Reagent-free and tunable, high-efficiency EAD fragmentation in the EAD cell, offering alternative fragmentation for both small and large molecules workflows
- New levels of specificity with various electron-based dissociation techniques
- Richer fragmentation for improved structural information.
- Greater than 5 orders of inter-scan linear dynamic range and 4 orders of intra-scan linear dynamic range in both MS and MS/MS modes
- Pre-optimized performance to easily switch between high flow, microflow and nanoflow rates with the OptiFlow Turbo V ion source

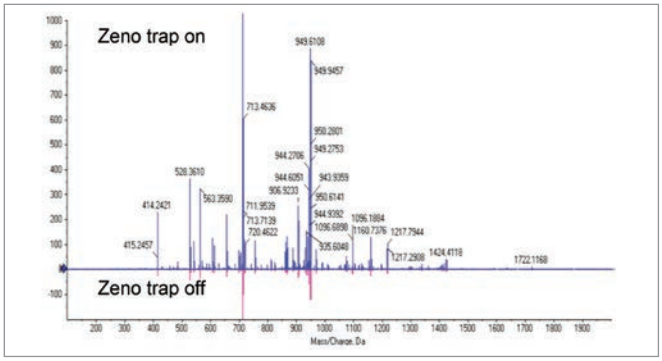


Figure 1. EAD MS/MS spectra of melittin with and without the Zeno trap activated. MS/MS spectra enhanced by using the Zeno trap (top, blue) shows 5 to 10 times improvement in sensitivity across the mass range compared to the EAD MS/MS spectrum acquired without the Zeno trap activated (bottom, pink), with negligible changes to noise. EAD yields significant sequence coverage for structural elucidation.

Zeno trap

Quadrupole time-of flight instruments most commonly use the orthogonal injection of ions coming from a quadrupole collision cell into the flight tube region, because this configuration maximizes TOF resolution, mass accuracy, and sensitivity for an entire spectrum without the need for scanning. This type of ion pulsing, however, suffers from a relatively low duty cycle. Typically, only 5 to 25% of ions are ejected with each pulse of the accelerator, depending on the geometry and m/z range. This is not usually an issue in the MS1 dimension, because the ion current as generated by modern sources (such as the Turbo V ion source), and transmitted by modern ion capture technology (such as the Q Jet ion guide), results in ion currents that need to be reduced to prevent saturation and to protect the longevity of TOF MS detectors. In the MS/MS dimension, however, an improvement in the duty cycle can lead to significant gains in sensitivity.

The ion losses are a result of the drift region between the collision cell and the TOF accelerator. This region behaves as a crude TOF separation, where low m/z ions migrate faster than high m/z ions and, as a result, a significant fraction over or under migrate to the accelerator region and are lost with each pulse. Previously, there have been many attempts to overcome this lack of synchronicity. It has only been achieved, however, either for narrow mass ranges or at low acquisition frequency. Use of the Zeno trap overcomes these technological barriers to recover duty cycle losses across the entire m/z at up to 100 Hz acquisition frequency. This is achieved using a linear ion trap, referred to as a Zeno trap, at the exit of the collision cell. The mechanism of trapping and releasing ions is highlighted in Figure 2. Ions enter the ion trap and are contained with potential barriers on the ZG and IQ3 lenses, while subsequent packages of ions are accumulated in the LINAC collision cell, preventing ion loss. The trapped ions are left to energetically cool and are subsequently released based on potential energy resulting in an ordered release generally ranging from high m/z to low m/z. In this way, each ion across the mass range reaches the center of the TOF accelerator simultaneously.

This simple trapping and releasing mechanism leads to significant gains in MS/MS sensitivity, as highlighted in Figure 3. MS/MS with the Zeno trap activated results in a 4- to 15-fold (or greater) gain in signal, with increased gains at low m/z fragments. The Zeno trap efficiency combined with precise ion-release timing yields ≥90% of the theoretical gain across the entire mass range. Due to the degree of selectivity afforded with high resolution MS/MS data, these improvements in signal are combined with negligible changes to noise, resulting in spectral and chromatographic signal-to-noise on the order of the gains observed in raw signal (Figure 4, 5).

