UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

SAREPTA THERAPEUTICS, INC.,

Petitioner

v.

GENZYME CORPORATION,

Patent Owner

U.S. Patent No. 12,298,313

"Methods for Detecting AAV"

IPR2026-00270

DECLARATION OF JOSHUA J. COON, PH.D., IN SUPPORT OF PETITION FOR *INTER PARTES* REVIEW OF U.S. PATENT NO. 12,298,313

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1. "A method of detecting post-translational modifications of one or more viral proteins (VPs) in a preparation of adeno-associated virus (AAV) particles, the method comprising" 110

2. "a) denaturing the AAV particles;" 112

3. "b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;" 113

4. "c) determining the masses of the one or more VPs; and" 114

5. "d) determining any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs that have not undergone post-translational modifications to detect a deviation in the compared masses," 115

6. "wherein the VPs comprise VP1, VP2 and VP3 capsid proteins, and wherein the method is performed in the absence of a gel separation step." 116

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1. "A method of determining the heterogeneity of viral particles in a preparation of adeno-associated virus (AAV) particles comprising VP1, VP2 and VP3 capsid proteins, the method comprising" 134

2. "a) denaturing the AAV particles;" 136

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3. "b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, thereby separating the peaks of the VP1, VP2 and VP3 capsid proteins;".....138

4. "c) deconvoluting the peaks of the VP1, VP2 and VP3 capsid proteins; and".....140

5. "d) determining the masses of one or more of the VP1, VP2 and VP3 capsid proteins and additional capsid proteins within one or more of the deconvoluted peaks," 141

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R. Claim 18: "The method of claim 11, wherein the liquid chromatography is reverse phase chromatography." 158

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1. "A method of preparing a pharmaceutical composition of adeno-associated virus (AAV) particles, the method comprising:".....161

2. "monitoring AAV particles for consistency and/or identity;".....163

3. "wherein the AAV particles comprise viral proteins (VPs) comprising VP1, VP2 and VP3 capsid proteins of an AAV particle capsid," 165

4. "wherein the AAV particle is monitored for consistency and/or identity by:" 166

5. "a) extracting an aliquot of an AAV particle preparation;".....168

6. "b) denaturing the AAV particles;" 169

7. "c) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;".....171

8. "d) determining the masses of one or more VPs of the AAV particles; and".....172

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10. "f) determining if there is any deviation of the determined masses of the one or more VPs from the theoretical masses of the corresponding VPs;" 174

11. "wherein the determination of any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs thereby monitors the AAV particles for consistency and/or identity;".....175

12. "wherein the monitoring for consistency and/or identity is performed in the absence of a gel separation step; and".....177

13. "wherein if less than an undesirable amount of deviation is determined during the monitoring for consistency and/or identity, the AAV particles are combined with one or more pharmaceutically acceptable excipients to form the pharmaceutical composition.".....179

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I, Joshua J. Coon, Ph.D., hereby declare as follows.

1. I have been retained as an expert witness on behalf of Sarepta Therapeutics, Inc. ("Sarepta") in connection with the above-captioned request for *inter partes* review ("IPR"). I am being compensated for my time in connection with this IPR at my standard rate, which is $600 per hour.

2. I understand that Sarepta is petitioning for *inter partes* review of U.S. Patent No. 12,298,313 ("the '313 patent") (EX1001) and requests that the United States Patent and Trademark Office cancel claims 1-27 of the '313 patent as unpatentable. The following discussion and analysis provides my opinions as to why claims 1-27 would have been obvious to a person of ordinary skill in the art ("POSA").

I. BACKGROUND AND QUALIFICATIONS

3. My research program is centered on the development and application of innovative chemical instrumentation and molecular analysis methodology, with a focus on advancing the fields of proteomics, structural biology, multi-omics, and systems biology.

4. I am dedicated to pushing the boundaries of analytical techniques, and work from my laboratory has led to significant advancements in mass spectrometry-based technologies for biomolecule characterization.

5. As a postdoctoral fellow, I co-invented electron transfer dissociation (ETD), a technique now widely used for protein sequencing and the identification of labile post-translational modifications.

6. Later I pioneered its coupling with Orbitrap technology, improved efficiency with both collisions and infrared photons, developed a negative ion analog (NETD), and applied these techniques to a wide variety of biological molecules.

7. This work led to commercial implementation, widespread adoption of our technology globally, and the ASMS Biemann Medal.

8. Our success in fundamental ion–ion chemistry proved a natural foundation for the expansion of my research program to include accelerated proteomics analysis, the development of the widely used targeted method parallel reaction monitoring (PRM), integrated multi-omics, and deep sequencing technologies, which recently allowed for the global mapping of how alternative splicing is incorporated into the proteome.

9. We have pioneered native mass spectrometry for structural characterization of biomolecules. In 2023, for example, we published the first three-dimensional reconstruction of mass analyzed protein–protein complexes.

