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Perspective

Keeping up with a Quickly Diversifying Pharmaceutical Landscape

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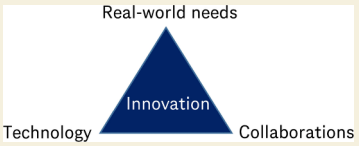
ABSTRACT: Small molecules and antibodies have dominated the pharmaceutical landscape for decades. However, limitations associated with therapeutic targets deemed "undruggable" and progress in biology and chemistry have led to the blossoming of drug modalities and therapeutic approaches. In 2023, a high number of 9 oligonucleotide and peptide products were approved by the Food and Drug Administration (FDA), accounting for 16% of all drugs approved. Additionally, for the first time, a clustered regularly interspaced short palindromic repeat (CRISPR)-Cas9 gene therapy product was approved for the treatment of sickle cell disease. New drug modalities possess a wide range of physicochemical properties and structures, which complicates their analytical characterization. Impurities are formed at each step of the oligonucleotide and peptide solid phase synthesis and during shelf life. Longer chain lengths lead to a higher number of closely related impurities that become increasingly more difficult to separate from the full-length product. Chemical modifications such as phosphorothioates (PS) result in the presence of diastereomers, which often require orthogonal methods for their profiling and strategies to prevent their interference with the separation of achiral impurities. In-vitro produced mRNA and plasmid DNA also present a variety of quality attributes that need to be determined, such as the polyA tail length or capping efficiency. Analytical challenges arise from the variety of drug modality physiochemical properties and attributes, fast turnaround times, and heightened level of characterization needed to enable data-driven decisions early in the drug development process. This perspective provides the author's views on the lessons learned and strategies employed in recent years.



KEYWORDS: *2D-LC, HILIC, multidimensional LC-MS, oligonucleotides, online bottom-up MS, peptides, synthetic molecules*

INTRODUCTION

A high number of 9 peptide and oligonucleotide products were approved by the Food and Drug Administration (FDA) in 2023.[1] Various drug modalities received FDA approval, including two antisense oligonucleotides (ASOs), one small interfering RNA (siRNA), and an aptamer.[2] In addition, for the first time, a clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 gene therapy product was approved for the treatment of sickle cell disease.[3] This first approval of a therapy based on the CRISPR technology, which received a Nobel Prize in 2020,[4] is one example that shows the appetite of drug makers to quickly adopt new technologies and accelerate drug development. An increasing number of manuscripts referring to the concept of druggability were published in the last two decades.[5] Consequently, drug discovery scientists now dispose of a variety of drug modalities to study a given target.[6]

The blossoming of drug modalities in research and early development requires analytical scientists to quickly adapt and address emerging needs. Fast turnaround times are needed to control the quality of drug candidates before in vivo testing and repurify them if the purity does not meet target values. While platform methods have become common for the characterization of small molecules[7] or antibodies[8], their application to new drug modalities might not be as straightfoward due to the wide range of physicochemical properties. In this Perspective, reflections are shared on technologies that have helped to expedite method development, automate analytical workflows, and assess difficult quality attributes. Current limitations and future outlooks are also discussed.

THE VERSATILITY OF 2D-LC

Large pharmaceutical companies have embraced the use of 2D-LC in recent years. For example, several studies were published between 2023 and 2024 on the peak purity analysis,[9] low level impurity quantification,[10] impurity analysis in therapeutic oligonucleotides,[11] and online sampling for the real time monitoring of monoclonal antibody (mAb) critical quality attributes (CQAs).[12]

2D-LC instrumentation can also be utilized to accelerate method development and determine the purity of drug candidates, simultaneously. For example, fast achiral−chiral and chiral−chiral 2D-LC methods were developed to separate the stereoisomers of a synthetic intermediate and different active

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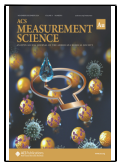

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pharmaceutical ingredients (APIs).[13] Later on, a versatile two-dimensional multicolumn liquid chromatography (LC-mLC) chiral-achiral approach was developed to expedite method development in less than 2 h.[14] The final conditions successfully separated all stereoisomers of an API containing 3 chiral centers.[14] In 2024, a multiplex platform was developed to expedite method development and routinely characterize therapeutic peptides using SEC-UV for aggregates, 2D-LC for achiral impurities, and HILIC-CAD for the quantification of trifluoroacetic acid (TFA).[15]