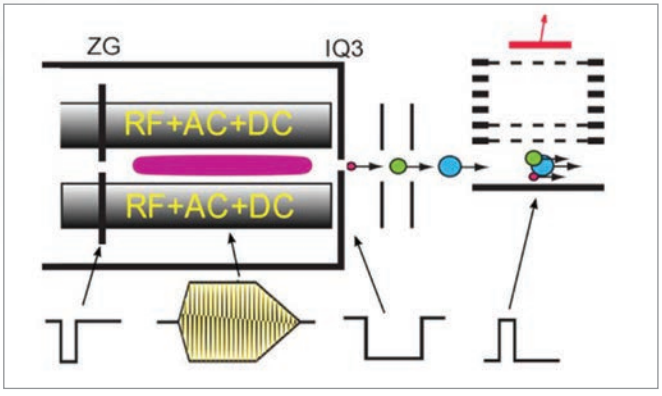


Figure 2: Timing diagram of gating voltages, AC ramp and TOF acceleration pulses. Ions are accumulated in a small ion trap at the exit of the collision cell, then released in reverse mass order to perfectly synchronize with each accelerator pulse.

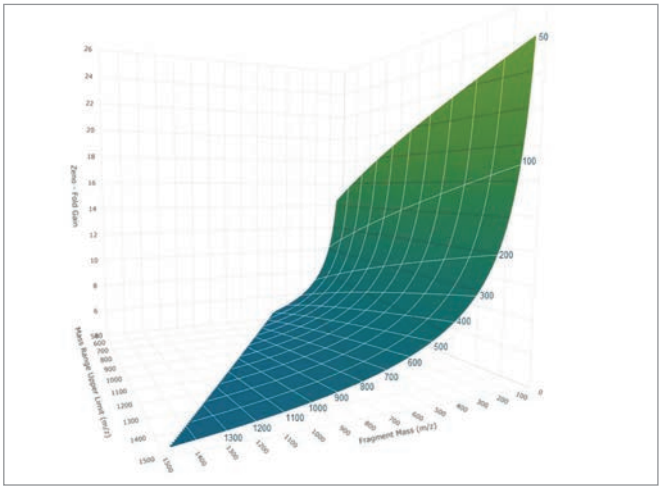


Figure 3: Theoretical sensitivity gains in MS/MS as a function of fragment m/z and acquisition mass range upper limit using the Zeno trap. Sensitivity gains are the result of the recovery of duty cycle losses that are a natural result of mating TOF analysis, a pulsed measurement technique, with the continuous beam coming from the quadrupole ion path. Greater ion losses occur as the upper limit of the MS/MS scan range increases. Zeno trap technology has the ability to recover >95% of these losses.

These improvements in MS/MS sensitivity not only have the ability to drastically improve LOQs for quantitative assays, but this additional sensitivity can be used to revolutionize entire workflows. With the Zeno trap activated, high-quality MS/MS spectra can be used for confirmation, identification, or library matching at much lower mass loading. This gives the ability to significantly dilute precious samples and improve ionization efficiency by minimizing matrix effects and improve instrumental robustness with lower mass loading.