10. I discuss some areas of my research in more detail below.

11. **Chemical Instrumentation and Tandem Mass Spectrometry.** A central theme of my research is to advance tandem mass spectrometry to provide

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faster and more complete proteome analysis. This technology provides the ability to sequence and identify proteins, map their sites of post-translational modificaiton (PTM), and assess their abundances. Since 2005, my group has published over 400 research articles, over half of which are from this area of research.

12. As I mentioned above, as a post-doc, I co-invented ETD and described the work in 2004. A few years later in my group, we pioneered the first coupling of ETD with the new Orbitrap technology.

13. By 2008, the commercial implementation of our technology began to reach researchers across the globe. Today well over 3,000 of these systems are in use worldwide and over 100 publications appear each year describing the use of our instrumentation to study a broad range of biomedical research applications.

14. Another key result was the discovery of negative ETD (NETD) in 2005. Since this time, we have published several manuscripts on NETD, a method for analysis of acidic PTMs such as glycosylation and histidine phosphorylation and therapeutic RNA molecules.

15. Automated instrument decision making is another theme to emerge from our instrumentation efforts and we have published several influential works in this area.

16. Our work on decision tree-driven mass spectrometry is one example that has been commercially developed. Related to this topic, we pioneered a new

type of targeted data acquisition called parallel reaction monitoring, a term we coined in a seminal manuscript describing the approach.

17. **Structural Biology**. We have developed innovative mass spectrometry-based technologies for cryogenic electron microscopy (cryo-EM) sample preparation through cryogenic soft landing.

18. Conventional plunge freezing for cryo-EM is limited by issues like protein denaturation at the air-water interface and preferred particle orientation, hindering structural analysis of challenging samples.

19. To overcome this, my group pioneered coupling a modified mass spectrometer with a cryogenic landing stage, enabling mass analysis and selection of protein complexes in the gas-phase for direct deposition onto a cryogenically cooled grid with subsequent deposition of amorphous ice.

20. We achieved the first-ever 3D reconstructions of mass-analyzed protein complexes, demonstrating that proteins can retain structural integrity after gas-phase manipulation and landing, while also improving the diversity of particle orientations.

21. To repair damage incurred during the process we most recently found that laser rehydration of cryo-landed particles restores native structure and should allow for atomic resolution of landed proteins.

22. This pioneering work, integrating mass spectrometry capabilities like purification and characterization with cryo-EM sample prep, aims to solve structures

currently inaccessible by conventional methods and pave the way for a high-throughput, proteome-scale structural biology platform.

23. **Native and Intact Protein Mass Spectrometry**. I have been interested in the field of native and intact protein mass spectrometry since my early career where I used ion/ion reactions to systematically characterize intact proteins on low resolution ion trap mass spectrometers.

24. Over the years, we have improved on these early works by coupling ETD with Orbitrap mass analyzers and the development of activated-ion ETD. We further demonstrated that activated ion-ETD improves fragmentation and sequencing for challenging intact proteoforms such as antibodies.

25. Native mass spectrometry complements intact protein analysis and has become a keen interest of my group. We recently used this approach to study intact macromolecular assemblies.

26. These innovations collectively aim to routinize sequencing and purification of intact and native proteins, solve structures currently inaccessible by conventional methods, and contribute to a high-throughput, proteome-scale understanding of protein structure and function.

27. **Proteomic Methodology: Deep Sequencing, PTMs, and Quantification**. The development of proteomic methodology is a major thrust of my program as evidenced by nearly 70 career publications in this space.

28. First, to complement our instrumentation advances, we have described strategies for comprehensive analysis of numerous PTM types, including acetylation, glycosylation, methylation, and phosphorylation, among others. An example of this synergy can be found in our 2019 manuscript profiling N-linked glycosylation in the brain.

29. Likewise, my laboratory has been a major pioneer in the field of quantitative proteomics.

30. We were among the first groups worldwide to reveal and explain reporter ion contamination in isobaric tagging. Our work demonstrated that dynamic range in isobaric tagging experiments was considerably compressed because of this problem, and we were the first to suggest a solution.

31. My group also innovated an entirely new method of protein quantification called neutron encoding – NeuCode.

32. We pursued methodology to allow deep, rapid sequencing of whole proteomes, publishing the deepest proteome on record in 2023.

33. In a recent exciting application, described in *Science*, we used our PTM and quantification technologies to reveal the regulatory role of N-glycosylation in signaling.

34. Also, in 2024 we published results demonstrating that we could analyze nearly the whole human proteome or phosphoproteome in approximately thirty minutes.

35. **Metabolism, Developmental, and Systems Biology**. We have applied our innovative tools to many cutting-edge biological projects spanning several areas as documented by nearly 200 such publications.