The screening of reaction conditions, such as chiral ligands, is commonly performed via high throughput experimentation (HTE) using 96-well plates. The determination of the reaction yield and enantiomeric excess is required to identify the best chiral ligands. A 2D-LC platform was developed to support multiple products generated via asymmetric reactions and determine the optimal reaction conditions.[16] The achiral-chiral platform has been used to screen more than 1000 reaction mixtures.[16]

ONLINE FRACTIONATION AND DIGESTION TO ANALYZE THERAPEUTIC ANTIBODIES AND RNAS AT THE SINGLE RESIDUE LEVEL WITH UNPRECEDENTED SPEED

Online bottom-up MS approaches previously used in the field of proteomics[17] have gained an important momentum in the pharmaceutical industry for the analysis of therapeutic proteins. A breakthrough was made in 2018 with the online fractionation and peptide mapping of impurities separated by cation exchange chromatography (CEC) for mAb products.[18] The same CEC-reduction-digestion-RPLC approach was then established at Genentech using modules from a different vendor.[19] Using the same instrumental setup, a SEC-reduction-digestion-RPLC method was then developed to study the drug-to-antibody ratio (DAR) content of antibody drug conjugates (ADC) size variants.[20] Later on, the multidimensional liquid chromatography−mass spectrometry (MD-LC-MS) platform was utilized to inform on cell culture,[21] determine multiple attributes for a variety of therapeutic proteins,[22] including sialyation, oxidation, deamidation, and glycosylation.[23] The CEC-reduction-digestion-RPLC method was implemented in additional laboratories and similar sequence coverages and levels of post translational modifications (PTMs) were obtained.[24] The various applications of the MD-LC-MS technology have been reviewed in 2024.[25] The evaluation of biologically relevant attributes via the use of immobilized neonatal fragment crystallizable receptor (FcRn) or FcγRIIIa cartridges has brought the MD-LC-MS technology to the next level with the integrated chemical and biological characterization of impurities separated by reference purity methods. In contrast, the characterization of therapeutic RNAs by MD-LC-MS has not attracted as much attention, which is likely due to the more recent interest of the field in these drug modalities.

The Limbach's group has made significant contributions to the sequencing of naturally occurring RNAs and their modifications (also referred to as RNA modification mapping), and in particular using bottom-up MS.[26] Their work laid the foundation of critical aspects involved in the sequencing of therapeutic RNAs by bottom-up MS, including ribonuclease digestions,[27] instrumentation,[28] and data treatment tools.[29] In 2020, we initiated a collaboration with an external partner to immobilize the ribonuclease (RNase) T1, A, and U2 on different cartridges for the nucleotide mapping of therapeutic RNAs by online bottom-up MS. We demonstrated their successful application to sequence guide RNAs (gRNAs),[30] mRNAs,[31] and determine isomeric phosphodiester (PO) impurities in gRNAs.[32] Additionally, in-solution RNase P1 or RNase H digestions have been used to sequence modified siRNAs,[33] compare the diastereomeric profiles of various ASO lots,[34] and determine mRNA capping efficiency.[35] Additionally, the RNase 4 improved the mRNA sequence coverage in comparison to the RNase T1.[36] Online digestions present numerous advantages over offline procedures due to the minimization of (i) lab work and surface decontamination procedures needed with sensitive RNA molecules such as mRNAs; (ii) variability due to the absence or lower number of pipetting steps; (iii) RNase contamination of the column and LC-MS system; and (iv) volume of sample needed to perform a nucleotide mapping. The likelihood to identify an impurity at the nucleotide level is also higher when using an online fractionation and digestion approach in comparison to traditional offline fractionation and digestion procedures due to a lower dilution. In the future, it is hoped that additional RNases, including MC1 and cusativin, and immobilized RNase cartridges, including RNase H, P1, 4, MC1 and cusativin will be commercialized. The next level analysis of therapeutic RNAs could integrate the online evaluation of biologically relevant attributes for fractionated impurities, similarly to therapeutic proteins.

ANALYSIS OF OLIGONUCLEOTIDES BY HILIC AND ULTRA WIDE PORE SEC

In 2020, we anticipated that HILIC would become a workhorse for the analysis of therapeutic oligonucleotides.[37] Since then, the utility of HILIC has been demonstrated for various oligonucleotide constructs such as ASOs and gRNAs.[38,39] For example, HILIC achieved the separation of isomeric PO impurities[32] and diastereomers.[40] Various studies suggested that hydrogen bonding interaction govern the separation of oligonucleotides using amide columns.[31,39−42] Some studies suggested the replacement of acetonitrile by methanol[39,41] with a potential benefit on the separation performance.[41] HILIC-MS can also be used to confirm the identity, sequence, and impurities in oligonucleotide products such as siRNAs.[43] Finally, a FDA laboratory validated a HILIC-MS method for the quantification of various process-related impurities.[44]

