THE APPLICATION NOTEBOOK
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Cover Photography: Getty Images
Comprehensive Analysis of C2–C8 PFAS Using a Novel LC Column

Restek Corporation

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

A unique, hybrid ion-exchange/HILIC column (Raptor Polar X) was used to develop a comprehensive LC–MS/MS method for the analysis of ultrashort-chain through long-chain, and alternative PFAS in water sources (tap, river, groundwater, and sewage effluent). The Raptor Polar X’s multimode retention mechanisms allow for retention with a single isocratic run.

Experimental
Chromatographic conditions are reported in Figure 1.

To avoid introducing background contamination, polypropylene vials and caps were used during sample preparation.

Each water sample of 250 μL was mixed with 250 μL of methanol and 5 μL of internal standard solution (10 ng/mL of 13C2-PFHxA, 13C2-PFOA, 13C3-PFBS, 13C4-PFOS in methanol).

Calibration standards were prepared by using deionized water and fortified with 14 analytes (see Figure 1) at a range of 10–800 ng/L. The calibration standard solutions were diluted 1:1 as above.

A Restek tap water sample, along with three water samples (river, ground, and sewage effluent) supplied by the United States Environmental Protection Agency were fortified at 40 and 160 ppt. Blank and fortified water samples were diluted 1:1 in methanol as above for chromatographic analysis. For TFA measurement in groundwater, the sample was diluted fivefold with deionized water before fortification due to its high TFA concentration.

Results and Discussion
All analytes were eluted in 4 min with good peak shapes (Figure 1). The overall analytical cycle time was 8 min to ensure no matrix-related interferences.
Method linearity from 20–800 ppt for trifluoroacetic acid (TFA) and 10–800 ppt for all other analytes provided $r^2$ values >0.996 and deviations <20% using a 1/x weighted quadratic regression.

Samples were fortified at the low and high concentrations of their calibration ranges and run in duplicate for each analytical batch. A total of three batches were measured on different days. Concentrations of fortified samples were adjusted to account for any observed background contamination in sample blanks. Results are presented in Table I.

Conclusions
These results demonstrate that switching to a mixed-mode LC column provides the capability to analyze currently monitored and emerging PFAS contaminants in a single, short, isocratic run, preparing laboratories for the future of PFAS testing.
Mono and disaccharides are of increasing importance in the growing industrialized food industry. With the rise of diabetes and other metabolic diseases, nutritional content has found itself at the forefront of consumer concern. Investigation of the constituents found on the packaging is now commonplace. Consumer concern, coupled with the increase in FDA regulation on consumer products, has brought about a change in individual ingredients reported on labels. Identification of the five most common mono and disaccharides found in packaged foods, lactose, maltose, sucrose, fructose, and glucose, is easily resolved on any of the RCX-30 family of columns offered by Hamilton Company.

Ultraviolet absorption, normally the detection method of choice for most chromatography separations, is unsuitable for carbohydrates. This means other analyte detection methods must be used, such as refractive index (RI), conductivity, and pulsed amperometric detection (PAD). Both RI and conductivity are excellent choices for detection if only isocratic methods are needed. PAD, however, offers the advantage of detection from either gradient or isocratic elution. An additional consideration when choosing a detector is sensitivity. RI and conductivity provide nanomolar detection levels while the detection limit for PAD is an order of magnitude lower.

The RCX-30 columns, powered by a propyl trimethylamine functionality, use a strong anion exchange mechanism to aid carbohydrate separation. Increasing saccharide retention is easily achieved by increasing pH or decreasing hydroxide concentration.

Similarly, initiating the opposite operations facilitate a decrease in retention and allow a fine-tuning of the individual analytes. The PS-DVB particles featured in all Hamilton polymeric columns provide exceptional column lifetime and reduced backpressure. Polymeric columns always provide excellent pH stability in alkaline mobile-phase conditions because there are no silanols to degrade, offering consistent performance, injection after injection.
The timsTOF Pro platform, introduced in 2017, already featured a sensitivity boost from the Parallel Accumulation Serial Fragmentation (PASEF®) technology, which provides time-and-space focusing of the ions in the Trapped Ion Mobility Spectrometry (TIMS) tunnel. The timsTOF SCP platform further enhances the sensitivity with robustly modified ion optic design. This boost in sensitivity enables measurement of low nanogram and sub-nanogram peptide loads, resulting in quantification of a few thousand protein groups per injection. Our data from as low as 200 pg of peptide loads demonstrates the applicability to unbiased true single cell proteomics in a routine fashion.

