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Articles

6  Breaking the Rules: Two- and Three-Dimensional Chromatography with Four Dimensions of Mass Spectrometry

William Craig Byrdwell

Two-dimensional liquid chromatography (2D-LC) allows much greater resolution of peaks than is possible in a classical single dimensional separation. For the next development in separations, we employed 2D-LC in two highly orthogonal dimensions of separation with four mass spectrometers for detection, with parallel detection in each dimension. We have further broken ground by using three dimensions of separation with four mass spectrometers, using two parallel second dimensions.

12  Six Key Differentiators Between Liquid Chromatography and High-Resolution Ion Mobility

Melissa Sherman and Laura Maxon

Using ion mobility, analytes that have the same molecular mass can be separated by their shape, centers of mass, and collision cross section, but challenges such as ion loss can still occur. A new development in ion mobility separation, high-resolution ion mobility (HRIM), addresses such problems, and is particularly well suited to challenging applications, such as glycosylation monitoring of biological drugs and vitamin D analysis.

15  Laser Desorption Postionization Mass Spectrometry

Teodora Zagorac and Luke Hanley

Supplementing short pulse lasers with laser postionization increases ionization yields for desorption and ablation of solid samples in mass spectrometry. Here, we give an overview of the mechanisms and technical requirements for molecular photoionization in femtosecond (fs) laser desorption postionization mass spectrometry (LDPI-MS).

20  A Quick Approach to Screen Oligomers from Extractables Studies Using Liquid Chromatography–Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

Bin Sun, Owen Perlowski, and Benben Song

Analysis of extractables and leachables (E&L) from plastic packaging is of great importance for pharmaceutical product safety. Accurate and rapid identification of unknown compounds in E&L is often complex and challenging. To address this challenge, we demonstrate a quick method for oligomer determination using LC–QTOF-MS.

Cover Art: Artistic rendition of injectable medication ampule with syringe
Cover image courtesy of Mr.Ikhn/stock.adobe.com
Breaking the Rules: Two- and Three-Dimensional Chromatography with Four Dimensions of Mass Spectrometry

Two-dimensional liquid chromatography (2D-LC) is now commercially available and is being implemented in an increasing number of laboratories around the world. Normally, 2D-LC is done using one or more detectors at the end of the second dimension, \(2^D\), and the first dimension, \(1^D\), is reconstructed from numerous slices across the \(1^D\) peaks. As a result, regular 2D-LC imposes severe constraints on the run times available in the second dimension and induces a number of other limitations. We started with a ground-up approach, adapted some principles taken as immutable rules, used uncommon approaches, and pioneered new techniques to accomplish separations that have never been possible before. We employed 2D-LC in two highly orthogonal dimensions of separation with four mass spectrometers for detection (employing different atmospheric pressure ionization types) with parallel detection in each dimension, referred to as \(LC2MS4\), with up to five other detectors for as many as nine detectors overall. We have further broken ground by using three dimensions of separation with four mass spectrometers, using two parallel second dimensions (\(2^D_S\)), for \(LC3MS4\). We used multicycle chromatography, employed parallel gradients instead of modulation period gradients, utilized transferred eluent dilution (TED) in both \(2^D_S\), and featured flow-rate programming to fine tune elution in the second \(2^D\). All systems are joined together using a wireless communication contact closure system that allows quick and easy switching between many combinations of chromatographs and detectors for maximum flexibility.

William Craig Byrdwell

Two-dimensional liquid chromatography (2D-LC) has been commercially available for several years now. An increasing number of laboratories are “taking the plunge” and implementing this advanced technology. 2D-LC allows much greater resolution of peaks than is possible in a classical single dimension separation because, at the maximum predicted by theory, the peak capacities are multiplicative (1,2). Some scientists at roundtables have discussed whether 2D-LC is “coming of age” (3), and review after review (>1750 reviews from Scopus) has appeared on the subject. For our purposes, we limit mention of a few reviews by the most recognized and prolific authors in the field: Schoenmakers (4–7), Pirok (5–7), Stoll (5,8,9), and Cacciola, Dugo, Mondello, and coworkers (10,11). While Stoll has perhaps become the most recognized face of 2D-LC, and has taken over the “LC Troubleshooting” column at LCGC, the work of Mondello and others has been mostly applied to food matrices; as a result, their work is the most relevant for our work. There are several excellent tutorials (6,7,12), with a highly useful one available from LCGC (13).

After reading the principles and learning the nomenclature, our laboratory explored using 2D-LC. In the past, we routinely used two, three, or four mass spectrometers simultaneously, for dual- (14), triple- (15), and quadruple (16)-parallel mass spectrometry (MS) attached to a single LC system, referred to as \(LC1MS2\), \(LC1MS3\), and \(LC1MS4\), respectively. These employed atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and atmospheric pressure photoionization (APPI) in different combinations. Multiple parallel mass spectrometry has been reviewed several times (17–20). For us, it seemed a natural evolution to apply multiple parallel mass spectrometers across multiple dimensions of LC separations. However, to accomplish our goals using our existing “legacy” instruments mixed with a few newer acquisitions, we needed to break many of the rules of conventional 2D-LC and take a different approach. This article describes our work combining LC\(_{-}\)LC with quadruple parallel mass spectrometry (MS4) for \(LC1MS2 \times LC1MS2 = LC2MS4\), and discusses our ongoing work using three dimensions of separation, comprised of two parallel second dimensions, combined with MS4 for \(LC1MS2 \times (LC1MS1 + LC1MS1) = LC3MS4\).
Materials and Methods

LC1MS2 × LC1MS2 = LC2MS4

Samples were seed oils, (African mobola plum (Parinari curatellifolia) seed oil, cherry kernel oil, and soybean oil) that were prepared using a modified version of the extraction process of Folch and others (21), as previously described (22). The instrument arrangement for the separation of naturally occurring trans fat–containing seed oils has been shown previously (22) and is reproduced in Figure S1 (in the Supplemental Information found online). We employed two conventional Inertsil ODS-2 (GL Sciences, Inc.) columns with the following dimensions: 25.0 cm x 4.6 mm, 5 μm particles, with a flow rate of 1.0 mL/min, and split the flow to send ~45 μL/min to the second dimension. The 1D gradient used methanol, ethanol, and dichloromethane (DCM), for non-aqueous reversed-phase (NARP) high performance liquid chromatography (HPLC). All LC–MS experiments described here employed sub-ambient column compartments at 10 °C. The 1D and 2D gradients, as well as all conditions for four mass spectrometers, plus five other detectors—UV x 2, fluorescence detector (FLD), charged aerosol detector (CAD), and evaporative light scattering detector (ELSD)—were given in the literature to our earlier report (22).

Two Thermo Scientific mass spectrometers, a TSQ Vantage EMR for APCI-MS and a QExactive for ESI-HRAM-MS, were used to monitor the 1D, and two more Thermo Scientific mass spectrometers, a TSQ Quantum Access Max for APPI-MS and an LCQ Deca XP for ESI-MS monitored the 2D. LC×LC software (GC Image, Inc.) was used to visualize the 2D chromatograms. All instruments and detectors were coordinated and controlled using the wireless communication contact closure system (WCCCS) previously described (23).

D_6-α-tocopherol was used as an internal standard for quantification of fat-soluble vitamins (FSVs) using EICs, selected ion monitoring (SIM), and selected reaction monitoring (SRM). Triacylglycerols (TAGs) were quantified by percent relative composition, using response factors developed from a calibrated gas chromatograph with flame ionization detection (GC-FID), as previously described (24).

LC1MS2 × (LC1MS1 × LC1MS1) = LC3MS4

Standard reference material 1849a, infant–adult nutritional formula was extracted using the modified extraction that Folch and others used (21), with vitamin D_3 added as an extraction internal standard, and vitamin K_2 (menaquinone) added as an analytical internal standard.

FIGURE 1 shows the arrangement of three liquid chromatographs (LC3) and four mass spectrometers (MS4) used for these experiments. The valve interface to the second 2D was constructed from available parts, as described elsewhere (25). The 1D separation employed the Agilent 1200 HPLC, with two Inertsil ODS-2 columns in series at 1.0 mL/min, as above. The NARP LC gradient was a methanol-acetonitrile:dichloromethane gradient (15,16), but the isocratic methanol time range was replaced with a methanol gradient (necessary for FSVs) combined with acetonitrile and dichloromethane, to shorten the analysis from 130 min to 75 min, as shown in Figure S2 (in the Supplemental Information online). The entire flow went through the UV and FL detectors before entering the splitter. The tee branch used to provide effluent to the 2D(1) supplied ~52 μL/min, while the branch to the 2D(2) gave ~67 μL/min, with additional transferred eluent dilution (TED) solvent (70% acetonitrile:50% water) added just before each valve.

The 2D(1) Agilent 1290 binary UHPLC system was operated with a Thermo Scientific Accucore C50, 50 mm x 2.0 mm, 2.6 μm column at 10 °C, using an acetonitrile:dichloromethane parallel gradient at 1.30 mL/min, shown in Figure S2b (in the Supplemental Information online). All of the 2D(1) flow went through the UV detector to an equal splitter, one half of which was directed to the Thermo Scientific LCQ Deca XP ion trap mass spectrometer in ESI-MS mode, and the other half was directed to waste.

An Agilent Infinity Flex II quaternary UHPLC pump, column compartment (at 10 °C), and UV detector were added to the arrangement of instruments to allow 2D(2), as shown in Figure 1. Separations were performed on a 100 mm x 3.0 mm, 2.6 μm particle Accucore C50 column using the acetonitrile:dichloromethane gradient shown in Figure S2c (in the Supplemental Information online). A Valco tee split the flow so the majority went to the ELSD and ~150 μL/min went to a Thermo Scientific QExactive HRAM-MS mass spectrometer operating in ESI-MS mode. Because of the highly correlated stationary phases and solvent systems, a

Figure 1: Arrangement of instruments for 3D-LC, LC1MS2 × (LC1MS1 × LC1MS1) = LC3MS4 experiments.
TED solvent was employed to “knock down” the solvent strength of the 1D solvent, thereby dramatically sharpening peaks in the 2D(1) and 2D(2).