The increased complexity and length of therapeutic nucleic acid oligonucleotides and adeno-associated viruses (AAVs) have fueled a new wave of innovation with the development of ultra wide pore SEC columns (≥1000 Å for mRNAs). In 2023, we demonstrated the separation of aggregates in mRNAs, topological forms in plasmid DNA and the separation of mRNAs from lipid nanoparticle (LNP) using 1300 Å prototype columns.[45] The breakthrough was followed by the commercialization of the GTxResolve Premier SEC 1000 Å 3 μm column in 2024. In the same year, ultra wide pore SEC columns have been commercialized by other vendors, for example, with the AdvanceBio SEC column 1000 Å 2.7 μm or Biozen dSEC-7 packed with 3.0 μm particles.

COLLABORATIONS

Tripartite collaborations between academia, the pharmaceutical industry, and vendors can provide an ideal environment that fosters innovation. Some academic groups have established numerous collaborations with industrial partners that led to

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impactful findings.[46,47] Additionally, the propelling analysts by removing analytical-, data-, instrument-, and sample-related encumbrances (PARADISE) project[48] provides a framework for several academic and industrial partners to collaborate on difficult real world challenges.[49] We established industrial collaborations with regards to column technology including biocompatibility, immobilization of enzymes, and improvement of software used to interpret the data generated from the digestion of therapeutic RNAs. In parallel, we partnered with academic institutions to explore new horizons in the characterization of complex therapeutics.

The immobilization of enzymes on cartridges is one key component of online bottom-up platforms. We initiated a fruitful collaboration to develop and test immobilized RNase T1 and A cartridges. These cartridges have been commercialized.[31] Subsequent collaborations were made with the same vendor to immobilize the Lys-C endoproteinase and perform parallel on-column trypsin and Lys-C digestions to identify an ethylene oxide adduct of a bispecific antibody.[50] We also partnered with the Guillarme's group of the University of Geneva to push the limits of existing analytical technologies.[51,52] In the future outlook, it will be valuable to have access to commercial devices that allow the online reduction of antibodies or denaturation of LNPs.

ORTHOGONAL TECHNOLOGIES AND DETECTION

Orthogonal technologies and detection can be critical to providing a more complete picture of impurity profiles and to ensure product quality and patient safety. For example, SEC is a reference technique for the determination of aggregates, which can trigger immunogenicity. However, SEC analyzes can suffer from adsorption issues that are often more pronounced for the high order species and can cause their underestimation. Limitations of existing technologies justify the need for continued advancement in analytical tools and capability development.

With the increasing amount of data generated, it is important to remain critical and assess the quality of the data, having in mind that analytical results do not always accurately represent the sample. Solid fundamental foundations are essential to consider the potential and limits of an analytical technique or column. For example, a SEC column packed with 200−300 Å particles will unlikely separate mRNA aggregates based on their size. A plethora of analytical artifacts can also introduce biases, such as (i) RNase contamination when reconstituting or diluting RNA samples and resulting in the presence of additional shortmer impurities; (ii) nonspecific adsorption on surfaces leading to the underestimation of early eluting peaks such as shortmers with acidic analytes[53] or aggregates with hydrophobic peptides[15] or large mRNAs;[52] (iii) adsorption occurring in the sample flow path and in particular the flow cell.[51] In the absence of a reference material, it is critical to use orthogonal methods to determine quality attributes. For example, the determination of drug encapsulation efficiency in nanoparticles often relies on indirect analyses. Various analytical techniques have been used to determine the free and total drug content, e.g., fluorescence-based or HPLC-based, but they may suffer from artifacts. Capillary electrophoresis is one orthogonal technique to HPLC but it has not attracted as much interest for the analysis of new drug modalities or delivery systems possibly due to a longer familiarization with the instrument, except for the analysis of plasmid DNA. Microfluidic (also referred to as micro capillary) electrophoresis helped to reduce the analysis time for mRNA and plasmid DNA.[54,55] The sequencing of large RNAs, including gRNA and mRNA, is another complex analytical task that can be performed using next generation sequencing (NGS). However, the library preparation can generate artifacts that may be confounded with sequence variants. Therefore, it is important to confirm the potential sequence variants by orthogonal techniques such as mass spectrometry.