Single-cell ‘omics in recent years has highlighted the microheterogeneity in clonal populations. Unlike other ‘omics technologies, single-cell proteomics is hindered by lack of amplification techniques for protein molecules. Single-cell proteomics is now beginning to get attention, and one common strategy is to multiplex labeled single cells together with carrier samples to boost the sensitivity in detection. Further improvements in sophisticated sample preparation techniques have resulted in increased peptide yield that is delivered to the liquid chromatography–mass spectrometry (LC–MS) instrumentation (1). While these improvements upstream of sample measurement have improved the analysis depth, the raw sensitivity requires improvement as well without compromising robustness. Parallel Accumulation and Serial Fragmentation (PASEF) (2) on the timsTOF Pro platform makes efficient usage of the ion beam and with intelligent precursor placement within a TIMS cycle achieves rapid-sequencing speed. In addition, the ions get focused in space and time within the TIMS cell, resulting in a significant boost in sensitivity. This enables the analysis of low sample amounts, in the range of low-nanogram peptide loads. The newly designed timsTOF SCP’s ion optics allow a 4–5× improvement in ion current by increasing the ion brightness while maintaining the robustness of the timsTOF Pro. As the yield of the electrospray ionization increases with lower flow rates, we further enhanced the experiment’s overall sensitivity by coupling the timsTOF SCP to an Evosep One (Evosep Biosystems) instrument operated with the new low-flow Whisper methods. We have characterized the resulting system’s performance by injecting and measuring peptide loads mimicking the amount resulting from a single-cell preparation.

Methods

The new ion optic design of the timsTOF SCP system includes switching the orientation of the ion optics with inclusion of an additional ion funnel and additional orthogonal turns of the ion beam to preserve the robustness of the instrument. The source contained a wider glass capillary orifice that draws more ions into an additional funnel housed in a multi-stage differentially pumped region. Our initial experiments...
demonstrated that in addition to the brighter ion beam these dedicated modifications were crucial to gain a factor five boost in ion current. For ultrahigh sensitivity measurements from 200 pg to a few nanograms of peptide, we coupled an Evosep One system (Evosep Biosciences) to the timsTOF SCP instrument and used a ~28 min gradient Whisper 40SPD method that offers a constant flow of 100 nL/min. Evotips were loaded with K562 (Promega) peptides according to the vendor instructions. Data were acquired in a DIA mode with window placements as shown (Figure 1B). All data were processed using Spectronaut software version 14 with default settings applying a hybrid library.

Results and Discussion
A dilution series of peptide load was performed starting from 200 pg to 25.6 ng in replicates using the ultralow flow method—Whisper 40SPD—from Evosep Biosoystems. This method delivers gradient at a flow rate of 100 nL/min further boosting the sensitivity of the platform. About 1200 protein groups could be quantified from the 200 pg loads, and that number increased to an excess of 4000 protein groups for 6.4 ng loads. Then 250 and 500 pg loads, mimicking the amount of peptides resulting from the digestion of one or two isolated cells, were used to test the accessible proteome depth. These samples were analyzed using the Whisper 40 samples per day (SPD) method applying dia-PASEF® methods with a 0.7 cycle time method that covers between 400 and 1000 m/z. The data were processed with a library consisting of 5200 protein groups and about 54,000 peptides. From 250 and 500 pg loads on average 1542 and 2146 protein groups were quantified, respectively.

Work done in the laboratories of Prof. Matthias Mann with the timsTOF SCP combined with robust low flow Evosep One when applied with efficient sample preparation yields exciting results on the biology of the cell cycle (3,4).