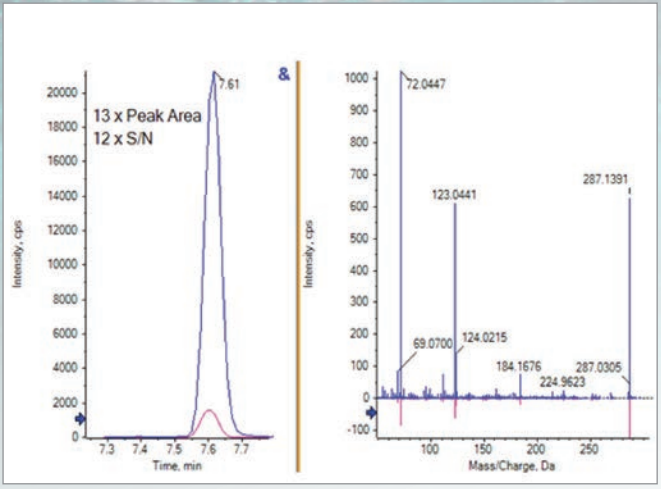


Figure 4: Sensitivity gains with the Zeno trap for difenoxuron with MRMHR acquisition. Due to the selectivity afforded by MRMHR, the gain in signal from the Zeno trap is accompanied by a minimal gain in noise. (Left) A 13-fold intensity gain results in a 12-fold signal to noise gain for the m/z = 72.044 fragment of difenoxurion. (Right) All peaks in MS/MS spectra show a sensitivity gain (6-13 fold) with use of the Zeno trap.

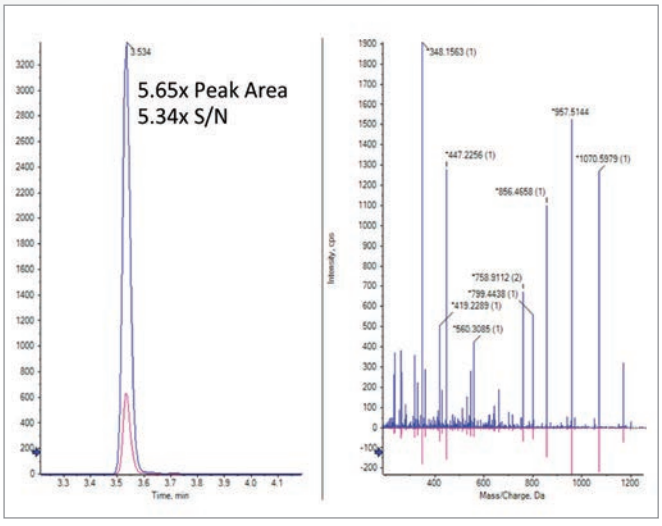


Figure 5: Sensitivity gains with the Zeno trap for SPYVITGPGVVEYK from PepCalMix with MRMHR acquisition. (Left) A 5.65-fold gain in peak area for m/z = 1070.50 fragment ion with a 5.34-fold gain in S/N. (Right) Gain observed for higher m/z peptide fragment ions is 5-7 fold with the Zeno trap on across the MS/MS mass range.

At the same sample loading, MS/MS with the Zeno trap on yields greater confidence in identifications while allowing for the ability to discover new metabolites, peptides biomarkers and contaminants at lower concentrations than ever before.

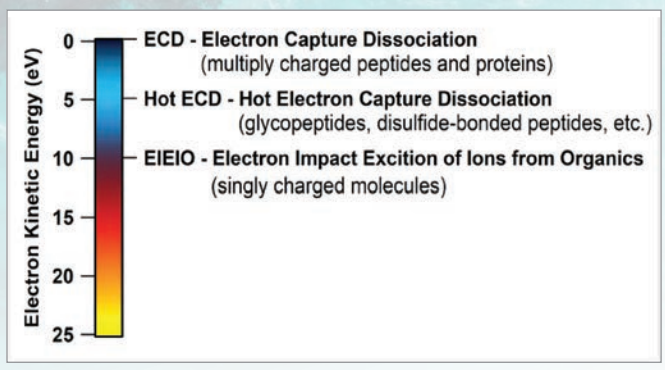


Figure 6: EAD family classified by precursor species and the kinetic energy of the electron beam. Common electron-based dissociation techniques and their typical applications are listed.

Precisely tunable electron activated dissociation (EAD) cell

Tandem MS is dominated by CID that generates ion fragments for quantification and identification of molecular species. With CID, ions are generated in an accelerated cell filled with a neutral gas species promoting molecular collisions that result in bond leavage, typically at the most labile sites. Although fast and efficient, CID can often result in few diagnostic fragments that are insufficient to elucidate structural information from unknown features or to differentiate isomeric species.

EAD, conversely, describes a family of free electron-based dissociation mechanisms characterized by the charge state of the precursor ion and the kinetic energy of the electron beam. EAD mechanisms are known to give complementary information to CID. The extent and location of bond cleavage differs for radical (EAD) and thermal (CID) techniques.