While modest progress has been achieved by separation techniques over the last five years, some innovative detectors have been designed and a renewed interest in historical ones has been observed. Mass photometry has shown promise for the detection of large drug modalities such as mRNAs.[54,56] Imaging techniques are desired to study the secondary structures of RNA and LNP structures. Atomic force microscopy (AFM) enabled the study of RNA structures under near physiological conditions.[57] Nuclear magnetic resonance (NMR) has been used to study the (polyethylene glycol) PEG structures (brush-like vs mushroom) located on the LNP surface.[58] A higher interest has been observed in the use of multiangle light scattering (MALS) to detect large molecules. For example, the coupling of MALS to SEC helped to identify LNP dimeric impurities.[45] Ion-mobility has shown promises in the separation of diastereomers.[40] The charged aerosol detector (CAD) has demonstrated important value for the detection of compounds lacking chromophores such as lipids, polymers[59] or ions.[60]

FUTURE OUTLOOK

MD-LC-MS reduces the burden associated with product-specific method development and enables the identification and quantification of impurities at the amino acid or nucleotide level. However, the instrumentation and software can be complex and prevent broader adaptation of the technology. For example, the initial instrument setup can often be discouraging but is critical for future use. It is therefore important for instrument vendors to invest in engineers able to build custom MD-LC-MS setups and provide training, in particular due to the absence of commercial 3D-LC-MS or 4D-LC-MS solutions at this time. Simplification of current HPLC instrumentation and software, and new features could also facilitate the broader adoption of more complex MD-LC-MS setups. Wouldn't it be helpful to purge the solvent lines or create instrument methods and sequences by voice control? Beyond instrument control, the improvement of chromatographic softwares is needed for the streamlined use of MD-LC-MS workflows. It could include the vizualization of the sample flow path and automated data treatment. It would be also helpful if a software could generate a visual representation of the peptide or oligonucleotide structure and depict the type, location and percentage of impurities. Additionally, data integrity principles would need to be considered for a broader implementation of the technology in regulated environments.