Conclusions
- timsTOF SCP provides robust proteome coverage with peptide loads in the range of 250 pg.
- Combination of timsTOF SCP with Whisper methods on the Evosep provide a robust and sensitive platform to perform single-cell proteomics.

References
4. A. Mund et al., https://doi.org/10.1101/2021.01.25.427969
Therapeutic antibody technology currently dominates the biologics market, and the last decade has seen drugs based on monoclonal antibodies (mAbs) progressively overtake their small-molecule alternatives. mAbs have shown remarkable efficacy to treat a plethora of indications, including cancers, infections, autoimmune disorders, and cardiovascular and neurological diseases. Because the whole antibody therapeutics platform is regarded as one of the most promising classes of pharmaceutical technology to date, there is growing interest in developing integrated analytical methods to provide layered information on the structure, purity, and stability of mAbs.

The rise of proteomics has generated remarkable technological advances in mass spectrometry (MS), especially in the field of high-resolution MS (HRMS), leading to an increased impact of MS on the field of structural biology. Electrospray ionization (ESI) has proven to be a very useful technique for obtaining multiply charged ions of intact proteins, providing the biopharmaceutical industry with a unique opportunity to characterize and control mAb quality during clinical development and commercial production. U/HPLC methods that allow hyphenation with MS are of particular interest for this purpose, providing users with an additional dimension in their mAb characterization (molecular weight determination and structural information). Although direct infusion in the mass spectrometer remains the most straightforward approach to conduct intact mAb characterization, it is typically hampered by the various contaminants (such as salts, stabilizers or detergents) present in the matrix of recovered and purified mAbs. Sample preparation before MS analysis is therefore essential to ensure accurate mass and structural characterization.

With the use of MS-compatible eluents, reversed-phase (RP) chromatography is particularly well suited for protein desalting prior to MS analysis. Using a gradient from low to high organic content, the protein binds to the column while MS-interfering contaminants are eluted. Additional benefits to RP desalting include the increased mobile-phase volatility at the source for an improved ionization process. Nevertheless, the use of organic solvent in the mobile phase causes denaturation of the protein, which results in increased charge \((z)\) values and often complicates MS data interpretation. In addition, mAb analysis by RP requires high temperatures (usually >50 °C) to reduce the strength of secondary interactions between mAb and the stationary phase, which can result in unwanted on-column fragments generation or loss of specific sugars from the glycan moieties.

Size-exclusion chromatography (SEC) remains the gold standard for determining the molecular weight (MW) distribution of mAbs expressed in mammalian cell culture. Obtaining structural information beyond the physical size (hydrodynamic volume) typically requires the combination of SEC with MS. Native ESI has proven particularly useful for generating multiply charged ions of intact proteins with lowered charge states, providing increased spectral resolution at higher \(m/z\) values. Still, the analysis remains challenging and involves biomolecule-specific optimization on both the chromatography and mass spectrometry side.

This application note describes the effective use of MS-compatible mobile-phase compositions in the analysis of mAbs using both RP (denaturing ESI) and SEC (native ESI) modes with in-line native ESI-MS detection on a hybrid Q-TOF instrument.

### Experimental Conditions

#### Reversed Phase Analysis (Denaturing)

- **Column:** TSKgel® Protein C-300, 3 μm, 2.0 mm ID × 5 cm
- **HPLC Instrument:** Nexera® XR UHPLC system
- **Mobile phase:**
  - A: H₂O containing 0.1% formic acid (FA)
  - B: acetonitrile (CH₃CN) containing 0.1% FA
- **Gradient:**
  - 0 to 1 min at 5% B from 1 (5% B) to 2.5 min (90% B)
  - 2.5 to 3.5 min at 90% B back to 5% B at 3.7 min
  - 3.7 to 8 min at 5% B
- **Flow rate:** 0.2 mL/min
- **Detection:** UV @ 280 nm
- **Temperature:** 40 °C
- **Injection vol.:** 2 μL
- **Sample:** NIST mAb @ 0.5 mg/mL
- **MS Instrument:** SCIEX X500B QTOF
- **Ionization mode:** Electrospray ionization, positive mode
- **MS mode:** Scanning, TOF MS \(m/z\) 900-4000
- **Ion source gas 1:** 45 psi
- **Ion source gas 2:** 45 psi
- **Curtain gas:** 30 psi
- **CAD gas:** 7 psi
- **Spray voltage:** 5000 V
- **Source temperature:** 450 °C
- **Declustering potential:** 275 V
- **Collision energy:** 20 V
**Results and Discussion**