The ZenoTOF 7600 system features the EAD cell, a new electron beam optic design that simultaneously and independently traps precursor ions and free electrons for efficient radical fragmentation. With the EAD cell, the system has the ability to tune electron energies above zero, which opens up radical fragmentation to applications beyond biomolecules. Tunable electron kinetic energy from 0-25 eV provides access to different fragmentation regimes (Figure 6), including electron capture dissociation (ECD), hot ECD, and electron impact excitation of ions from organics (EIEIO). These advances enable fast, precise and quantitative dissociation of various analytes ranging from singly charged small molecules to multiply protonated proteins. Also, at higher energies, reaction times are reduced allowing for these dissociation techniques to be used on a chromatographic timescale. It is the combination with the Zeno trap that allows EAD to now have the sensitivity and specificity needed for routine use.

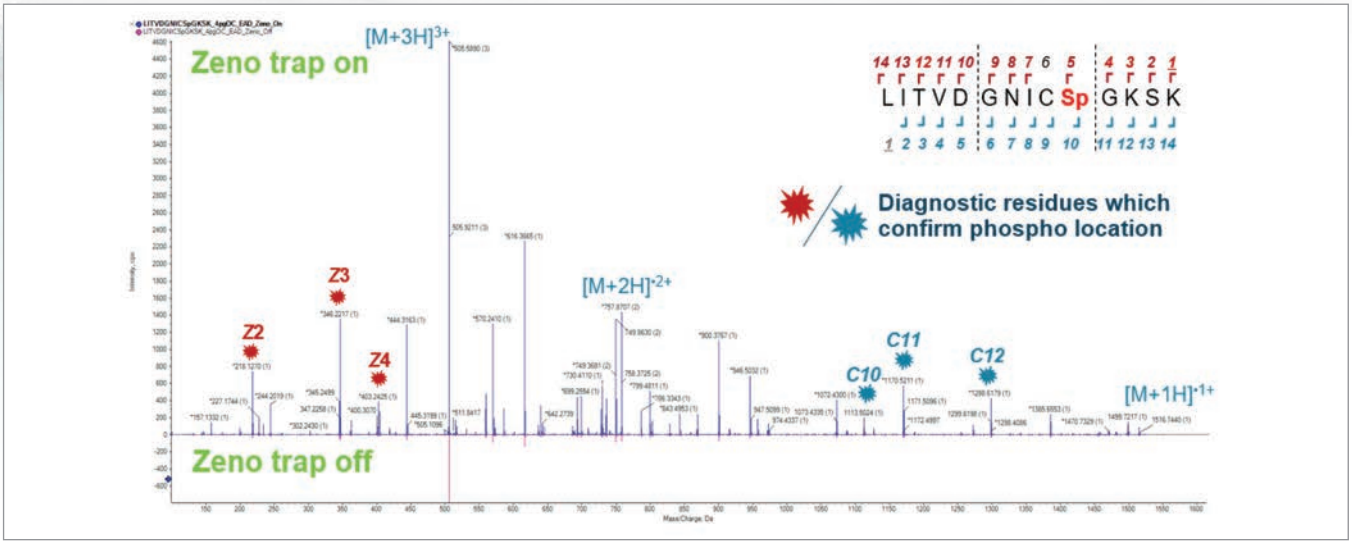


Figure 7. Phosphorylated peptide, LITV, analyzed using EAD-MS/MS. (Top) LITV EAD-MS/MS spectrum with Zeno trap on. (Bottom) LITV EAD MS/MS spectrum with Zeno trap off. MS/MS sensitivity is significantly enhanced with the Zeno trap activated. With the Zeno trap on, 100% sequence coverage is achieved with phosphorylation site location confirmed on multiple c- and z- ion series.