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Notes

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■ REFERENCES

(1) Al Shaer, D.; Al Musaimi, O.; Albericio, F.; de la Torre, B. G. 2023 FDA TIDES (Peptides and Oligonucleotides) Harvest. *Pharmaceuticals (Basel)* **2024**, *17* (2), 243.
(2) Mullard, A. 2023 FDA Approvals. *Nat. Rev. Drug Discovery* **2024**, *23* (2), 88−95.
(3) Commissioner, O. of the. *FDA Approves First Gene Therapies to Treat Patients with Sickle Cell Disease*. FDA. https://www.fda.gov/news-events/press-announcements/fda-approves-first-gene-therapies-treat-patients-sickle-cell-disease (accessed 2024−08−30).
(4) *Nobel Prize in Chemistry 2020*. NobelPrize.org. https://www.nobelprize.org/prizes/chemistry/2020/press-release/ (accessed 2024−08−30).
(5) Yang, W.; Gadgil, P.; Krishnamurthy, V. R.; Landis, M.; Mallick, P.; Patel, D.; Patel, P. J.; Reid, D. L.; Sanchez-Felix, M. The Evolving Druggability and Developability Space: Chemically Modified New Modalities and Emerging Small Molecules. *AAPS J.* **2020**, *22* (2), 21.
(6) Blanco, M.-J.; Gardinier, K. M.; Namchuk, M. N. Advancing New Chemical Modalities into Clinical Studies. *ACS Med. Chem. Lett.* **2022**, *13* (11), 1691−1698.
(7) Tarafder, A.; Miller, L. Chiral Chromatography Method Screening Strategies: Past, Present and Future. *J. Chromatogr. A* **2021**, *1638*, 461878.
(8) D'Atri, V.; Imiołek, M.; Quinn, C.; Finny, A.; Lauber, M.; Fekete, S.; Guillarme, D. Size Exclusion Chromatography of Biopharmaceutical Products: From Current Practices for Proteins to Emerging Trends for Viral Vectors, Nucleic Acids and Lipid Nanoparticles. *J. Chromatogr. A* **2024**, *1722*, 464862.
(9) Lawler, J.; Lesslie, M.; Randstrom, C.; Zhao, Y.; Breitbach, Z. A Standardized 2D-LC Screening Platform for Peak Purity Determination in Pharmaceutical Analysis. *LC GC N. Am.* **2023**, *41*, 220−224.
(10) Lin, Z.; Wang, Q.; Zhou, Y.; Shackman, J. G. Trapping Mode Two-Dimensional Liquid Chromatography for Quantitative Low-Level Impurity Enrichment in Pharmaceutical Development. *J. Chromatogr. A* **2023**, *1700*, 464043.
(11) Stoll, D.; Sylvester, M.; Meston, D.; Sorensen, M.; Maloney, T. D. Development of Multiple Heartcutting Two-Dimensional Liquid Chromatography with Ion-Pairing Reversed-Phase Separations in Both Dimensions for Analysis of Impurities in Therapeutic Oligonucleotides. *J. Chromatogr. A* **2024**, *1714*, 464574.
(12) Dunn, Z. D.; Bohman, P.; Quinteros, A.; Sauerborn, B.; Milman, F.; Patel, M.; Kargupta, R.; Wu, S.; Hornshaw, M.; Barrientos, R.; Bones, J.; Tayi, V. S.; Abaroa, N.; Patel, B.; Appiah-Amponsah, E.; Regalado, E. L. Automated Online-Sampling Multidimensional Liquid Chromatography with Feedback-Control Capability as a Framework for Real-Time Monitoring of mAb Critical Quality Attributes in Multiple Bioreactors. *Anal. Chem.* **2023**, *95* (49), 18130−18138.
(13) Barhate, C. L.; Regalado, E. L.; Contrella, N. D.; Lee, J.; Jo, J.; Makarov, A. A.; Armstrong, D. W.; Welch, C. J. Ultrafast Chiral Chromatography as the Second Dimension in Two-Dimensional Liquid Chromatography Experiments. *Anal. Chem.* **2017**, *89* (6), 3545−3553.
(14) Lin, J.; Tsang, C.; Lieu, R.; Zhang, K. Fast Chiral and Achiral Profiling of Compounds with Multiple Chiral Centers by a Versatile Two-Dimensional Multicolumn Liquid Chromatography (LC-mLC) Approach. *J. Chromatogr A* **2020**, *1620*, 460987.
(15) Goyon, A.; Wang, S.; Hofmann, K.; Nguyen, D. N.; Yehl, P.; Zhang, K. Unified and Versatile Multiplex Platform for Expedited Method Development and Comprehensive Characterization of Therapeutic Peptides. *Anal. Chem.* **2024**, *96* (34), 14011−14019.