**Denaturing vs. Native ESI-MS Analysis**

The main difference between traditional denaturing and native ESI–MS is the composition and pH of the employed mobile phases (see Experimental Conditions). Denaturing solutions, containing organic solvents and non-neutral pH conditions, produce unfolded proteins by disrupting noncovalent interactions, causing molecules to unfold and expose sites of protonation. Conversely, native solutions at neutral pH (~7) preserve noncovalent interactions, as these molecules transition to the gas phase. Charge state and intensity during electrospray ionization are mainly determined by the number of ionizable sites. ESI–MS of denatured species typically produces a Gaussian distribution of highly charged ions in a wider charge state distribution, while native ESI produces ions with lower charge states and a narrower charge state distribution. This is largely due to the globular nature of folded proteins, limiting solvent accessible residues to only those on the surface of the protein. This is illustrated in Figure 1, which depicts the charge state envelopes obtained for the NIST mAb in both denaturing (RP-MS) (Figure 1B) and native (SEC-MS) (Figure 1E).

Both RP (on a TSKgel Protein C4-300 column) and SEC (on a TSKgel UP-SW3000 column) analysis of the ~150 kDa NIST mAb coupled to a HRMS QTOF mass spectrometer led to robust separation and detection of the mAb. The main mAb RP peak eluting at 6 min (Figure 1A) exhibits a mass spectrum (Figure 1B) made of a charge distribution ranging from ~40+ to ~60+, with the Gaussian distribution representative of its unfolded form centered around m/z ~2900. In contrast, the main mAb SEC peak eluting at 10.5 min (Figure 1D) exhibits a less complex mass spectrum made of a charge distribution ranging from 24+ to 30+, with the Gaussian distribution representative of its native, folded form centered around m/z ~5300 (Figure 1E).

Intact protein mass values—also called the zero-charge state—from these pseudo-molecular ions can be determined using deconvolution of protein charge states. MS deconvolution results for the denatured and native NIST mAb are presented in Figures 1C and F, respectively, exhibiting low mass errors (average: <10 Da) and achievement of MS resolution for the major glycoforms of the NIST mAb.
Buffer Consideration for Intact Protein Analysis by LC–MS
Making your liquid chromatography (LC) method compatible with MS is a crucial first step in your method development or transfer. When using MS detection, your analytes first must be “transferred” from the liquid phase into the gas phase, which is quite different from most optical detectors classically hyphenated with LC (for example, UV and fluorescence detectors) where your sample is maintained in the liquid phase. Hyphenation with MS involves the process of ionization, which occurs in the ion source of your MS equipment, making your mobile phase (and all its additives) a crucial part of a successful method development and/or transfer.

RP is easily amenable to MS compatibility, with the focus being on the acidifier additive. Traditional RP-UV methods have used trifluoroacetic acid (TFA), which needs to be replaced by a volatile acidifier such as formic acid (FA) or difluoroacetic acid (DFA). In the case of SEC, although they are best suited to maintain the native folded structure of your mAb of interest, phosphate-based buffers cannot be used as they will contaminate your ion source. Switching to volatile buffers (such as ammonium acetate, ammonium formate or ammonium bicarbonate) is required. When doing so, considering your mobile phase from both the SEC and the MS point of view is crucial, as both LC performance (retention times, peak shape, resolution) and MS performance (ionization efficiency, maintenance of proteins’ native structures) can be affected by buffer type and buffer concentration.