In contrast to LC2MS4 experiments, the LC3MS4 experiments employed only NARP separations in all three dimensions. Thus, instead of the shifted gradients used in LC2MS4 experiments, 3D LC3MS4 experiments utilized parallel gradients, inspired by discussions with T. Górecki (26) at the 2019 Eastern Analytical Symposium. The 2D(2) used a parallel gradient that did not elute analytes in one modulation period, but instead intentionally kept analytes on-column through multiple modulation periods, or cycles, which we refer to as multicycle comprehensive multidimensional LC (MC-CMDLC). LC3MS4 used flow rate programming (as shown in Figure S2c in the Supplemental Information online), to adjust flow rates that minimized the elution of major peaks across modulation period boundaries.

Results and Discussion
Our work may be referred to as split-flow comprehensive multidimensional LC (SF-CMDLC), specifically split-flow comprehensive 2-D LC (SF-C2DLC) and split-flow comprehensive 3-D LC (SF-C3DLC).

\[ LC1MS2 \times LC1MS2 = LC2MS4 \]

The data from LC2MS4 runs are summarized in Figure 2, which shows dual parallel mass spectrometry, LC1MS2, (APCI-MS and ESI-HRAM-MS) in the 1D and dual parallel mass spectrometry, LC1MS2, (APPI-MS and ESI-MS) in the 2D. The first benefit of the LC2MS4 approach is that we completely bypassed the problem of undersampling, since the 1D was not reconstructed from multiple 2D modulations across the 1D peaks. This gave us much greater flexibility and longer run times in the 2D.

Another benefit of the LC2MS4 approach was the ability to use conventional quantification for SIM and SRM analyses of fat-soluble vitamin analytes, as shown in Figure 2 (22) and Figure 3 (27) using APCI-MS on a relatively slower scanning tandem sector quadrupole (TSQ) instrument. Quantification of 2D-LC chromatograms based on the blobs in contour plots is improving, but it is still not as simple or reproducible as integrating a conventional SIM or SRM chromatogram in the 1D.

Furthermore, we were able to readily identify oxo-TAGs (Figure 3 (22)). Then, we used conventional extracted ion chromatograms (EICs) of diacylglycerol-like fragment ions, [DAG]+, and protonated molecules, [M+H]+, by APCI-MS to quantify the percent relative composition of normal and oxo TAGs in the 1D. APCI-MS mass spectra in Figure 2 showed protonated molecule peaks and some [DAG]+ fragments, depending on the degree of unsaturation (28), while ESI-HRAM-MS gave ammonium adducts. Figure 2 shows average mass spectra across the whole range of TAGs, as well as an ESI-HRAM-MS/MS spectrum of m/z 896.766, oleoyl,linoleyl,oleoestearin (OLEI), one of the most abundant TAGs in P. curatellifolia, as well as a typical single APCI-MS mass spectrum of ELEIO, all from the 1D.

Figure 2 also shows 2D-LC chromatograms by APPI-MS and ESI-MS. The more saturated colors in the LC×LC chromatogram from ESI-MS indicates its larger peak heights and greater sensitivity than APPI-MS. These chromatograms both display good peak shapes, indicating that the orthogonal natures of the two stationary phases and mobile phase solvent systems led to minimal peak broadening.

Regioisomers
TAGs have their fatty acids (FAs) arranged in different ways on the three-carbon glycerol backbone (stereospecific numbering sn-1, sn-2, and sn-3), giving rise to regioisomers. While TAG regioisomers do not separate in the 1D, and when silver-ion LC is used as the 2D, the location of unsaturation in the TAGs allows them to be separated by 2D-LC. If the unsaturation was located in the sn-2 position, it was less accessible for coordination with the silver ion, and was retained more poorly (eluted earlier) than when the unsaturation was in the outer sn-1 and sn-3 positions.

Figure S3 (in the Supplemental Information online) illustrates the dramatic separation of regioisomers using silver-ion UHPLC in the 2D of a SF-C2DLC separation. Figure S3a (in the Supplemental Information online) shows a single sharp peak in the 2D EIC for the TAG made of two oleostearic acid (El = 9Z, 11E, 13E octadecatrienoic acid, 18:3) moieties and one palmitic acid (P. hexadecanoic acid, 16:0), EIEP/PEIEI/EIEPI, by NARP-HPLC-APCI-MS at m/z 851.71.
The dual parallel MS 2D chromatograms prior to transformation into modulation periods are shown in FIGURES S3b and S3e (in the Supplemental Information online), and after transformation in the 3D chromatograms in FIGURE 2, with the peaks marked by red asterisks. In the expanded time range from 60 to 70 min for the EICs of the [M+H]+ at m/z 851.71 in FIGURE S3c (in the Supplemental Information online), and the EIC of the [M+NH4]+ at m/z 868.74 in FIGURE S3f (in the Supplemental Information online), the single peak was split into three peaks, with the last two peaks being separated by ~1.91 min. The first peak in each set is the earliest eluting regioisomer pair, EIEIP and PEIEl, which can only be separated by lengthy chiral chromatography. The second and third peaks are the later eluting isomer EIElP, which was split into two modulation periods, because of elution during a valve switch.

Because the raw 2D EICs are simple (FIGURES S3c and S3f in the Supplemental Information online), the peak areas were readily integrated and quantified, and the peak areas are shown in FIGURES S3c and S3f (in the Supplemental Information online). These areas lead to the conclusion that the EIEIP/PEIEl/EIElP regioisomers, which theoretically should be evenly distributed at 33.3%, were actually composed of EIEIP + PEIEl = 53% and EIElP = 67.0% by APPI-MS and EIEIP + PEIEl = 35.6% and EIElP = 64.4% by ESI-MS. This means there was twice as much EIElP as statistically expected. The differences between quantification of regioisomers by APPI-MS and ESI-MS have been discussed in detail elsewhere (29) and an example of 2D-LC for identification of TAG regioisomers in Jacaranda mimosifolia seed oil has been shown elsewhere (27).

LC1MS2 × (LC1MS1 + LC1MS1) = LC3MS4
Milk and infant formula analyses presented unique challenges compared to the seed oils described above. First, milk and formula contain many saturated (no double bonds in FAs) short-chain (SC) FAs that are essentially unretained on a silver-ion column, which depends on coordination of double bonds with the silver ions. Thus, silver-ion UHPLC as the 2D, described above, is ineffective for the saturated SC-FAs. Second, the 1D peaks for TAGs with SC-FAs are composed of typically one to four isomers made of different combinations of SC-FAs. Many of these are not separable using 1D NARP-HPLC, so a different approach was required for milk and formula.

We used National Institute of Standards and Technology (NIST) standard reference material 1849a adult–infant formula as a model for milk and formula analysis. After testing multiple columns with varying stationary phases on standard reference material 1849a, we found that no alternative chemistries that provided better separation in the 2D than a C30 column. FIGURE S4 shows typical 1D chromatograms from parallel APPI-MS (FIGURE S4a, S4b, and S4d in the Supplemental Information online), ESI-MS (FIGURE S4e–h in the Supplemental Information online), and the CAD (FIGURE S4c in the Supplemental Information online). The APCI-MS mass spectra of SC saturated TAGs in FIGURE S4d1 (in the Supplemental Information online) clearly showed the intact [M+NH4]+ ion at m/z 628.5 (as well as the [M+Na]+ at m/z 633.5).

Fat-Soluble Vitamins (FSVs) in the 1D
FIGURE S5 (in the Supplemental Information online) shows the chromatograms obtained from SRM of FSVs, as well as inset panels showing the resulting calibration lines from both SRM and SIM. Vitamin D₂ was added to samples before extraction, as an extraction internal standard (EIS), and menaquinone, vitamin K₂, was added to extracts before analysis as an analysis internal standard (AIS). As can be seen in FIGURE S5, most FSVs gave sharp peaks with good peak shapes. Peaks were integrated using conventional software, which overcame the problems with quantification using “blobs” in 2D chromatograms.

TAGs in 2D(1)
The first 2D used the conventional approach of having peaks elute in one modulation period. FIGURE 3 shows the contour plot and 3D plot of the 2D(1). The first feature to notice is the sharp

Figure 3: Contour plot and 3D chromatogram of first second dimension separation, 2D(1), on 5.0 cm C30 column with detection by ESI-MS on LCQ Deca XP mass spectrometer. See abbreviations in Figure 4.
and rather symmetric peaks. The TED solvent was very effective at sharpening peaks, which were broad and indistinct in the absence of TED. The 2D(1) separation did a better job of separating overlapped TAGs containing short-chain FAs from TAGs having long-chain FAs.

**TAGs in 2D(2)**

The second 2D employed a multicyle elution strategy that is analogous to twin-column recycling chromatography (TCRC) (30), which employs two columns with a switching system that repeatedly transfers analytes from one column to another to effectively increase the length of column used without increasing the back pressure.

Instead of using two different physical columns and switching between them, our approach was to employ a single column, but allow analytes to remain on the column through multiple modulation cycles, which we refer to as multi-cycle chromatography, also known as “constructive wraparound chromatography.” In these experiments, triolein eluted in the 1D in the twenty-third modulation period and eluted from the 2D(2) in the twenty-seventh modulation period.

**Figure 4** shows the contour plot for the 2D(2) separation of NIST standard 1849a TAGs, while **Figure 5** shows the associated 3D chromatogram. Even though several modulation periods elapsed, the components still produced fairly sharp and symmetric peaks, showing that the TED solvent was very effective at minimizing peak broadening despite large injection volumes and extended time on the column.

**TAG Regioisomers**

Unlike silver-ion chromatography, the NARP-UHPLC used in the 2D(2) was not effective to resolve regioisomers. Instead, critical ratios (CR) defined previously (28,29,31–33) were used for structural analysis of TAGs. CR 2 allowed us to identify regioisomers using the API-MS and ESI-MS/MS mass spectra. For instance, C10:0,C6:0,C10:0 (CaCoCa) gave a value below 50% for [CaCa]⁺, so Byrdwell CR analysis (BCRA) indicated that it was the sn-1,3 isomer. On the other hand, the TAG LaLaM (12:0;12:0;14:0) gave a [LaLa]⁺ fragment greater than 50%, so BCRA indicated that it was not the sn-1,3 isomer. It was the 1,2 and 2,3 isomers. BCRA also revealed that if “B” was shorter than “A”, then “B” was in the sn-2 position, and the isomer was ABA. Conversely, if “A” was shorter than “B”, then “B” was in the 1 or 3 position, and the isomer was AAB/ABA. Thus, the shortest FA was always in the sn-2 position, and if there were short FAs, one was always in the sn-2 position and one was in an outside (1,3) position.