(16) Goyon, A.; Masui, C.; Sirois, L. E.; Han, C.; Yehl, P.; Gosselin, F.; Zhang, K. Achiral-Chiral Two-Dimensional Liquid Chromatography Platform to Support Automated High-Throughput Experimentation in the Field of Drug Development. *Anal. Chem.* **2020**, *92* (22), 15187−15193.
(17) Nadler, T.; Blackburn, C.; Mark, J.; Gordon, N.; Regnier, F. E.; Vella, G. Automated Proteolytic Mapping of Proteins. *J. Chromatogr. A* **1996**, *743* (1), 91−98.
(18) Gstöttner, C.; Klemm, D.; Haberger, M.; Bathke, A.; Wegele, H.; Bell, C.; Kopf, R. Fast and Automated Characterization of Antibody Variants with 4D HPLC/MS. *Anal. Chem.* **2018**, *90* (3), 2119−2125.
(19) Goyon, A.; Dai, L.; Chen, T.; Wei, B.; Yang, F.; Andersen, N.; Kopf, R.; Leiss, M.; Mølhøj, M.; Guillarme, D.; Stella, C. From Proof of Concept to the Routine Use of an Automated and Robust Multi-Dimensional Liquid Chromatography Mass Spectrometry Workflow Applied for the Charge Variant Characterization of Therapeutic Antibodies. *J. Chromatogr A* **2020**, *1615*, 460740.
(20) Goyon, A.; Kim, M.; Dai, L.; Cornell, C.; Jacobson, F.; Guillarme, D.; Stella, C. Streamlined Characterization of an Antibody-Drug Conjugate by Two-Dimensional and Four-Dimensional Liquid Chromatography/Mass Spectrometry. *Anal. Chem.* **2019**, *91* (23), 14896−14903.
(21) Camperi, J.; Dai, L.; Guillarme, D.; Stella, C. Fast and Automated Characterization of Monoclonal Antibody Minor Variants from Cell Cultures by Combined Protein-A and Multidimensional LC/MS Methodologies. *Anal. Chem.* **2020**, *92* (12), 8506−8513.
(22) Camperi, J.; Dahotre, S.; Guillarme, D.; Stella, C. Monitoring Multiple Quality Attributes of a Complex Fc-Fusion Protein during Cell Culture Production Processes by mD-LC-MS Peptide Mapping. *Talanta* **2022**, *246*, 123519.
(23) Camperi, J.; Dai, L.; Guillarme, D.; Stella, C. Development of a 3D-LC/MS Workflow for Fast, Automated, and Effective Characterization of Glycosylation Patterns of Biotherapeutic Products. *Anal. Chem.* **2020**, *92* (6), 4357−4363.
(24) Camperi, J.; Grunert, I.; Heinrich, K.; Winter, M.; Özipek, S.; Hoelterhoff, S.; Weindl, T.; Mayr, K.; Bulau, P.; Meier, M.; Mølhøj, M.; Leiss, M.; Guillarme, D.; Bathke, A.; Stella, C. Inter-Laboratory Study to Evaluate the Performance of Automated Online Characterization of Antibody Charge Variants by Multi-Dimensional LC-MS/MS. *Talanta* **2021**, *234*, 122628.
(25) Bouvarel, T.; Camperi, J.; Guillarme, D. Multi-Dimensional Technology - Recent Advances and Applications for Biotherapeutic Characterization. *J. Sep Sci.* **2024**, *47* (5), No. e2300928.
(26) Herbert, C.; Valesyan, S.; Kist, J.; Limbach, P. A. Analysis of RNA and Its Modifications. *Annu. Rev. Anal Chem.* **2024**, *17* (1), 47−68.
(27) Thakur, P.; Abernathy, S.; Limbach, P. A.; Addepalli, B. Chapter One - Locating Chemical Modifications in RNA Sequences through Ribonucleases and LC-MS Based Analysis. In *Methods in Enzymology*; Jackman, J. E., Ed.; RNA Modification Enzymes; Academic Press: Cambridge, MA, 2021; Vol. *658*, pp *1*−24.
(28) Yoluç, Y.; Ammann, G.; Barraud, P.; Jora, M.; Limbach, P. A.; Motorin, Y.; Marchand, V.; Tisné, C.; Borland, K.; Kellner, S. Instrumental Analysis of RNA Modifications. *Crit. Rev. Biochem. Mol. Biol.* **2021**, *56* (2), 178−204.
(29) D'Ascenzo, L.; Popova, A. M.; Abernathy, S.; Sheng, K.; Limbach, P. A.; Williamson, J. R. Pytheas: A Software Package for the Automated Analysis of RNA Sequences and Modifications via Tandem Mass Spectrometry. *Nat. Commun.* **2022**, *13* (1), 2424.
(30) Goyon, A.; Scott, B.; Kurita, K.; Maschinot, C.; Meyer, K.; Yehl, P.; Zhang, K. On-Line Sequencing of CRISPR Guide RNAs and Their Impurities via the Use of Immobilized Ribonuclease Cartridges Attached to a 2D/3D-LC-MS System. *Anal. Chem.* **2022**, *94* (2), 1169−1177.
(31) Goyon, A.; Scott, B.; Yehl, P.; Zhang, K. Online Nucleotide Mapping of mRNAs. *Anal. Chem.* **2024**, *96* (21), 8674−8681.