The effects of MS-compatible volatile SEC buffers on mAb elution profiles are illustrated in Figure 2, where the separation of Humira® biosimilar on the TSKgel UP-SW3000 column was conducted using four different buffers at 50 mmol/L concentration. A sharp monomer peak at 5.7 min was observed when using a non-volatile buffer (MES) containing 150 mmol/L NaCl. When testing three volatile, MS compatible buffers at 50 mmol/L, three different peak shapes and retention times were observed, with 50 mmol/L ammonium formate offering the best chromatographic performance.
Experimental Considerations for a Successful Intact mAb Characterization by LC–ESI–MS

Below are the three main steps of how to optimize a successful intact mAb analysis using LC–MS:

1. Consider the various aspects of chromatographic separation and how to make them compatible with MS analysis.
2. Electrospray ionization: Identify the various parameters that can influence ionization efficiency and desolvation when you hyphenate LC with MS.
3. The MS detection and analysis: What parameters to consider on your MS instrument.

Table I presents a non-exhaustive summary of the various parameters and variables to consider when developing intact mAb ESI–MS experimental workflow, including MS platform-specific factors.

Conclusions

Monoclonal antibodies are highly complex biomolecules, requiring high resolution, precision, and dynamic range to fully characterize them with confidence. This application note illustrates an intact mAb analysis workflow solution integrating U/HPLC technologies, high-resolution mass spectrometry on a QTOF instrument and software for automatic data processing.

The workflow permits rapid and accurate mass characterization of mAbs, using either denaturing ESI using reversed-phase chromatography or native ESI using size-exclusion chromatography, leading to excellent mass accuracy for glycoform distribution. Detailed information was obtained about the heterogeneous composition of mAb proteins, with minimal sample preparation involved.

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Analytical toxicology involves methods for comprehensive screening of biological matrices for the presence of abused drugs. Routine analysis of samples in clinical and forensic settings demands quick and efficient extraction procedures. Smaller sorbent amounts utilized by solid-phase extraction (SPE) products allow scaling-down of starting sample size and minimize the total solvent volumes required to wash matrix components and elute the target analytes. A 2 mg or less measure of sorbent particles embedded in a disc membrane allows for sample enrichment and high throughput processing. As compared to loose sorbent, disk format eliminates channeling effects and reduces dead volume. Removal of the evaporation step from the procedure also decreases overall turn-around time.

In this application note, methods for extracting a large drugs of abuse panel from urine and plasma using UCT’s Micro-Prep HLB (W96-XTMC-HLB) and MMCX (W96-XTMC-MMCX) microelution plates have been described. HLB consists of a highly retentive uncharged hydrophilic and lipophilic sorbent which can effectively retain a range of acids, neutrals and bases via reverse-phase. The mixed-mode cation exchange chemistry of MMCX allows extraction of polar and non-polar analytes from aqueous samples. HPLC separation was carried out using UCT’s Selectra® PFPP column prior to detection by LC–MS/MS. The pentafluorophenylpropyl phase can undergo dipole-dipole, and pi-pi interactions, imparting unique selectivity and retention mechanisms to the column that distinguish it from a traditional biphenyl phase. Water and methanol consisting of 5 mM ammonium formate and 0.1 % formic acid were used as mobile phase. The total run time was 13 min at a 0.4 mL/min flow rate.

### Results

HLB microelution plate utilized to extract urine and plasma quality control samples yielded excellent recoveries for a majority of the analytes in the panel. From a total of 47 drugs, >80% recoveries were achieved for 37 drugs fortified at 5 ng/mL and for 43 drugs spiked at 50 ng/mL. Corresponding RSD values were <10% at both concentration levels. From a total of 50 drugs extracted on the MMCX microelution plate, 45 and 48 drugs showed >80% recoveries at 5 ng/mL and 50 ng/mL respectively. The RSD values for both concentrations were <20%.

### Conclusion

The use of UCT Selectra PFPP UH PLC column resulted in excellent peak shape and good linear calibration curves for all the analytes. Excellent recoveries and relative standard deviation (RSD) values confirm both the microelution extraction methods to be efficient. In addition to using minimal wash and elution solvent volumes, the elimination of the drying step reduced the overall processing time to approximately less than 30–40 min. The potential for automation and the option to load the collection plate directly on to the autosampler make this extraction technique very convenient for high throughput forensic and clinical laboratories.