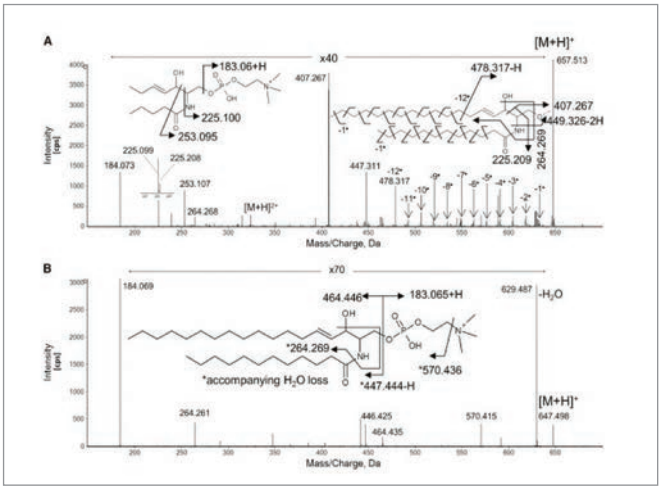


Figure 8: Comparison between dissociation product spectra by EIEIO (A) and CID (B). The sample was a synthesized standard SM, SM(d18:1,12:0).

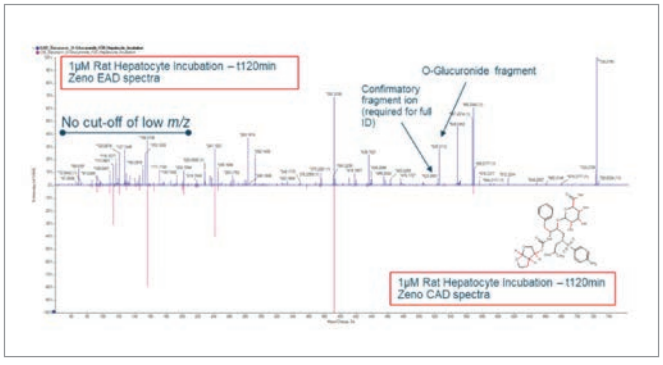


Figure 9: EAD (top) and CID (bottom) spectra of O-glucuronide conjugated darunavir. EIEIO creates unique fragments characteristic of the glucuronide conjugation positioning.

ECD and hot ECD for multiply charged peptides, proteins and biotherapeutic molecules

Post-translational modifications (PTMs) are widely important for various protein functions, including protein conformation, signaling and activity. Some PTMs can be difficult to characterize using MS, however, when they are labile, CID is used as the dissociation technique. Radical dissociation techniques have the ability to maintain these PTMs, which allows for peptide backbone mapping, while simultaneously elucidating the identity and location of the PTM. Figure 7 shows the example of a phosphorylated peptide, LITV, using hot ECD (KE = 7 eV). Here, not only is nearly the entire peptide sequenced with hot ECD, but the location of the phosphorylation is maintained (4).

Electron impact excitation of ions from organics (EIEIO) for singly charged molecules

Having the ability to tune electron kinetic energies in the 5 to 15 eV range opens up EAD to the realm of singly charged molecules. Electron capture, resulting in neutralization, is reduced at this kinetic energy, allowing the electrons to induce dissociation through radical mechanisms. Figure 8 shows the comparison between CID and EIEIO for the fragmentation of a sphingomyelin lipid species. EIEIO gives spectral information for nearly every bond to elucidate head group identity, backbone type, carbon chain lengths, double bond positions and double bond stereoisomerism. In a