https://doi.org/10.1021/acsmeasuresciau.4c00050
ACS Meas. Sci. Au 2024, 4, 615−619

(32) Goyon, A.; Nguyen, D.; Boulanouar, S.; Yehl, P.; Zhang, K. Characterization of Impurities in Therapeutic RNAs at the Single Nucleotide Level. *Anal. Chem.* **2022**, *94* (48), 16960−16966.
(33) Jones, J. D.; Grassmyer, K. T.; Kennedy, R. T.; Koutmou, K. S.; Maloney, T. D. Nuclease P1 Digestion for Bottom-Up RNA Sequencing of Modified siRNA Therapeutics. *Anal. Chem.* **2023**, *95* (9), 4404−4411.
(34) Li, Z.; Tong, F.; Xiao, L.; Larson, N. R.; Zhou, X.; Zhang, Y.; Immel-Brown, J. P.; Bou-Assaf, G. M. Establishing Stereochemical Comparability in Phosphorothioate Oligonucleotides with Nuclease P1 Digestion Coupled with LCMS Analysis. *Analyst* **2023**, *148* (21), 5361−5365.
(35) Beverly, M.; Dell, A.; Parmar, P.; Houghton, L. Label-Free Analysis of mRNA Capping Efficiency Using RNase H Probes and LC-MS. *Anal Bioanal Chem.* **2016**, *408* (18), 5021−5030.
(36) Wolf, E. J.; Grünberg, S.; Dai, N.; Chen, T.-H.; Roy, B.; Yigit, E.; Corrêa, I. R., Jr Human RNase 4 Improves mRNA Sequence Characterization by LC-MS/MS. *Nucleic Acids Res.* **2022**, *50* (18), No. e106.
(37) Goyon, A.; Yehl, P.; Zhang, K. Characterization of Therapeutic Oligonucleotides by Liquid Chromatography. *J. Pharm. Biomed. Anal.* **2020**, *182*, 113105.
(38) Goyon, A.; Zhang, K. Characterization of Antisense Oligonucleotide Impurities by Ion-Pairing Reversed-Phase and Anion Exchange Chromatography Coupled to Hydrophilic Interaction Liquid Chromatography/Mass Spectrometry Using a Versatile Two-Dimensional Liquid Chromatography Setup. *Anal. Chem.* **2020**, *92* (8), 5944−5951.
(39) Wei, B.; Wang, J.; Dai, L.; Chen, B.; Zhang, K. Characterization of Synthetic Guide Ribonucleic Acids through Hydrophilic Interaction Chromatography Coupled with Mass Spectrometry. *J. Chromatogr. A* **2023**, *1710*, 464414.
(40) Goyon, A.; Blevins, M. S.; Napolitano, J. G.; Nguyen, D.; Goel, M.; Scott, B.; Wang, J.; Koenig, S. G.; Chen, T.; Zhang, K. Characterization of Antisense Oligonucleotide and Guide Ribonucleic Acid Diastereomers by Hydrophilic Interaction Liquid Chromatography Coupled to Mass Spectrometry. *J. Chromatogr A* **2023**, *1708*, 464327.
(41) Lardeux, H.; D'Atri, V.; Guillarme, D. Recent Advances and Current Challenges in Hydrophilic Interaction Chromatography for the Analysis of Therapeutic Oligonucleotides. *TrAC, Trends Anal. Chem.* **2024**, *176*, 117758.
(42) Goyon, A.; Scott, B.; Kurita, K.; Crittenden, C. M.; Shaw, D.; Lin, A.; Yehl, P.; Zhang, K. Full Sequencing of CRISPR/Cas9 Single Guide RNA (sgRNA) via Parallel Ribonuclease Digestions and Hydrophilic Interaction Liquid Chromatography-High-Resolution Mass Spectrometry Analysis. *Anal. Chem.* **2021**, *93* (44), 14792−14801.
(43) Huang, M.; Xu, X.; Qiu, H.; Li, N. Analytical Characterization of DNA and RNA Oligonucleotides by Hydrophilic Interaction Liquid Chromatography-Tandem Mass Spectrometry. *J. Chromatogr. A* **2021**, *1648*, 462184.
(44) Islam, R.; Abdullah, A. M.; Sommers, C.; Rodriguez, J.; Zhang, D.; Kozak, D.; Yang, K. Validation of HILIC-HRMS Method for Oligonucleotide Analysis. https://www.fda.gov/media/168594/download (accessed 2024−08−30).
(45) Goyon, A.; Tang, S.; Fekete, S.; Nguyen, D.; Hofmann, K.; Wang, S.; Shatz-Binder, W.; Fernandez, K. I.; Hecht, E. S.; Lauber, M.; Zhang, K. Separation of Plasmid DNA Topological Forms, Messenger RNA, and Lipid Nanoparticle Aggregates Using an Ultrawide Pore Size Exclusion Chromatography Column. *Anal. Chem.* **2023**, *95* (40), 15017−15024.
(46) Liu, Z.; Zhou, Y.; Wang, Q.; Foley, J. P.; Stoll, D. R.; Shackman, J. G. Development of Tandem-Column Liquid Chromatographic Methods for Pharmaceutical Compounds Using Simulations Based on Hydrophobic Subtraction Model Parameters. *J. Chromatogr. A* **2023**, *1695*, 463925.