### HLB Sample Extraction Procedure

1) **Sample Preparation**
   - 300 µL sample + ISTD
   - 300 µL 100 mM pH 10.0 Sodium car/bicarbonate buffer
2) **Condition (Optional)**
   - 100 µL MeOH
   - 100 µL 100 mM pH 10.0 Sodium car/bicarbonate buffer
3) **Apply sample**
   - Load 400 µL sample onto the microelution plate
4) **Wash column**
   - 100 µL 5% MeOH in DI H₂O
5) **Elute**
   - 50 µL 2% Formic acid in MeOH
6) **Post Elution (Optional)**
   - Evaporate & Reconstitute in mobile phase or
   - Add 50 µL DI H₂O

### MMCX Sample Extraction Procedure

1) **Sample Preparation**
   - 300 µL sample + ISTD
   - 300 µL 100 mM pH 6.0 Phosphate buffer
2) **Condition (Optional)**
   - 100 µL MeOH
   - 100 µL 100 mM pH 6.0 Phosphate buffer
3) **Apply sample**
   - Load 400 µL sample onto the microelution plate
4) **Wash column**
   - 100 µL 100 mM Glacial acetic acid in DI H₂O
   - 100 µL 40% MeOH
5) **Elute**
   - 50 µL 2% NH₄OH in MeOH
6) **Post Elution (Optional)**
   - Evaporate & Reconstitute in mobile phase or
   - Add 50 µL 2% Formic acid in DI H₂O
Once thought of as garbage bins, extracellular membrane vesicles full of cellular remnants responsible for the possible spread of many diseases, exosomes are viewed now as potential vehicles for regenerative medicine and targeted therapies for chronic and degenerative diseases, certain genetic disorders, musculoskeletal pain, and even Alzheimer's disease. Targeted therapies may use a variety of targeting or signaling molecules such as RNA (messenger RNA and small interfering RNA, for example), DNA fragments, peptides, proteins, and lipids (1).

However, it is important to separate and purify the desired exosome from impurities during production. In this example of exosome (EV) analysis, the EV preparation process from cell culture supernatant was followed by a combination of polymer-based aqueous SEC (GFC) column OHpak SB-806 HQ and various detectors. Ultraviolet (UV) at 280 nm covers general culture-derived impurities, and fluorescence (Ex at 280 nm and Em at 348 nm) responds mainly to proteins via tryptophan residue fluorescence. In addition, MALS scattered light (LS) provides a highly sensitive response especially for large objects like nanoparticles. Moreover, MALS gives an estimate of the target RMS (root mean square) radius. The fraction consisted mainly EV was separated from many culture-derived impurities and was found around 8 min. While UV and fluorescence provide important insights into the progress and efficacy of the purification process and profiling of purified products, they are less sensitive to EVs mostly composed of lipid membranes and containing trace amounts of protein/nucleic acid cargo. Light scattering (LS) is an effective EV tracking method, especially in the early stages of purification. SB-806 HQ is a high-performance aqueous SEC (GFC) column suitable for bioproducts with a sufficient pore size to hold and separate EV-class nanoscale objects. Combined with a variety of detectors, a comprehensive analysis of the complex bio-nano target preparation process is achieved.

EV fraction preparation conditions (1) Concentration step: Commercially available centrifugal ultrafiltration membrane 100 kDa (40 times concentrated) (2) Affinity step: Commercial affinity purification kit (equivalent to 10-fold concentration), sample preparation by Showa Denko Materials Co., Ltd.

Reference

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**SEC–MALS Analysis of Exosomes Using the Shodex OHpak SB-806 HQ**

Ronald Benson* and Hirobumi Aoki†, *Showa Denko America, Inc., †Showa Denko K.K, Japan

Once thought of as garbage bins, extracellular membrane vesicles full of cellular remnants responsible for the possible spread of many diseases, exosomes are viewed now as potential vehicles for regenerative medicine and targeted therapies for chronic and degenerative diseases, certain genetic disorders, musculoskeletal pain, and even Alzheimer’s disease. Targeted therapies may use a variety of targeting or signaling molecules such as RNA (messenger RNA and small interfering RNA, for example), DNA fragments, peptides, proteins, and lipids (1).