For Type III TAGs, BCRA used the fact that the [DAG]⁺ with the lowest abundance was the sn-1,3 isomer (34), so was labeled as [AC]⁺. Both Type II and Type III TAGs had structures in which the shortest FAs were in the sn-2 positions.

**Flow Rate Programming**

To make the appearance of 2D (and 3D) chromatograms more appealing, flow rate programming was used to make late-eluting peaks elute a little earlier and early eluting peaks elute a little later to avoid the edges of the 2D and 3D chromatograms, as shown in **Figure 4**.

**Institutional Limitations**

Only a binary UHPLC could be configured and used in 2D OLCS version C.01.09. Also, the 2D-LC software did not allow flow rate programming like that used in the standalone 2D(2). One of the most important shortcomings, though, was the limit of 100 steps allowed in a method in the standalone 2D(2) (and the 1D), which limited our ability to emulate the shifted gradient used previously. Two 2D(2) methods had to be “stitched together” for each 1D run (data not shown). The use of a parallel gradient alleviated this shortcoming. Finally, a limit was also encountered in the complexity of 2D(1) runs using the 2D OLCS software. Complex 2D(1) methods failed to implement the gradient; it simply remained isocratic, because it overtaxed the 2D(1) binary pump.

**Conclusions**

The LC1MS2 × LC1MS2 = LC2MS4 algorithm data above clearly indicated that the combination of conventional 1D LC with dual parallel mass spectrometry, combined with SF-C2DLC allowed...
conventional quantification in the 1D using EICs, SIM, SRM, and UV. In the 2D, silver-ion UHPLC allowed separation of TAGs based on the degree of unsaturation and location, as well as type (cis/trans). But silver-ion UHPLC was not useful for short-chain saturated TAG isomers. Samples like milk and infant formula appeared to have better separations of TAGs, we had to push instrument capabilities past their designed limits. Overall, these LC–MS experiments demonstrate several benefits of split-flow comprehensive multidimensional LC. The use of a TED solvent in an open split-flow system was very easy and inexpensive to implement, while being extremely effective at keeping peaks sharp and well resolved in the 2D. The SF-CMDLC approach allowed the use of up to six detectors simultaneously in the 1D, meaning that conventional quantification could be used, and that the problems with under-sampling the first dimension to reproduce the second dimension were completely bypassed. This allowed much greater flexibility and longer run times in the 2D runs, opening new opportunities for innovative separation strategies.

**Supplemental Information**

Additional information for this article, including Figures S1 through S5, is available online at https://www.chromatographyonline.com. Please also see the end of this article for the QR code link to the supplemental information.

**REFERENCES**


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Ion mobility separations are based on fundamental principles that differ from liquid chromatography (LC) in that several parameters of ionized molecules—size, charge, shape, and structure—come into play simultaneously. As a result, analytes that have the same molecular mass can be separated by their shape, charge and collision cross section (CCS). A new development in ion mobility separation, high-resolution ion mobility (HRIM), overcomes the problem of ion loss in other ion mobility separation techniques. HRIM also provides excellent structural resolution and high reproducibility. The technique is particularly well suited to challenging applications in the pharmaceutical and clinical fields, such as glycosylation monitoring of biological drugs and vitamin D analysis.

**Melissa Sherman and Laura Maxon**

Truly disruptive advances catapult onto the scientific stage when a convergence between an emerging need and innovation occurs, thus thrusting a new technology into the limelight. High-resolution ion mobility (HRIM) based on structures for lossless ion manipulation (SLIM), originally invented in the laboratory of Dr. Richard D. Smith at Pacific Northwest National Laboratory (PNNL), provides the unprecedented capability to separate and identify molecular structures that are practically indistinguishable using traditional methods such as liquid chromatography (LC). Some of these inherently difficult to detect analyte classes include peptides, proteins, lipids and glycans. In fact, HRIM’s separation capability is powerful enough to separate and detect isobaric molecules; one particularly relevant example of this is glycan analysis of the novel coronavirus spike protein (1).

Classical analytical separation techniques, like LC, are often too slow, too experimentally challenging, or not powerful enough to resolve and structurally characterize molecules to meet the current demands for fast or high throughput commercial applications. HRIM offers an alternative that should be evaluated where appropriate to help meet the demand for fast, efficient analyses, including biologic therapeutic development and biomarker analysis.

**What is Ion Mobility?**

Ion mobility separations are based on fundamental principles that differ from liquid chromatography, in that several parameters of ionized molecules—size, charge, shape and structure—come into play simultaneously. As a result, analytes that have the same molecular mass can be separated by their shape, charge and collision cross section (CCS).

Ion mobility separation has been available for decades, but there remains concern about ion loss leading to a reduction in sensitivity, the mass range per analysis is limited, and the length of the separation path is not sufficient enough to achieve high-resolution separation. The distinguishing feature of HRIM compared with other ion mobility separation techniques is that separations are achieved essentially losslessly on very long pathlengths implemented with serpentine electrode patterns on conventional printed circuit board (PCB) technology. Digitizing separations, so to speak, on PCBs with nearly limitless pathlengths addresses the ion loss and resolution limitations of incumbent technology. With HRIM technology, ion manipulation and separation are achieved by applying electric fields to electrodes on two parallel PCBs to create an ion conduct through which ions travel without striking physical surfaces and thereby avoiding neutralization and loss that are typical to other ion mobility technologies.

The serpentine separation path design packs a single pass 40-foot ion path (in the first commercial product) into a device about the size of a laptop, addressing the resolution limitations inherent to shorter pathlength ion mobility instruments, whether multi-pass cyclic or linear, and ultimately achieves performance that enables characterizations that were previously impossible. The long pathlengths achieved with HRIM, and the foundational separation mechanism of ion mobility technology, provide additional structural information not otherwise gleaned from other separation techniques.
HRIM–MS produces higher quality data more quickly, resulting in savings for development costs and improvement of safety and efficacy for potentially faster regulatory approvals. Shown above, a 15-min LC–MS generated chromatogram of IgG control with N-glycan identifications noted, and the extracted ion mobility drift plots (or mobiligrams) from the 2-min HRIM–MS method demonstrating resolution of glycoforms not identified using the LC–MS method.

**Figure 1:** HRIM–MS produces higher quality data more quickly, resulting in savings for development costs and improvement of safety and efficacy for potentially faster regulatory approvals. Shown above, a 15-min LC–MS generated chromatogram of IgG control with N-glycan identifications noted, and the extracted ion mobility drift plots from the 2-min HRIM–MS method demonstrating resolution of glycoforms not identified using the LC–MS method.

### The Differentiators Between High-Resolution Ion Mobility and Liquid Chromatography

**Mechanism of Separation**

HRIM separates ions based on the difference in collision cross-section, size, charge density and overall shape. The sample of interest is dispersed in the gas phase using conventional electrospray ionization. As the ions enter the separation chamber, voltages applied to electrodes on the PCBs provide the lossless separation conduit through which the ions traverse along the separation pathlength. Separation is achieved because smaller CCS molecules travel faster than the larger CCS molecules and hit the detector first. Because CCS is an inherent physical property, highly reproducible results are achieved as long as pressure and temperature are controlled within the system. With separation occurring in the gas phase compared to the liquid phase with LC, separations and data collection are achieved in milliseconds not minutes or hours. Thus, dozens of samples could be processed in the time required for a single LC run.

### Analyte Agnostic: No Column Changes for Greater Instrument Uptimes

“Those LC guys need to understand a bit of magic,” is the way this author has heard it phrased. LC often necessitates matching column size, length, and packing material to specific separations and their intended purposes. In many cases, columns are dedicated to particular types of analytes. For example, one set of protein samples will be run on a different column than a set of small molecule samples. Similarly, an operator might use different column materials (positively or negatively charged, polar or non-polar) to separate different types of molecules. LC very often relies on adjusting the solvent (liquid) system to the task at hand, which may require complicated calculations, trial runs, algorithms for step or gradient changes, and so on. In reality, scientists are running an entirely different set of experiments, each time, for each analyte type, complete with lengthy experimentation to validate the method development.

Scientists who work with LC are often extremely experienced operators, sometimes like a magician in their ability to utilize workarounds and nuances to overcome stumbling blocks and achieve successful results.

In contrast, HRIM allows scientists to resolve multiple classes of analytes without component changes, as there are no hardware changes to make. Less experience is required to run experiments, and often, with less than a week’s worth of training, the experiments can be up and running. In a laboratory setting where different sample types (such as glycans, peptides, proteins, small molecules) are the mainstay, the same HRIM instrument can be used across the board. Running samples of varying analytes back-to-back is practical and simple, significantly increasing lab productivity.

### Reproducibility

With HRIM, reproducibility and achieving consistent data collection depends less on the experience of the operator. HRIM achieves separation of ionized molecules based on the physical parameters of the analytes themselves.

Since HRIM does not make use of specific columns or solvent systems, and separations are analyte agnostic, there are fewer variables to control, and the process for running HRIM requires much less user input. HRIM technology lends itself to more reproducible results because the separation mechanism is an inherent physical property with fewer variables than LC based methods. Enabling more predictable and more reproducible results across different laboratories, LC’s reproducibility is highly dependent on the preparation of buffers, solutions, and samples, method development, solvent amounts, column, flow rate, and sample size can each affect the reproducibility of the results. In fact, data reproducibility has been the most consistent comment received from HRIM’s beta users.

### Higher Peak Capacity and Resolution

To put the comparison simply, the biggest difference between HRIM and LC is the level of resolution that is achievable in a short amount of time. In situations where multiple molecules have similar elution times on the chromatogram, LC separation can take more than an hour to distinguish them effectively, whereas HRIM routinely achieves the most challenging separations in two to five minutes. HRIM eliminates the common trade-off between speed for resolution or vice versa, resolution for speed. With HRIM you can have both speed and resolution without sacrifice.

HRIM outperforms in areas that have been notoriously challenging: specifically, lipid and glycan analysis. In fact, study of these biomolecular classes has suffered from the difficulty to closely follow their biosynthesis, structural distribution, and metabolism, largely due to isomeric natures and vast structural diversity (2–4).
HRIM has come to the forefront to meet these needs. Biosimilar development provides one example of the importance of glycan analysis, where in order to achieve regulatory approval, the biosimilar glycan profile must match the originator (5). The more detailed the glycan profiles for the purpose of comparison, the higher likelihood of approval.