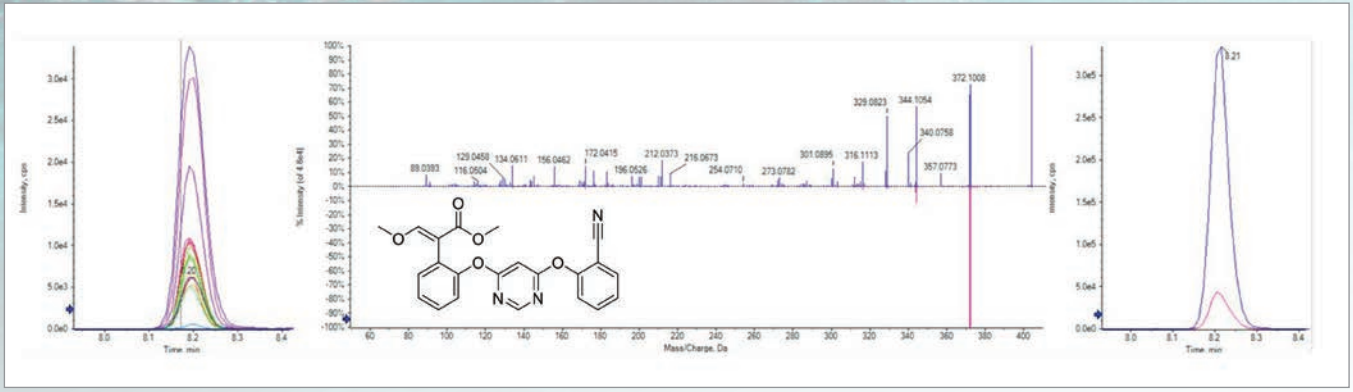


Figure 10: Comparison between CID and EAD MS/MS spectra for azoxystrobin. EIEIO (middle top, blue) creates roughly 100x the number of fragments with S/N >10 compared to CID (middle bottom, pink). The XICs for EAD (left) and CID (right) highlight the flexibility of EAD for ion ratio-based confirmation.

Figure 11: Linear dynamic range of the 4-channel NCP detector with ADC signal processing. Greater than 5 orders of inter-scan linear dynamic range can be achieved in both positive ion (top) and negative ion (bottom) modes, for both MS (left) and MS/MS (right) acquisitions.

similar fashion, EIEIO can be used to differentiate between isomers of small molecules. Figure 9 highlights the unique, characteristic fragment for O-glucuronide conjugated darunavir compared to its N-glucuronide isomer, allowing for more precise molecular information within metabolite identification workflows. EIEIO fragmentation also opens up additional specificity for non- targeted and suspect screening workflows. Figure 10 highlights the difference between EIEIO and CID for the fragmentation of azoxystrobin, a fungicide. The CID spectrum is dominated by two main fragments, whereas the EIEIO spectrum contains over 200 peaks with S/N >10, which allows for significantly improved confidence during library matching and structural elucidation.

Linear dynamic range (LDR)

LDR is important in many applications where the analyte concentration varies widely. The ZenoTOF 7600 system is equipped with a 4-channel MCP detector with ADC signal processing, resulting in greater than 5 orders of inter-scan linear dynamic range in both positive and negative ion modes, and for both MS and MS/MS acquisitions (Figure 11). Further, the ADC detector is capable of 4 orders if intra-scan linear dynamic range, allowing for the simultaneous detection of both high- and low-level analytes without a loss of data quality.

Acquisition speed

The ZenoTOF 7600 system comes equipped with a high-speed LINAC collision cell that allows for fast acquisition rates,