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**Table I**

<table>
<thead>
<tr>
<th>Column</th>
<th>Shodex™ OHpak SB-806 HQ (8.0 mm I.D. x 300 mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eluent</td>
<td>PBS (-)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL/min</td>
</tr>
<tr>
<td>Detector</td>
<td>UV (280 nm) Fluorescence (Ex = 280 nm, Em = 348 nm), MALS (DAWN8+ produced by Wyatt Technology Corp.)</td>
</tr>
<tr>
<td>Column temperature</td>
<td>25 °C</td>
</tr>
</tbody>
</table>

**Figure 1:** Sample 1: Cell culture supernatant 50 μL inj. 2: Concentrate 50 μL inj. 3: Crude product 15 μL inj. Process: supernatant concentrated by ultrafiltration; crude product obtained by affinity chromatography.
Liposomes and lipid nanoparticles are often used as nanocarriers to encapsulate fragile nucleic acids and hydrophobic or highly toxic drugs, and to safely deliver them to target tissue. During drug nanocarrier product and process development, as well as quality control, it is of great importance to monitor liposome size distributions accurately while also verifying drug encapsulation. FFF-MALS-DLS, consisting of field-flow fractionation (FFF) combined with multi-angle light scattering (MALS) and dynamic light scattering (DLS), is a powerful tool for characterizing the size, concentration, and structure of large nanoparticle ensembles.

**Method**
Encapsulation might cause changes in liposomal dimensions, but that is not always the case and other effects could cause such changes. Therefore, a more sophisticated analysis is warranted than mere size. Here, we report the analyses of two liposome samples, one empty and one filled with drug, by means of an Eclipse™ FFF system followed by a DAWN® MALS detector with embedded WyattQELS™ DLS module. The FFF separation method was optimized with the aid of Wyatt’s proprietary FFF simulation software. ASTRA software was used to collect and analyze the light scattering data to determine size and concentration (number density).

**Results and Discussion**
FFF separates particles according to hydrodynamic radius. Thanks to upstream separation, quantitative size distributions by FFF-MALS-DLS provide far more resolution and quantification than batch (unfractionated) DLS. Online DLS directly measures the hydrodynamic radius, $R_h$, sequentially for each eluting size fraction, while MALS simultaneously measures the root-mean square radius, $R_g$. The shape factor $\rho$, which is defined as the ratio $R_g/R_h$, provides important structural information: it can discriminate between empty and filled shells or quantify the axial ratio of a uniform ellipsoid.

Both $R_h$ and $R_g$ are plotted against elution time in Figure 1. The results from duplicate runs demonstrate clean separation and excellent reproducibility of the FFF-MALS-DLS method. Figure 1(a) shows that the $R_g$ values for both empty and filled liposomes are well-overlaid, which is expected since FFF separates according to hydrodynamic size. However, as shown in Figure 1(b), $R_h$ values for these two liposomes do not overlay, which indicates different internal structures.

Figure 2(a) plots $R_g$ against $R_h$; $\rho$ is the slope of the linear fit. Interpretation of $\rho$ requires an assumption about the shape of the particles; here we make use of a priori knowledge that they are spherical. The values of $\rho$ for these two populations then correlate precisely to empty and filled liposomal structures.

In addition to size and structure, FFF-MALS can determine quantitative size distributions (number density vs. size) of size-fractionated nanoparticles if the refractive index of the constituent material is known. For lipids this is quite straightforward and Figure 2(b) provides the quantitative nanoparticle concentration analysis.

**Conclusion**
For liposomes or other nanoparticles, FFF-MALS-QELS provides an easily adaptable yet powerful characterization tool to obtain information on particle size, size distribution, particle count, as well as structure—all without making assumptions about the particles or their composition. FFF-MALS-DLS instrumentation is essential for robust drug nanocarrier development and quality control.

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