Collectively, scientists using HRIM can achieve unprecedented glycan separation, including distinct elucidation of isomers and other molecules with highly similar composition. Advances in glycan characterization are more important now, more than ever, given the need to understand glycosylation of human and animal viruses such as influenza, HIV, and coronaviruses. HRIM is currently being used to detail the glycosylation microheterogeneity in the spike glycoprotein that decorates the surface of the SARS-CoV-2 (Covid-19) viral capsid. It is expected that this information will aid understanding of the heterogeneity in glycosylation on this surface protein, help explain how the virus binds to its target, and aid the development of an effective treatment. This particular application of HRIM makes clear its enormous potential, especially in conditions where full resolution and fast analysis are necessary.

Method Development
Method validation is the process of proving that an analytical method is acceptable for use. Verifying that an analysis procedure will accurately and consistently deliver a reliable measurement of an active ingredient in a compounded preparation requires that the method be specific, accurate, and precise over a usable range. Furthermore, it must have an acceptable limit of detection and quantitation, and be robust enough for the demands of the experimental conditions.

Software platforms can assist with method development for LC; however, trial runs and adjustments to a myriad of variables can consume hours, if not days. In the end, the method may not be valid for all sets of conditions, or all the desired analytes.

HRIM method development is driven by software that provides real time visualization of adjustments being made to any preprogrammed workflow. Adjustments are made live, in process, significantly reducing method development time. Method development is not only faster, but methods are more easily and consistently transferred from laboratory to laboratory.

Operator Training Time
While HRIM is based on highly sophisticated chemical and electronic properties, such as collision cross-section and ion polarity, less sophisticated operators can still achieve useful separation results. Generally, scientists are able to run the instrument after only two days of training.

Good sample handling methods, understanding the experimental goals, and overall interpretation of the analytical results are always important, for both LC and HRIM. However, HRIMs ease of use allows greater focus on interpretation and analytical result evaluation, rather than the grind of running samples.

Conclusions
Liquid chromatography is the laboratory workhorse for straightforward, high throughput applications, and quantitative bioanalysis of analytes of interest.

For some routine separations and analyses, the superior resolving power of HRIM technology may not be required. Still, for other applications, HRIM’s superior resolution, straightforward method transfer, greater instrument uptime, and 5x to 60x faster analysis make it well worth evaluating. Since 2014, more than 35 papers have been published describing the scientific theory behind SLIM, as well as the evidence in support of its adoption as the new gold standard in separations technology.

The most recent publications from 2021 that demonstrate the capabilities of HRIM-MS include work from the McLean group at Vanderbilt University, where near baseline resolution of several biochemical isomers with as little as 0.6% difference in CCS was achieved (6). Another from Dr. Kim Ekoos discusses a 2-minute, LC free method to separate isomeric species from the ganglioside lipids with superior resolution and reproducibility (7). Finally, a workflow targeting biopharmaceutical characterization that demonstrates a fourfold decrease in chromatographic method times while resolving Post Translational Modifications that were undetected with LC-MS alone (8). The future of ion mobility has arrived.

Author Note
MOBIlion is the exclusive licensee of the SLIM technology for commercialization purposes.

References

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Laser Desorption Postionization Mass Spectrometry

Photoionization has long been used to probe molecular structure on a fundamental level, but it has also been used in many mass spectrometry applications. This overview describes the mechanisms of and technical requirements for molecular photoionization based on single photon, multiphoton, and ultrashort pulse strategies under analyte conditions ranging from vacuum to atmosphere. One application of photoionization is the use of laser-based single photon ionization to enhance the signal while minimizing differential detection in analyses that probe solid samples by laser desorption or ablation. This laser desorption postionization method offers the possibility of foregoing matrix application to the sample while achieving higher lateral resolution. Results are presented for femtosecond (fs) laser desorption postionization mass spectrometry (LDPI-MS) using 7.9 eV single photon ionization for the detection of drug compounds that have previously been found to have low ionization efficiencies by secondary ion mass spectrometry. Geological applications of LDPI-MS are also discussed. Finally, a few commercial implementations are mentioned.

Teodora Zagorac and Luke Hanley

Short pulse lasers with a wide range of wavelengths have been popular in mass spectrometry for desorption/ablation of solid samples. Supplementing these methods with laser postionization increases ionization yields, improves lateral resolution in mass spectrometry imaging, and generally expands analytical opportunities.

Popular Methods in Mass Spectrometry (MS) Imaging

A major goal in mass spectrometry (MS) imaging is analyzing sub-micrometer features and integrated depth profiling, but these are limited by the trade-off in established methods between chemical information and lateral resolution. The most widely used MS imaging technique is matrix assisted laser desorption–ionization (MALDI), which ionizes samples by mixing them with an organic matrix, then irradiating the resultant film with nanosecond (ns) laser pulses. Typical lateral resolution in MALDI is ~20 µm, with no capacity for in situ depth profiling, although a new implementation of atmospheric pressure MALDI has improved the resolution to a few micrometers. Although less popular than MALDI, secondary ion mass spectrometry (SIMS) was the first MS imaging method to demonstrate molecular imaging and permit depth profiling. Pulsed cluster ion beam sources have pushed the lateral resolution in SIMS of molecular species to slightly below one micrometer. Nano-SIMS uses atomic ion probes that enable much higher lateral resolution of ~50 nm, but is limited to the detection of only atomic ions, CN−, and other small fragment ions that nevertheless have been used for sophisticated molecular analyses. Desorption electrospray ionization (DESI) has been limited to ~10 µm lateral resolution without any capability for direct depth profiling. MALDI, SIMS, and DESI have all been shown to be quite powerful for MS imaging, but they also suffer from various shortcomings that can sometimes be addressed by the addition of laser postionization.

Volatilizing Analytes by Laser Desorption or Ablation

The molecular analysis of tissue, biofilms, and synthetic organic films can be achieved by laser desorption or ablation coupled with laser postionization to facilitate MS imaging with enhanced detection of neutrals. We begin by considering the initial step, where the term laser desorption (LD) generally refers to a lower energy emission of analyte into the gas phase whereas the term laser ablation (LA) refers to an explosive ejection. It should be noted that LD and LA are often used interchangeably by the MS community (for example, LD and LA are used interchangeably in MALDI, which proceeds by a more explosive mechanism).

The mechanism of analyte volatilization in LD, LA, MALDI, and laser desorption ionization (LDI) vary with wavelength, fluence, and pulse length, along with the optical properties of the sample. Pulse lengths can vary from ~10 ns to less than 100 fs; all can be used for ablation or desorption with varying results. Mechanisms depend in part on the optical properties of the target: ultraviolet nanosecond–laser desorption (UV ns-LD) of metal targets often proceeds by laser-induced thermal desorption whereas semiconductor targets can additionally undergo...
desorption induced by electronic transitions. MALDI most commonly employs UV ns-LD, but proceeds by a more complex desorption-ionization mechanism.

A more universal ablation occurs when utilizing ultrashort, <100 fs laser pulses that can ablate any material regardless of its optical properties while imparting little to no thermal damage to the underlying sample (8,10). These unique characteristics of femtosecond laser ablation (fs-LA) have led to several popular applications. Sub-100 fs lasers are used to perform fs-LA on biological cells and tissues in laser surgery with minimal damage to the adjacent tissue (10). Inductively coupled plasma-mass spectrometry (ICP-MS) also sometimes employs fs-LA to volatilize geological and biological samples for elemental analysis (11). Finally, fs-LA has been coupled to gas chromatography–mass spectrometry (GC–MS) for analyzing petroleum-bearing fluid inclusions (12). These prior applications of fs-LA have inspired its use in sampling for MS imaging, which can occur when the energy of an incident photon exceeds the ionization energy (19). This similarity implies that it may be possible to use EI-MS libraries to reliably identify LDPI-MS performed with SPI.

**Current Trends in Mass Spectrometry**

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**Time-of-flight or other mass analyzer**

**Laser desorption / ablation by 10 ns to 100 fs, UV to near IR laser pulses**

**Pulsed VUV, UV, or near IR laser postionization of neutrals firing ~10 µs after laser desorption pulse**

**Figure 1**: Schematic representation of laser desorption postionization mass spectrometry (LDPI-MS). The purple arrow follows the path of a pulsed laser beam that desorbs a solid sample to volatile neutrals that are postionized by a pulsed laser, as represented by the green arrow.

Ion Formation and Introduction to Postionization

We have so far ignored the process of ion formation in LD, LA, MALDI, and LDI. Laser desorption, ablation, and ionization are inextricably linked in MALDI and LDI. MALDI and SIMS ion yields are typically <10^{-5} (4,8,9), leaving behind considerable sample undetected and limiting lateral resolution. MALDI also requires extensive and often complex sample preparation, including matrix application, that often varies with the sample type. Ion formation in other ns-LDI-MS strategies varies widely with the sample type and preparation (8). MS imaging with fs-LDI has also been demonstrated for lipids from pancreatic tissue (13), but limited molecular ion formation makes fs-LDI-MS most promising for elemental analysis (8,14).

LDPI-MS, also known by the terms two laser MS, two-step laser MS, or laser ablation laser ionization MS (7,8,15), is an MS imaging technique that uses one laser pulse to desorb or ablate molecules from a solid sample held under vacuum and a second, temporally delayed laser pulse to photoionize the resultant plume of gaseous species, as shown in **FIGURE 1**. Non-optical methods of postionization, such as electrospray, chemical, and plasma-based ionization, have also been coupled with LD and LA to enhance ion yields beyond what is possible with MALDI or ns-LDI (16).

**Laser Postionization via Single Photon Ionization**

A common optical postionization strategy has been single photon ionization (SPI), which can occur when the energy of an incident photon exceeds the ionization energy of a gas phase molecule (8). Most organic compounds have ionization energies below ~10 eV and will undergo SPI by irradiation with vacuum ultraviolet (VUV) light of a similar or higher photon energy. One trend within organic homologous compounds is that ionization energies decrease as molecular weight increases (15). SPI has a simple mechanism and relatively low limits of detection that can reach down to the mid-low parts-per-billion range (15).