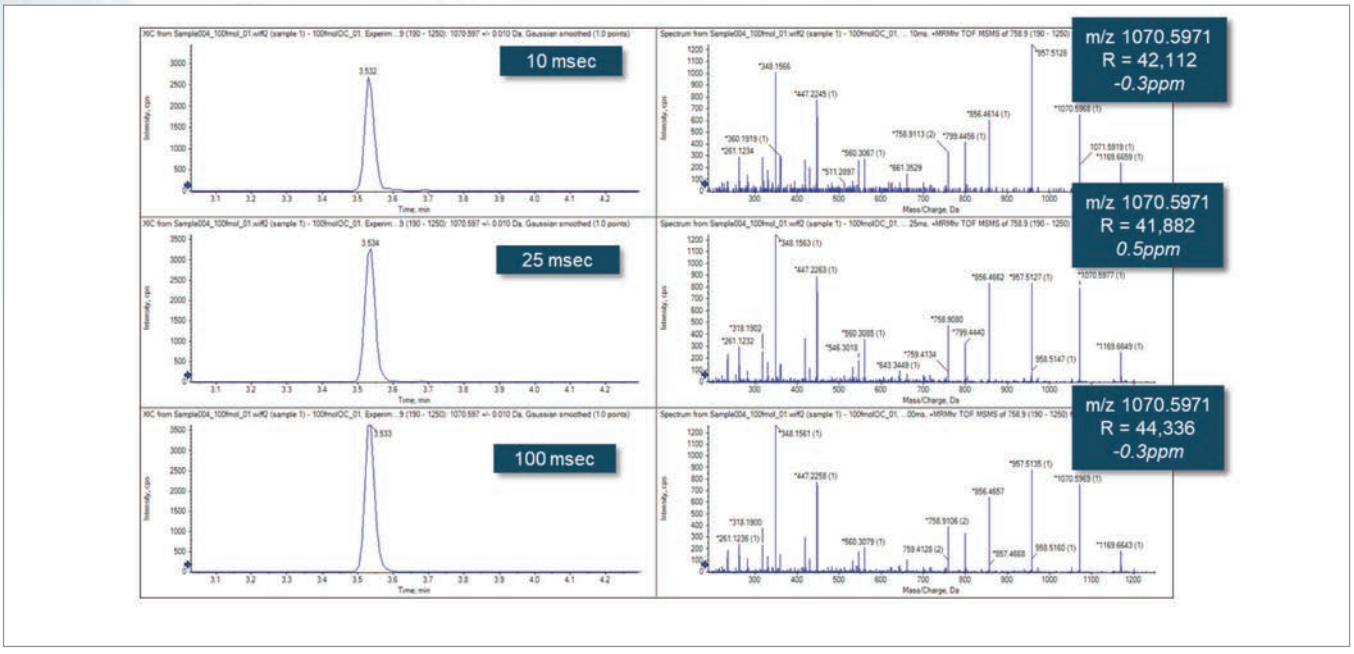


Figure 12: Peak intensity, mass resolution and mass accuracy across accumulation times. Faster accumulation times do not come at a sacrifice of mass resolution or mass accuracy with the ZenoTOF 7600 system. Mass resolution is consistent across the entire mass range.

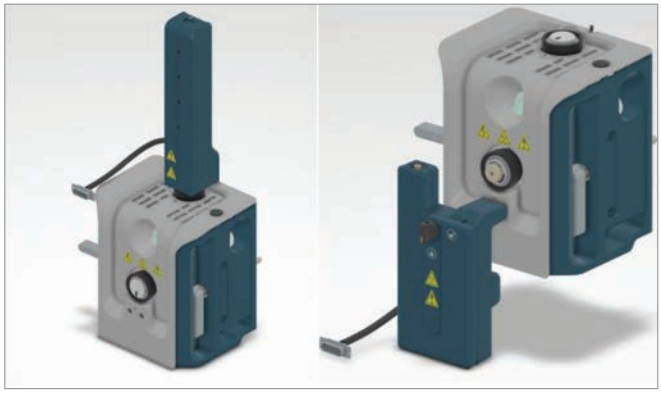


Figure 13: OptiFlow source configurations. (Left) Microflow configuration. (Right) Nanoflow configuration.

suitable for highly multiplexed experiments and rapid LC separations. Combined with the high speed of the MCP detector, the ZenoTOF 7600 system is capable of >100 Hz acquisition rates, with accumulation times as low as 5 msec, without sacrificing mass accuracy and resolution. Figure 12 highlights the maintenance of mass accuracy, mass resolution and peak intensity across a range of accumulation times.

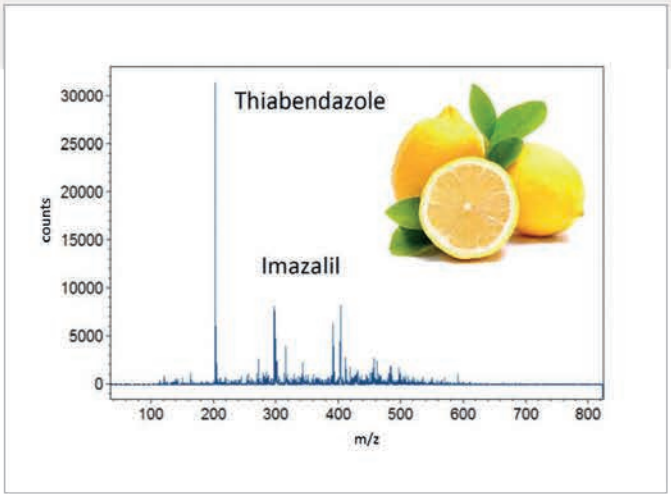
OptiFlow Turbo V ion source for low flow chromatography