(47) Lardeux, H.; Stavenhagen, K.; Paris, C.; Dueholm, R.; Kurek, C.; De Maria, L.; Gnerlich, F.; Leek, T.; Czechtizky, W.; Guillarme, D.; Jora, M. Unravelling the Link between Oligonucleotide Structure and Diastereomer Separation in Hydrophilic Interaction Chromatography. *Anal. Chem.* **2024**, *96* (24), 9994−10002.
(48) Amsterdam, U. van. *PARADISE: bringing analytical science to the next level - HIMS*. Van't Hoff Institute for Molecular Sciences - University of Amsterdam. https://hims.uva.nl/content/news/2020/02/paradise-bringing-analytical-science-to-the-next-level.html (accessed 2024−08−28).
(49) Verduin, J.; Tutiš, L.; Becking, A. J.; Famili, A.; Zhang, K.; Pirok, B. W. J.; Somsen, G. W. Characterization of Dye-Loaded Poly(Lactic-Co-Glycolic Acid) Nanoparticles by Comprehensive Two-Dimensional Liquid Chromatography Combining Hydrodynamic and Reversed-Phase Liquid Chromatography. *Anal. Chem.* **2023**, *95* (51), 18767−18775.
(50) Oezipek, S.; Hoelterhoff, S.; Breuer, S.; Bell, C.; Bathke, A. mD-UPLC-MS/MS: Next Generation of mAb Characterization by Multidimensional Ultraperformance Liquid Chromatography-Mass Spectrometry and Parallel On-Column LysC and Trypsin Digestion. *Anal. Chem.* **2022**, *94* (23), 8136−8145.
(51) Lardeux, H.; Goyon, A.; Zhang, K.; Nguyen, J. M.; Lauber, M. A.; Guillarme, D.; D'Atri, V. The Impact of Low Adsorption Surfaces for the Analysis of DNA and RNA Oligonucleotides. *J. Chromatogr. A* **2022**, *1677*, 463324.
(52) D'Atri, V.; Lardeux, H.; Goyon, A.; Imiołek, M.; Fekete, S.; Lauber, M.; Zhang, K.; Guillarme, D. Optimizing Messenger RNA Analysis Using Ultra-Wide Pore Size Exclusion Chromatography Columns. *Int. J. Mol. Sci.* **2024**, *25* (11), 6254.
(53) Gilar, M.; DeLano, M.; Gritti, F. Mitigation of Analyte Loss on Metal Surfaces in Liquid Chromatography. *J. Chromatogr. A* **2021**, *1650*, 462247.
(54) Camperi, J.; Lippold, S.; Ayalew, L.; Roper, B.; Shao, S.; Freund, E.; Nissenbaum, A.; Galan, C.; Cao, Q.; Yang, F.; Yu, C.; Guilbaud, A. Comprehensive Impurity Profiling of mRNA: Evaluating Current Technologies and Advanced Analytical Techniques. *Anal. Chem.* **2024**, *96* (9), 3886−3897.
(55) Coll De Peña, A.; Gutterman-Johns, E.; Gautam, G. P.; Rutberg, J.; Frej, M. B.; Mehta, D. R.; Shah, S.; Tripathi, A. Assessment of pDNA Isoforms Using Microfluidic Electrophoresis. *Electrophoresis* **2024**, 1−10.
(56) De Vos, J.; Morreel, K.; Alvarez, P.; Vanluchene, H.; Vankeirsbilck, R.; Sandra, P.; Sandra, K. Evaluation of Size-Exclusion Chromatography, Multi-Angle Light Scattering Detection and Mass Photometry for the Characterization of mRNA. *J. Chromatogr. A* **2024**, *1719*, 464756.
(57) Ding, J.; Lee, Y.-T.; Bhandari, Y.; Schwieters, C. D.; Fan, L.; Yu, P.; Tarosov, S. G.; Stagno, J. R.; Ma, B.; Nussinov, R.; Rein, A.; Zhang, J.; Wang, Y.-X. Visualizing RNA Conformational and Architectural Heterogeneity in Solution. *Nat. Commun.* **2023**, *14* (1), 714.
(58) Wang, M. M.; Wappelhorst, C. N.; Jensen, E. L.; Chi, Y.-C. T.; Rouse, J. C.; Zou, Q. Elucidation of Lipid Nanoparticle Surface Structure in mRNA Vaccines. *Sci. Rep* **2023**, *13*, 16744.
(59) Tang, S.; Pederson, Z.; Meany, E. L.; Yen, C.-W.; Swansiger, A. K.; Prell, J. S.; Chen, B.; Grosskopf, A. K.; Eckman, N.; Jiang, G.; Baillet, J.; Pellett, J. D.; Appel, E. A. Label-Free Composition Analysis of Supramolecular Polymer-Nanoparticle Hydrogels by Reversed-Phase Liquid Chromatography Coupled with a Charged Aerosol Detector. *Anal. Chem.* **2024**, *96* (15), 5860−5868.
(60) Zhang, K.; Dai, L.; Chetwyn, N. P. Simultaneous Determination of Positive and Negative Pharmaceutical Counterions Using Mixed-Mode Chromatography Coupled with Charged Aerosol Detector. *J. Chromatogr. A* **2010**, *1217* (37), 5776−5784.

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