It is instructive to compare SPI with electron impact (EI), as shown in **FIGURE 2** as coupled to gas chromatography and for SPI, laser sampling. Both methods initially generate radical cations, M^+, which have long been considered to be less stable than the protonated molecules, MH^+, typically generated by electrospray and chemical ionization. For decades, EI has been used for identifying molecular species by comparison of the mass spectral fragmentation patterns of separated compounds to those of known compounds in EI-MS libraries (17). However, SPI has higher ionization efficiency than EI as well as lower fragmentation when performed with photons no more than a few eV above the ionization energy (15). This may partially explain why there have been few reports of postionization of molecular species by electron impact of molecules volatilized by LD or LA.

A recent study showed that three drug compounds that could not be easily detected by SIMS were observed using LDPI-MS, where ablation was performed with ~75 fs, 800 nm laser pulses, and SPI with ~7 ns, 7.9 eV laser pulses (see below) (18). The LDPI mass spectrum of imipramine displays higher intact precursor ion M^+ compared with its corresponding EI mass spectrum in **FIGURE 3**. Furthermore, although the most intense peaks from EI-MS were replicated by LDPI-MS, the latter showed overall less fragmentation. The similarity between LDPI-MS and EI-MS was observed with the two other compounds successfully examined in this study (18). SPI-MS often displays major fragments as similar to those seen in EI-MS, but almost always displays the M^+ that can be missing from EI-MS of labile compounds and additionally leads to a lower overall extent of fragmentation (19). This similarity implies that it may be possible to use EI-MS libraries to reliably interpret LDPI-MS performed with SPI.
The use of SPI for postionization in LDPI-MS is limited by the performance of sources of pulsed VUV radiation. The most convenient laboratory VUV source for LDPI-MS is the 157 nm fluorine excimer laser. Fluorine lasers are reasonably priced and reliable. Their intensities are in the range of ~1 mJ/pulse, allowing high efficiency detection via saturation of SPI. Furthermore, their pulse repetition rate is ~500 Hz, high enough for rapid imaging of relatively large sample areas. However, the fluorine laser photon energy is only 7.9 eV, generally limiting SPI to molecules such as polyaromatic hydrocarbons, tertiary amines, some fused ring drug compounds (15), and certain clustered ion species (20).

SPI of the wider array of organic compounds with higher ionization energies is possible with a VUV source that displays a correspondingly higher photon energy. Third harmonic generation of the 555 nm output from a standard ns pulsed Nd:YAG laser produces 118 nm wavelength (10.5 eV photon energy) laser pulses that serve as the more universal source for SPI alluded to above (15). However, this strategy produces <1 µJ/pulse that cannot saturate SPI and leads to low overall ionization efficiency. The low repetition rates of the high power Nd:YAG lasers needed for VUV generation also limit imaging speed. Finally, the low intensity VUV beam is difficult to manipulate for inexperienced personnel and requires considerable engineered improvements before it can be readily used for general analytical applications.

Overall, expanding the use of SPI in LDPI-MS requires a more robust laboratory source of pulsed VUV radiation with ~10 eV photon energy, ~1000 pulse rate, and at least 100 µJ/pulse. High harmonic generation from ultrashort pulse lasers might one day fulfill these requirements if considerably higher intensity VUV pulses can be generated (21).

**Postionization Using Multiphoton to Strong-Field Ionization**

An alternative postionization to SPI is to instead utilize multiphoton strategies. SPI yields are linearly dependent on the number of photons, but resonance-enhanced multiphoton ionization (REMPI) becomes possible with exponentially higher laser intensities, in the range of $10^7$–$10^{11}$ W/cm$^2$ (9,22). Furthermore, the excitation pathway in REMPI proceeds through at least one intermediate excited state.

**FIGURE 4a** depicts the simplest form of REMPI: Two photon resonant ionization where one photon excites to an intermediate state via a strong optical absorption (which can be observed in UV-vis absorption spectroscopy), then a second photon excites out of the intermediate state to form a radical cation. An example of a resonant two photon ionization would be using 266 nm, ns laser pulses to excite a $\pi$-$\pi^*$ transition in a polyaromatic hydrocarbon like anthracene, then excite out of the $\pi^*$ state to form an ion. The two photons collectively impart 9.5 eV of energy to the polyaromatic hydrocarbon, well in excess of its ionization energy. The specific molecular electronic structure determines the types of REMPI pathways possible, the requisite laser wavelengths, and the extent of dissociation of the precursor.

REMPI is traditionally performed with ns-laser pulses, but transitioning to shorter laser pulses can reduce fragmentation that often occurs through other pathways out of the intermediate states (9,22). Femtosecond-laser MS is performed with pulses shorter than a picosecond that lead to less dissociation in REMPI and can open up new ionization pathways less dependent on specific molecular structure (23). Femtosecond laser MS can also proceed by non-resonant multiphoton ionization (NRMPI) when yet higher laser intensities ~$10^{15}$ W/cm$^2$ are used and has the added advantage that it does not require the laser wavelength to be resonant with an excited state (**FIGURE 4b**) (9).

The higher laser intensities that allow NRMPI, when accessed via sub-100 fs IR laser pulses, can also lead to strong-field ionization (9,24). Strong-field ionization can occur by tunneling through the barrier to ionization via high intensity electric field-induced distortion of molecular electronic states induced by ultrashort laser pulses (9). Pulse duration, laser intensity, wavelength, and molecular electronic structure control how a specific molecule will undergo strong-field ionization. **FIGURE 5** shows how structural isomers can be quantified in mixtures using a dual pulse fs IR laser technique in which a pump fs laser pulse ionizes the mixture and creates a radical cation in a ground state, then a time-delayed probe laser pulse (from the same laser) induces dissociation (24). The nitrotoluene ion signal varies with pump-probe delay time for its structural isomers $\sigma^*$, $m^*$, and $p^*$-nitrotoluene (ONT, MNT, and PNT, respectively). Preliminary results found that the relative concentration of each isomer in binary and ternary mixtures could be determined to better than 10% accuracy without any preliminary use of chromatographic separation. Such a strategy would be very powerful when applied to LDPI-MS imaging, which like most MS imaging methods lacks a chromatographic front end. Femtosecond-laser MS also potentially allows coupling with sophisticated computational modeling of the ionization event that might find predictive utility in analysis (24).

**Applications of LDPI-MS**

Nanosecond-LDPI-MS has found applications in analysis of biofilms, tissue samples, organic electronics, and geological samples (7,8,15,16). For example,
LDPI-MS was able quantify antibiotics in biofilms and differentiate biofilms of different strains of E. coli (7,8).

A version of LDPI-MS is under consideration for incorporation into space flight instruments being developed for analysis of icy moons, comets, and carbonaceous asteroids (25). The molecular analyzer for complex refractory organic-rich surfaces (MACROS) is an instrument package utilizing ns-pulse lasers that has been developed at the NASA Goddard Space Flight Center. One of MACROS’ four basic operation modes for in situ sample analysis uses ns pulsed 266 nm IR laser for desorption and a ns pulsed laser for postionization via REMPI. L2MS is considered a “detailed” mode in MACROS because it does not require sample preparation, has a lower limit of detection, and is highly sensitive to detection of aromatic organic molecules of great interest to the mission.

The application of LDPI-MS using fs-laser ablation is at an earlier stage (8), but has already been shown to allow imaging of an organic electronic compound at a lateral resolution of 2 µm by taking advantage of the nonlinearity of fs-LA with laser intensity (8). Alternatively, the application of experimentally robust fs laser postionization to LDPI-MS will allow faster imaging and analysis of a wide range of compounds. Femtosecond laser MS has been coupled with gas chromatography for the ultrasensitive analysis of explosives (23,24) and pesticides (26), indicating the promise of its use in postionization for LDPI-MS.

**Postionization via Photoionization at Elevated Pressures**

We have focused on LDPI-MS performed with the sample, desorption, ablation, and postionization all occurring under high vacuum. However, there are several advantages to performing the analogous analysis at atmospheric pressure (8). The sample does not dehydrate and does not need to undergo the slow evacuation process required in traditional mass spectrometers. The plume of species desorbed or ablated from a sample immediately collides with air molecules, which enhances dissociation of a larger species, slowing down molecular velocities and possibly leading to oxidation (this final event also possibly being a disadvantage). Atmospheric pressure photoionization is most commonly initiated with SPI by VUV radiation and subsequently leads to chemical–ionization-like events that can result in the formation of protonated molecules instead of radical cations (27).

Such protonation processes show great potential for expanding the breadth of LDPI-MS applications and may have driven the application of non-laser based postionization (16). Finally, most commercial mass spectrometers currently have atmospheric pressure interfaces, so coupling a LDPI front end to them avails one of their exquisite performance in terms of mass resolution and accuracy as well as tandem capabilities.

### Commercial Instruments

Utilizing any analytical method relies on its commercial availability, but such options for LDPI-MS remain limited as of this writing. MALDI-2 is a method that uses a ns-pulse UV postionization laser to induce secondary ionization in the gas phase of plumes ablated by MALDI (28). Postionization in MALDI-2 occurs at intermediate pressures within an ion mobility device, leading to the de-clustering of higher mass species formed by MALDI. The pressure-dependent mechanism of MALDI-2 is probably more akin to that of atmospheric pressure photoionization than direct laser photoionization of a molecule isolated in vacuum. Perhaps foremost amongst the impressive advantages of UV laser postionization in MALDI-2 is that it enhances signal intensities of membrane lipids and other species detected in a tissue sample by up to two orders of magnitude over those observed by tra-
The development of LDPI-MS and related methods will continue to be driven by the rapid improvement and lowered costs of sub-ns-pulsed lasers, but requires coupling to higher performance mass analyzers to increase user acceptance. Readers interested in a more rigorous discussion of laser postionization are referred to a recent book on the fundamentals and the entire range of applications of photoionization in mass spectrometry (9).