The ZenoTOF 7600 system has the option for the OptiFlow Turbo V ion source, which is designed for microflow and nanoflow chromatography (Figure 13) (5). Probe and electrode combinations are pre-optimized for sensitivity and robustness from 0.1 to 200 µL/min. This means no manual adjustments are needed to maximize performance. SecurityLink tubing and fittings are used such that all fittings are finger tight, leak free and have zero dead volume. For microflow chromatography, the exit of the column connects directly to the electrospray probe, minimizing post-column broadening for enhanced S/N. The instrument comes configured with the OptiFlow interface, so switching between high flow or microflow to nanoflow is a toolless change, without the requirement of breaking vacuum on the system.

References

1. *ELECTRON ACTIVATED DISSOCIATION: A new paradigm for tandem mass spectrometry. SCIEX publication RUO-MKT-19-13372.*
2. *ZENO TRAP: Defining new levels of sensitivity. SCIEX publication RUO-MKT-19-13373.*
3. *PTM site localization and isomer differentiation of phosphorylated peptides – Tunable electron activated dissociation (EAD) MS/MS using the ZenoTOF 7600 system. SCIEX technical note RUO-MKT-02-13174-A.*
4. *Single source solution for low flow chromatography. SCIEX technical note RUO-MKT-02-9701-A.*

Detection of Residual Pesticides on Fruits and Vegetables Using PortabilityTM Miniature Mass Spectrometer



Mass spectrometry can now be deployed for on-site pesticide screening in real time

A case study for residual pesticide screening on fruits and vegetables is reported. All produce was purchased from a local market in San Jose, California and immediately analyzed by TD-ESI coupled to the PortabilityTM mass spectrometer without any sample preparation. The portable analyzer was able to detect ppm levels of pesticides such as thiabendazole, imazalil, flutolanil, and permethrin. Featuring light weight and compact size, BaySpec's novel mass analyzers based on linear iontrap technology are the most sensitive portable devices available on the market with parts-per-trillion detection sensitivity. These extremely compact instruments are simple to operate and maintain, and they are ideal for a variety of bulk or trace on-site detection in real time. Learn how you can bring the lab to the sample with portable analytical tools from BaySpec by reading our educational application note for pesticide screening of produce.

Read the full app note at http://tas.txp.to/1018/ANBaySpec

timsTOF SCP

Unbiased, quantitative single cell 4D-Proteomics (SCP) research

Expanding the horizons of single cell research

Mass spectrometry-based proteomics has become an important tool for modern research in understanding biological function and disease mechanisms. Healthy or diseased tissues that seem homogenous are composed of cells with a variety of different proteomes. Deciphering the proteome of each single cell is key to fully understanding its function and has traditionally presented a major challenge.

- **4D-Proteomics™** - Unbiased single cell, immunopeptidomics and CCS-enabled PTM analysis (4D-Epiproteomics)
- **PASEF® acquisition** - PASEF enables acquisition at >100 Hz with ion focusing and removal of chemical noise for clean MS and MS/MS
- **Single Cell Sensitivity** - New ion source concept coupled to the PASEF principle

For more information please visit www.bruker.com

TIMS-QTOF MS

Innovation with Integrity

FOUR Qs ARE BETTER THAN QQQ

Perfect for challenging applications in semiconductors, geosciences, biomonitoring, and more, the cutting-edge, multi-award-winning **NexION® 5000 ICP-MS** is the industry's first four-quadrupole system, combining the simplicity of a reaction/collision cell with multi-quadrupole technology that transcends traditional triple quad. That means superior interference removal, stability, and matrix tolerance – all in a system that virtually eliminates routine maintenance, for unsurpassed instrument uptime.

The NexION 5000 multi-quadrupole ICP-MS:
This is performance to the power of four.

For more information on our NexION 5000 ICP-MS, visit www.perkinelmer.com/nexion5000

PerkinElmer®
For the Better