Acknowledgments

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Figures

Figure 5: Time dependent pump-probe experiments used for quantification of structural isomers in a mixture. Nitrotoluene ion signal varies with pump-probe delay time for its structural isomers α-, m-, and p-nitrotoluene (ONT, MNT, and PNT, respectively).
A Quick Approach to Screen Oligomers from Extractables Studies Using Liquid Chromatography–Quadrupole Time-of-Flight Mass Spectrometry (LC–QTOF-MS)

Examination of extractables and leachables (E&L) is of great importance to patient protection and regulatory requirements. Accurate and rapid identification of unknown compounds in E&L is often difficult due to the wide range of additives, oligomers, and degradation products from the plastic material. Oligomers often exist at high percentages in extracted samples, making them a critical aspect of data analysis. Furthermore, oligomers can exhibit either linear or cyclic forms with a variable ratio of composing monomers, making it difficult to identify oligomers with a general formula. Toward this end, we propose a screening method for categorizing polymeric oligomers, utilizing their common fragment structure ions. First, a total ion chromatogram (TIC) of samples in both MS and elevated energy MS (MS^E) mode are acquired. Next, oligomers’ characteristic fragment ions with known mass ion values, are extracted to give extracted ion chromatograms (EICs). These oligomer peaks are quickly categorized and identified without library matching. Known polyethylene terephthalate (PET) fragments including [OCC_6H_4CO_2]^+ and [OCC_6H_4CO_2C_2H_4OH]^+ were utilized. Out of a total of nineteen extractable peaks, thirteen PET oligomers were identified with this oligomer screening approach. This new approach greatly optimizes the accurate identification of categorized oligomers and enables a more precise E&L safety assessment.

Bin Sun, Owen Perlowski, and Benben Song

The implementation of single-use technology (SUT) has been a growing trend across the field of pharmaceutical production, due to cost effectiveness and operating efficiency advantages over traditional stainless-steel systems. At the same time, extractables and leachables (E&L) from plastic single-use components pose risks of leaching into solutions, potentially compromising process conditions and safety for the patient. Bis(2,4-di-tert-butylphenyl) phosphate (bDtBPP), a common antioxidant additive in plastic bioreactors, showed toxicity to cell culture growth. Levels as low as 1 mg/L of bDtBPP in solution led to over 90% cell growth reduction (1). E&L are getting more attention in the biopharmaceutical industry, to the point where general requirements for E&L safety assessments have been mandated by regulatory agencies. USP (2) and BioPhorum Operations Group (BPOG) (3) have published standardized extractables protocols and industry guidelines on E&L testing and safety assessments. With increased concern and regulation, E&L testing systems and safety assessments are being developed by pharmaceutical industry members and CRO companies (4,5). As raw materials and manufacturing processes of single-use components and systems change, it is critical to evaluate the safety profile and potential impacts of new E&L compounds.
As a supplier and integrator of SUT, Pall Corporation takes on increased responsibilities to ensure delivery of both high quality and safe materials to end-users and patients. We established a standardized E&L testing protocol and risk assessment strategy based on USP and BPOG guidelines. This protocol covers the extract sample preparation, extraction conditions, data recording, and results reporting. A full analytical chemistry testing procedure has been created to analyze organic volatiles and non-volatiles, as well as trace elements. This procedure includes ultraviolet-visible spectrophotometry (UV-vis), Fourier transform infrared spectroscopy (FT-IR), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS), and inductively coupled plasma–mass spectrometry (ICP-MS).

An array of polymer resins is used for SUT including polyethylene (PE), polypropylene (PP), polycarbonate (PC), polyethersulfone (PES), silicones, and fluoropolymers. Typical E&L are from plastic additives, rinsing solvents, colorants, lubricants, and other compounds related to the materials of construction in single-use components. With such a large pool of E&L compounds, an extensive internal library is needed for analytical screening. As single-use components and systems contain plastics, sterilization procedures, such as heat and gamma irradiation, can cause degradation to these materials. These processes produce oligomers, such as nylon 6,6 oligomers and polyethylene terephthalate oligomers (FIGURE 1). The degraded compounds may end up in the process solution and pose risks to drug potency and safety.

After gamma irradiation or heat sterilization of the single-use components, these degraded oligomers bear a variety of functional groups. In some cases, there are no comprehensive E&L libraries nor literature resources for these oligomeric structures. Additionally, an interference is seen between the oligomers and other plastic additives in chromatography. All these factors make compound identification and structure elucidation of the oligomers challenging. With the structures of certain E&L compounds remaining uncertain, suppliers and drug manufacturers expend more resources on advanced analytical data, E&L safety assessments, and toxicological studies with patient safety and process efficacy at the forefront.

Here, we propose an oligomer screening approach to group and identify these oligomeric compounds based on type of polymer and fragment ions. Unequivocal identification of specific oligomers against the library or by structure elucidation is conducted in the subsequent step. This new approach greatly optimized the accurate identification of categorized oligomers. It increases confidence in identification to facilitate toxicological and safety assessment.

![Figure 1: Examples of oligomers/degradants from plastic resins: (a) polyethylene terephthalate oligomers; (b) nylon 6,6 oligomers.](image)

![Figure 2: Pall extraction set-up (PFA= perfluoro-alkoxy alkane); figure from reference (6).](image)
risk assessment of oligomeric extractables. A screening of PET oligomers in a filter extracted sample is used as a case study to demonstrate this approach.

Materials and Methods
A 50% ethanol solution was selected as extraction solvent for a nylon filter with polyester drainage and support layer. It is an aggressive extraction solvent, where high level of oligomeric species has been found in the extracted samples. The majority of these oligomers come from the filter membranes or the support and drainage layer materials, as they constitute the largest surface area in contact with the extraction solvent. The extraction system is set up with temperature control and proper thermal isolation (Figure 2). Both the pump and tubing are made from inert materials to avoid introducing contamination. The extraction solvent was recirculated through the filter and the system. A separate extraction was concurrently performed without filters to generate a negative control. The extract samples are then injected to Waters Xevo G2S QTOF MS (UHPLC) for further analysis. Chromatograms with MS and MS² information under low and high collision energy (CE) are acquired. The high CE chromatogram with MSE provides us fragmentation details of the analytes.

Results and Discussion
Polyethylene terephthalate (PET) is one of the most common thermoplastic polymer resins of the polyester (PE) family (9), composed of repeating unit $\text{C}_10\text{H}_8\text{O}_4$ (192.0423 Da). As we investigated the mass spec-

Figure 3: The mass spectra of (a) $[\text{TG}]_3$ and (b) $[\text{TG}]_2$-$\text{G}$ in 50% ethanol extract sample under high collision energy (CE) mode. The x-axis label is observed mass (m/z) and y-axis is intensity (counts).

Figure 4: Chromatograms of an extract sample of filter comprising PET resin: (a) total ion chromatogram under low collision energy (CE); (b) extracted ion chromatogram (EIC) of 149.0231 m/z $\text{C}_8\text{H}_5\text{O}_3^+$ under high CE; (c) EIC of 193.0495 m/z $\text{C}_{10}\text{H}_9\text{O}_4^+$ under high CE; (d) EIC of 385.0914 m/z $\text{C}_{20}\text{H}_{17}\text{O}_8^+$ under high CE.
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**Table I: Proposed structures of PET oligomers in 50% ethanol extract sample**

<table>
<thead>
<tr>
<th>PET Oligomers</th>
<th>Structure</th>
<th>Formula</th>
<th>MW</th>
<th>Molecular ion (m/z)</th>
<th>Fragment ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis(2-hydroxyethyl) terephthalate</td>
<td>H-G-[TG]-OH</td>
<td>C_{12}H_{14}O_{6}</td>
<td>254.0790</td>
<td>255.0862</td>
<td>149.0231, 193.0495</td>
</tr>
<tr>
<td>G capped PET linear dimer</td>
<td>H-G-[TG]_{2}-OH</td>
<td>C_{22}H_{22}O_{10}</td>
<td>446.1213</td>
<td>447.1285</td>
<td>149.0231, 193.0493, 385.0914</td>
</tr>
<tr>
<td>G capped PET linear trimer</td>
<td>H-G-[TG]_{3}G-OH</td>
<td>C_{32}H_{30}O_{14}</td>
<td>638.1636</td>
<td>639.1710</td>
<td>149.0233, 193.0494</td>
</tr>
<tr>
<td>PET linear monomer</td>
<td>H-[TG]-OH</td>
<td>C_{16}H_{16}O_{5}</td>
<td>210.0528</td>
<td>211.0596</td>
<td>149.0231, 193.0495</td>
</tr>
<tr>
<td>PET linear dimer</td>
<td>H-[TG]-OH</td>
<td>C_{20}H_{18}O_{9}</td>
<td>402.0951</td>
<td>403.1019</td>
<td>149.0231, 193.0491, 385.0913</td>
</tr>
<tr>
<td>PET linear trimer</td>
<td>H-[TG]_{2}-OH</td>
<td>C_{30}H_{26}O_{13}</td>
<td>594.1373</td>
<td>595.1451</td>
<td>149.0232, 193.0492, 385.0914</td>
</tr>
<tr>
<td>PET cyclic dimer</td>
<td>[TG]_{2}</td>
<td>C_{20}H_{16}O_{5}</td>
<td>384.0845</td>
<td>385.0911</td>
<td>149.0231, 193.0491</td>
</tr>
<tr>
<td>PET cyclic trimer</td>
<td>[TG]_{3}</td>
<td>C_{30}H_{24}O_{12}</td>
<td>576.1268</td>
<td>577.1347</td>
<td>149.0232, 193.0495, 341.0643</td>
</tr>
<tr>
<td>PET cyclic tetramer</td>
<td>[TG]_{4}</td>
<td>C_{40}H_{32}O_{16}</td>
<td>768.1690</td>
<td>769.1772</td>
<td>149.0231, 193.0491</td>
</tr>
<tr>
<td>G inserted PET cyclic monomer</td>
<td>[TGG]</td>
<td>C_{12}H_{12}O_{5}</td>
<td>236.0685</td>
<td>237.0755</td>
<td>149.0233, 193.0494</td>
</tr>
<tr>
<td>G inserted PET cyclic dimer</td>
<td>[TGG]_{2}</td>
<td>C_{24}H_{24}O_{10}</td>
<td>472.1369</td>
<td>473.1440</td>
<td>149.0231, 193.0491, 341.0647</td>
</tr>
<tr>
<td>Single G inserted PET cyclic dimer</td>
<td>[TG]_{3}G</td>
<td>C_{22}H_{20}O_{9}</td>
<td>428.1107</td>
<td>429.1180</td>
<td>149.0234, 193.0495, 385.0917</td>
</tr>
<tr>
<td>Single G inserted PET cyclic trimer</td>
<td>[TG]_{4}G</td>
<td>C_{32}H_{26}O_{13}</td>
<td>620.1530</td>
<td>621.1608</td>
<td>149.0234, 193.0495, 385.0897</td>
</tr>
</tbody>
</table>

* T refers to terephthalate unit -(OOC(H)CO)-; G refers to ethylene glycol unit -(OCH(OH)CH)-
* The mass error between the measured value and calculated value is within 2 mDa

**Figure 5:** A stream map of oligomer screening approach.

---

The mass spectrometry profile of the 50% ethanol extract sample, thirteen structures in a series of PET oligomers have been proposed based on accurate mass of parent ions and fragment ions summarized in **Table I** with literature backup (10). The mass error between the measured value and calculated value was within 2 mDa. PET cyclic trimer [TG]_{3} was found at 6.80 min with parent ions of [M+H]^+ and [M+Na]^+ under low CE mode. Fragment ions of [TG]_{3}, 149.0231 m/z C_{16}H_{12}O_{4}^+, 193.0495 m/z C_{10}H_{9}O_{4}^+, and 385.0914 m/z C_{20}H_{17}O_{8}^+ were found with high intensity in the high CE mode (FIGURE 3). PET cyclic dimer with one ethylene glycol unit inserted into the PET cyclic dimer ([TG]_{2}-G) was also observed in the form of H^+ and Na^+ adducts under low CE mode, with identical C_{8}H_{6}O_{3}^+, C_{12}H_{11}O_{4}^+, and C_{20}H_{17}O_{8}^+ fragments in high CE mode. These three fragments were also seen in several other peaks under high CE mode. A 2 µg/mL PET cyclic trimer [TG]_{3} authentic standard was spiked and injected. The presence of C_{20}H_{12}O_{4}^+, C_{10}H_{9}O_{4}^+, and C_{20}H_{17}O_{8}^+ fragments have further confirmed them as characteristic fragments of PET oligomers. Knowing these characteristic fragments, we can rapidly screen out PET oligomers in extract samples of filters comprising its polymer resin. An example is shown in **FIGURE 4**. Extracted ion chromatograms (EICs) were obtained by extracting C_{20}H_{12}O_{4}^+, C_{10}H_{9}O_{4}^+, and C_{20}H_{17}O_{8}^+ characteristic fragment ions (±0.5 Da range) in the chromatogram, respectively. Comparing the TIC with the EICs, these peaks between 4.8 and 6.6 min in TIC (in green) were shown to have intense C_{8}H_{6}O_{3}^+, C_{12}H_{11}O_{4}^+, and C_{20}H_{17}O_{8}^+ fragment ion peaks in EIC under high CE mode. This comparison demonstrates that they produce characteristic fragments of PET oligomers and bear the oligomeric functional groups in structure. These compounds were then identified to be **PET-related oligomer**. Further library matching of parent ions and structure elucidation was conducted in a subsequent step to unequivocally identify each PET-related oligomer. Additionally, this oligomer screening approach is not limited to PET oligomers; it also applies to siloxane and nylon oligomers (11).
samples in both MS and MSE mode are acquired. Second, oligomers’ characteristic fragment ions are proposed from polymer structure or looked up in literature. With known mass ion values of the characteristic fragment ions, EICs can be obtained. The peaks in EIC may suggest the presence of oligomeric functional groups in structure. Further identification of the compounds can be conducted with the help of library search and structure elucidation. If proposed library matches yield incomplete or inconsistent results in the subsequent step, these oligomer peaks can be quickly categorized and identified based on family of polymers, even though it is not an unequivocally ID of each PET oligomer. This new approach significantly decreases processing time on oligomer screening, and optimizes the accurate identification of categorized oligomers. It yields thorough screening and accurate identification of E&L compounds, and then provides meaningful E&L risk assessments. Furthermore, a good E&L risk assessment gives SUS suppliers and their customers confidence in their product portfolio.

Chemical safety risk assessment is the process by which the potential adverse patient safety impact of E&L is determined and quantified. A patient’s total daily intake (TDI) level is determined by considering E&L exposure from the clinical use of the drug product. Based on the leachable’s toxicity, a permitted daily exposure (PDE) is established. If the TDI is less than the PDE, the leachable is deemed to be safe. Equations 1 and 2 above show examples of a safety assessment on PET related oligomer with a total of 4 µg/mL in the extract sample from an extractables study. The sample was extracted from one filter at 30 °C for 24 h, which brackets end-users process conditions for manufacturing of a drug product intended for 10 mL/day administration. TDI was calculated considering the design of the extraction study, dilution volume in the manufacturing process, and daily dosage (2). Toxicologists determine the PDE for human exposure based on available toxicological information, body weight, and applicable modifying factors (12). In this example, the PDE of a PET oligomer is 2000 µg/day, which is significantly higher than the estimated TDI 16 µg/day. The margin of safety (PDE/TDI) is greater than 1 for a PET-related oligomer identification, yielding a negligible adverse impact. If this compound were an unknown, it would have to be identified as higher than acceptable safety concern thresholds (for example, 1.5 µg/day).

Conclusion
With this oligomer screening approach, 13 PET oligomers and 3 nylon oligomers were identified successfully by extracting common fragments. These oligomer peaks are quickly screened and categorized before library matching. This new approach greatly optimized the accurate identification of categorized oligomers. It enables a more precise and meaningful E&L safety assessment. Our future work is to expand this oligomer screening approach to a wider range of polymeric materials and build a comprehensive E&L library of oligomers from SUT.

Acknowledgments
The authors would like to thank Gilbert Tumambac for helpful technical reviews of the manuscript. The authors are also grateful to Wenan Lu, Emily Volk, Kuang-Wei Yang and Jin Ren for their contributions in the early stages of this project. The authors would like to give a final thanks to Rebecca Kale for facilitating the revision process.

References


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Interested in more content like this? Subscribe to our newsletters!
A mobile proton transfer reaction–time of flight–cube design (PTR-TOF QB) analyzer was deployed to map smoke plume from landfill fire, tracking air pollution where and when it matters. Ionicon real-time volatile organic compound (VOC) analyzers are frequently operated in mobile-measurement laboratories, contributing to local air quality networks and public safety.

University of Oslo’s Ionicon PTR-MS laboratory tracks impact of toxic organic gases
Landfill fires occur frequently and toxic emissions from such fires often constitute a major public health concern. In the outskirts of Stockholm, a 100,000-ton landfill was on fire in late 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.

After being contacted by their Swedish colleagues, researchers from the University of Oslo (UiO) in Norway offered rapid help, overcoming borders closed 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 laboratory smoke plume monitoring
The measurements revealed high concentrations of benzene and styrene, and 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 analyzer for this important, time-critical measurement campaign. The PTR-TOF QB allows for straightforward integration for urgent deployment into makeshift mobile laboratories such as regular passenger cars. Of course, the PTR-TOF QB can also be integrated into existing 19-in racks in 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.

Ionicon Analytik GmbH
Eduard-Bodem-Gasse 3, 6020 Innsbruck, Austria
Tel.: +43 512-214-800
Website: www.ionicon.com
Oligonucleotides are becoming more prevalent in biotechnological research and development and in commercial production. However, the separation of the active or desired oligonucleotide sequence and length requires highly sensitive instrumentation such as a liquid chromatography system coupled with mass spectrometry (LC–MS). Moreover, separation must be performed with an efficient chromatography column. Previously, ion pair reagents were used in conjunction with reverse phase chromatography. However, these reagents can cause several issues including difficulty flushing the reagent from the LC system, lower system-to-system reproducibility for validated methods and ion pair reagents may interfere with separations of labeled oligonucleotides (1).

The new Shodex™ HILICpak™ VN-50 series columns are polyvinyl alcohol-based columns with diol functional terminal groups. A new method was developed using the VN-50 2D (2.0 × 150 mm) wherein the diol groups successfully perform the separation without the need of an ion pair reagent. A gradient mixture of acetonitrile and relatively low concentration volatile solvent was used.

The separation was performed using a Shodex HILICpak VN-50 2D column on a Shimadzu Nexera LCMS-8030 Plus. A photodiode array detector was used in conjunction with the mass spectrometer in SIM negative mode. A 1 uL sample of several synthesized oligonucleotides were injected at a concentration of 1 mg/mL and introduced into the MS via electrospray ionization (ESI). Samples included oligo-DNA (20-mer), phosphorothioated oligo-DNA (20-mer), and phosphorothioated oligo-RNA (19-mer, 20-mer and 21-mer), all synthesized. The mobile phase consisted of a mixture of 50 mM aqueous ammonium formate (pH 9.8) and acetonitrile. A gradient was used for elution. Mixture A consisted of 64% acetonitrile progressing linearly to 57% acetonitrile from 10 to 20 min and back to 64% acetonitrile at 25 minutes. The flow rate was 0.3 mL/min.

The column successfully separated all three lengths of phosphorothioated oligo-RNA. Moreover, both oligo-DNA and phosphorothioated oligo DNA were successfully separated. The overlay in Figure 1 shows the complete separation of all species by PDA at 260 nm. Most impressively, the column was able to separate the phosphorothioated oligo-DNA from the phosphorothioated oligo RNA due to the higher retention of the oligo-RNA. All compounds were separated by increasing degree of polymerization.

The Shodex HILICpak VN-50 series, polymer-based diol type HILIC columns, demonstrated highly selective LC/UV/ESI-MS measurements. The oligonucleotides were separated and eluted in the order of smaller to higher degree of polymerization. The gradient setting with a mixture of 50-mM aqueous ammonium formate solution and acetonitrile enabled the analysis without a need of ion-pair reagents for reversed phase mode or highly concentrated salt for ion exchange mode generally used.

The developed method will also be beneficial for preparative purposes, as it does not require de-salting process. Phosphorothioated oligonucleotides are more often chosen for oligonucleotide therapeutics due to their higher stability compared to regular oligonucleotides. The high sensitivity of this technique will be invaluable for preparative phosphorothioated oligonucleotide separations. The results presented in this work proved that the VN-50 series columns are expected to be effective tools for the development and quality control of oligonucleotide therapeutics.

Reference
Analysis of Fentanyl and Its Analogues in Human Urine by LC–MS/MS

Shun-Hsin Liang and Frances Carroll, Restek Corporation

Abuse of synthetic opioid prescription painkillers such as fentanyl, along with a rapidly growing list of illicit analogues, is a significant public health problem. In this study, we developed a simple dilute-and-shoot method that provides a fast 3.5-min analysis of fentanyl and related compounds (norfentanyl, acetyl fentanyl, alfentanil, butyryl fentanyl, carfentanil, remifentanil, and sufentanil) in human urine by liquid chromatography–tandem mass spectrometry (LC–MS/MS) using a Raptor Biphenyl column.

In recent years, the illicit use of synthetic opioids has skyrocketed, and communities worldwide are now dealing with an ongoing epidemic. Of the thousands of synthetic opioid overdose deaths per year, most are related to fentanyl and its analogues. With their very high analgesic properties, synthetic opioid drugs such as fentanyl, alfentanil, remifentanil, and sufentanil are potent painkillers that have valid medical applications; however, they are also extremely addictive and are targets for abuse. In addition to abuse of these prescription drugs, the current opioid crisis is fueled by a growing number of illicit analogues, such as acetyl fentanyl and butyryl fentanyl, which have been designed specifically to evade prosecution by drug enforcement agencies.

As the number of opioid drugs and deaths increases, so does the need for a fast, accurate method for the simultaneous analysis of fentanyl and its analogues. Therefore, we developed this LC–MS/MS method for measuring fentanyl, six analogues, and one metabolite (norfentanyl) in human urine. A simple dilute-and-shoot sample preparation procedure was coupled with a fast (3.5 min) chromatographic analysis using a Raptor Biphenyl column. This method provides accurate, precise identification, and quantitation of fentanyl and related compounds, making it suitable for a variety of testing applications, including clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

### Experimental Conditions

#### Sample Preparation

The analytes were fortified into pooled human urine. An 80 µL urine aliquot was mixed with 320 µL of 70:30 water–methanol solution (fivefold dilution) and 10 µL of internal standard (40 ng/mL in methanol) in a Thomson SINGLE StEP filter vial (Restek cat. #25895). After filtering through the 0.2 µm PVDF membrane, 5 µL was injected into the LC–MS/MS.

#### Calibration Standards and Quality Control Samples

The calibration standards were prepared in pooled human urine at 0.05, 0.10, 0.25, 0.50, 1.00, 2.50, 5.00, 10.0, 25.0, and 50.0 ng/mL. Three levels of QC samples (0.75, 4.0, and 20 ng/mL) were prepared in urine for testing accuracy and precision with established calibration standard curves. Recovery analyses were performed on three different days. All standards and QC samples were subjected to the sample preparation procedure described.

LC–MS/MS analysis of fentanyl and its analogues was performed on an ACQUITY UPLC instrument coupled with an ACQUITY UPLC–Q-TOF MS system (Waters Corp.). The LC conditions were optimized for separation of all target compounds. The mobile phase consisted of ultra-pure water and methanol (70:30) with 0.1% formic acid, and the flow rate was set at 0.3 mL/min. The column used was a Raptor Biphenyl column (Restek Corporation). The temperature was maintained at 40°C, and the injection volume was 5 µL. The ionization source was set to electron-spray ionization in positive mode, and the acquisition parameters were optimized to obtain the best signal-to-noise ratio for all compounds.

### Table I: Analyte transitions

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Precursor Ion</th>
<th>Product Ion Quantifier</th>
<th>Product Ion Qualifier</th>
<th>Internal Standard</th>
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<tbody>
<tr>
<td>Norfentanyl</td>
<td>233.27</td>
<td>84.15</td>
<td>56.06</td>
<td>Norfentanyl-D$_5$</td>
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<tr>
<td>Acetyl fentanyl</td>
<td>323.37</td>
<td>188.25</td>
<td>105.15</td>
<td>Acetyl fentanyl-$^{13}$C$_6$</td>
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<tr>
<td>Fentanyl</td>
<td>337.37</td>
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<td>105.08</td>
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<td>Butyryl fentanyl</td>
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<td>188.20</td>
<td>105.15</td>
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<tr>
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<td>317.30</td>
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<td>84.15</td>
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<td>—</td>
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<tr>
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<td>329.37</td>
<td>188.25</td>
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<td>—</td>
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<tr>
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<td>188.27</td>
<td>—</td>
<td>—</td>
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<tr>
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<td>238.25</td>
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<td>—</td>
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<td>400.40</td>
<td>340.41</td>
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</table>
with a Waters Xevo TQ-S mass spectrometer. Instrument conditions were as follows, and analyte transitions are provided in Table I.

Analytical column: Raptor Biphenyl (5 µm, 50 mm x 2.1 mm; cat. #9309552)
Guard column: Raptor Biphenyl EXP guard column cartridge, (5 µm, 5 mm x 2.1 mm; cat. #930950252)
Mobile phase A: 0.1% Formic acid in water
Mobile phase B: 0.1% Formic acid in methanol
Gradient
<table>
<thead>
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<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
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<tr>
<td>2.50</td>
<td>70</td>
</tr>
<tr>
<td>2.51</td>
<td>30</td>
</tr>
<tr>
<td>3.50</td>
<td>30</td>
</tr>
</tbody>
</table>
Flow rate: 0.4 mL/min
Injection volume: 5 µL
Column temp.: 40 °C
Ion mode: Positive ESI

Results

Chromatographic Performance
All eight analytes were well separated within a 2.5-min gradient elution (3.5-min total analysis time) on a Raptor Biphenyl column (Figure 1). No significant matrix interference was observed to negatively affect quantification of the fivefold diluted urine samples. The 5-µm particle Raptor Biphenyl column used here is a superficially porous particle (SPP) column. It was selected for this method in part because it provides similar performance to a smaller particle size fully porous particle (FPP) column, but it generates less system back pressure.

Linearity
Linear responses were obtained for all compounds and the calibration ranges encompassed typical concentration levels monitored for both research and abuse. Using 1/x weighted linear regression (1/x² for butyryl fentanyl), calibration linearity ranged from 0.05 to 50 ng/mL for fentanyl, alfentanil, acetyl fentanyl, butyryl fentanyl, and sufentanil; from 0.10 to 50 ng/mL for remifentanil; and from 0.25 to 50 ng/mL for norfentanyl and carfentanil. All analytes showed acceptable linearity with r² values of 0.996 or greater and deviations of <12% (<20% for the lowest concentrated standard).

Accuracy and Precision
Based on three independent experiments conducted on multiple days, method accuracy for the analysis of fentanyl and its analogues was demonstrated by the %recovery values, which were within 10% of the nominal concentration for all compounds at all QC levels. The %RSD range was 0.5–8.3% and 3.4–8.4% for intraday and interday comparisons, respectively, indicating acceptable method precision (Table II).

Conclusions
A simple dilute-and-shoot method was developed for the quantitative analysis of fentanyl and its analogues in human urine. The analytical method was demonstrated to be fast, rugged, and sensitive with acceptable accuracy and precision for urine sample analysis. The Raptor Biphenyl column is well suited for the analysis of these synthetic opioid compounds, and this method can be applied to clinical toxicology, forensic analysis, workplace drug testing, and pharmaceutical research.

<table>
<thead>
<tr>
<th>QC Level 1 (0.750 ng/mL)</th>
<th>QC Level 2 (4.00 ng/mL)</th>
<th>QC Level 3 (20.0 ng/mL)</th>
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<tbody>
<tr>
<td>Analyte</td>
<td>Average Conc. (ng/mL)</td>
<td>Average % Accuracy %RSD</td>
</tr>
<tr>
<td>Acetyl fentanyl</td>
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<td>102</td>
</tr>
<tr>
<td>Alfentanil</td>
<td>0.733</td>
<td>97.6</td>
</tr>
<tr>
<td>Butyryl fentanyl</td>
<td>0.741</td>
<td>98.9</td>
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<tr>
<td>Carfentanil</td>
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<tr>
<td>Fentanyl</td>
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<tr>
<td>Norfentanyl</td>
<td>0.768</td>
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</tr>
<tr>
<td>Remifentanil</td>
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</tr>
<tr>
<td>Sufentanil</td>
<td>0.752</td>
<td>100</td>
</tr>
</tbody>
</table>
Monoclonal antibodies (mAbs) are an important class of therapeutics with the immense capacity to treat multiple diseases. Because of 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 three 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 that immobilizes the recombinant FcγRIIIA ligand bonded to porous 10 μm polymethacrylate particles that 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.

**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 |

Figure 1: Novel workflow for analysis of released glycans
Results and Discussion

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 a mass spectrometer 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 (G0F).

Conclusion

This two-step workflow, consisting of the combination of semi-preparative TSKgel FcR-III 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, 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.
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Adenosine Triphosphate (ATP) is the universal energy currency in biological systems. ATP is synthesized from adenosine mono and di-phosphate substrates. The relative ratio of ATP, ADP, and AMP is an important indicator of metabolic standing: the energy charge (Figure 1) is considered a quantitative measure of energy status, and serves as a barometer of sorts, as aberrant change in EC is associated with onset of numerous pathological states. Measurement of adenosine nucleotides, therefore, represents an important and insightful diagnostic.

Column Information

<table>
<thead>
<tr>
<th>Packing Material</th>
<th>PRP-1, 5 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions</td>
<td>2.1 x 150 mm</td>
</tr>
<tr>
<td>P/N</td>
<td>79366</td>
</tr>
</tbody>
</table>

Chromatographic Conditions

<table>
<thead>
<tr>
<th>Gradient</th>
<th>0 min. 1% B</th>
<th>3 min. 1% B</th>
<th>10 min. 15% B</th>
<th>15 min. 55% B</th>
<th>16 min. 95% B</th>
<th>19 min. 95% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>50 °C</td>
<td></td>
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<tr>
<td>Injection Volume</td>
<td>5 µL</td>
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<tr>
<td>Detection</td>
<td>UV at 254 nm</td>
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<tr>
<td>Sample Concentration</td>
<td>0.02 mM</td>
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</tbody>
</table>

In the present study, an ion pair reversed phase HPLC method was developed to separate ATP, ADP, and AMP from like-phosphorylated guanosine metabolic products (e.g., GTP, GDP, GMP) enabling quantification of these nucleotides from tissue extracts.

Figure 1. Energy Charge

\[
\text{EC} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}
\]

Compounds:
1. Guanosine Monophosphate
2. Guanosine Diphosphate
3. Adenosine Monophosphate
4. Guanosine Triphosphate
5. Adenosine Diphosphate
6. Adenosine Triphosphate

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