36. With our fast and quantitative multi-omic technologies we are partnering with worldwide leaders to probe large genetic reference populations toward resolving the genetic factors that influence complex traits.

37. This approach is exemplified in publications where we used mass spectrometry to map the proteomes, lipidomes, and metabolomes of hundreds of yeast and human cell lines, each lacking a single gene related to mitochondrial biology. From these data we predicted molecular functions, mapped gene-specific perturbations that reflect protein functions, and resolved several cases of undiagnosed human disease, resulting in a high profile publication in the journal Nature (2023).

38. My group first became active in this multi-omics space in 2010 with our groundbreaking work to couple the Orbitrap mass analyzer with gas chromatography separations. The system we developed was commercialized by Thermo Scientific in 2015.

39. Liquid chromatography mass spectrometry small molecule assays are also critical and we have developed and applied a number of lipidomic assays for the study of biological systems.

40. Further, compared to proteomics, where software routinely quantifies thousands of proteins with minimal manual validation, discovery lipidomics is much less mature.

41. Given this major technology bottleneck, we developed a freely available software suite to unify and automate all stages of lipid identification – LipiDex. Building from LipiDex we developed another tool to use gene loci to identify unannotated tandem mass spectrometry spectra.

42. And just this year, we described the combined analysis of peptides and lipids in a single shot format for large scale analysis of lysosomal storage diseases.

43. **Research Team and Additional Work**. My research team currently consists of 13 graduate students, three postdocs, two technicians, and two Ph.D. level staff scientists.

44. We have a rapidly growing cohort of alumni (39 PhDs and 12 postdoctoral researchers to date) who are contributing to biological and technological research in academia and industry. Our graduates are known for being "technology innovators" rather than just users, with many taking leading roles at companies like Thermo Fisher Scientific where they design the next generation of

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mass spectrometry hardware and software. Others have moved into high-level positions at pharmaceutical giants like Genentech, Pfizer, and Eli Lilly, where they apply these advanced technologies to drug discovery and vaccine characterization. In academia, former lab members have successfully established their own research programs at universities across the globe including University of California-San Francisco, University of Washington-Seattle, Colorado University-Boulder, Duke, and University of North Carolina-Chapel Hill, aomong others, ensuring that our lab's unique emphasis on blending chemical instrumentation, coding, and biology continues to influence the wider scientific community.

45. We have established a one-of-its-kind, weeklong North American Mass Spectrometry Summer School that has trained ~1,500 scientists from 44 states and beyond over the past nine years. This program equips participants, particularly early-career scientists and non-experts, with the fundamental knowledge and skills needed for biological mass spectrometry, ultimately promoting the wider adoption and application of these powerful technologies in biomedical research.

46. With the evolution of our research program into structural biology, we spearheaded a new annual conference called MS+M, which focuses on the intersection of mass spectrometry and microscopy. Now going into its third occurrence, this international conference has been held at Oxford, UK, Madison, WI,

and now the Netherlands, attracts ~100 scientists each year, and has catalyzed rapid growth in this nexus of fields.

47. I also am the inventor on nineteen issued U.S. patents.

48. EX1004 is a copy of my *curriculum vitae* setting forth additional information concerning my background, credentials, publications, and awards.

II. MATERIALS CONSIDERED

49. In formulating my opinions, I considered all of the references cited in this Declaration, which are set out in the table below, including the following documents:

- U.S. Patent No. 12,298,313 ("the '313 patent") (EX1001);
- Satkunanathan *et al*., "Establishment of a Novel Cell Line for the Enhanced Production of Recombinant Adeno-Associated Virus Vectors for Gene Therapy," *Human Gene Therapy* 25.11 (2014): 929-941 (EX1005);
- Shytuhina *et al*., "Development and application of a reversed-phase high-performance liquid chromatographic method for quantitation and characterization of a Chikungunya virus-like particle vaccine," *Journal of Chromatography A* 1364 (2014): 192-197 (EX1006);
- Yuan *et al*., "Reversed-phase high-performance liquid chromatography of virus-like particles," *Journal of Chromatography A* 816.1 (1998): 21-28 (EX1037).

Exhibit Number	Description
EX1001	U.S. Patent No. 12,298,313 ("the '313 patent")
EX1002	Prosecution history of U.S. Patent No. 12,298,313 ("the '313 prosecution history")

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EX1003	Expert Declaration of Joshua J. Coon, Ph.D.
EX1004	*Curriculum vitae* of Joshua J. Coon, Ph.D.
EX1005	Satkunanathan *et al*., "Establishment of a Novel Cell Line for the Enhanced Production of Recombinant Adeno-Associated Virus Vectors for Gene Therapy," *Human Gene Therapy* 25.11 (2014): 929-941 ("Satkunanathan")
EX1006	Shytuhina *et al*., "Development and application of a reversed-phase high-performance liquid chromatographic method for quantitation and characterization of a Chikungunya virus-like particle vaccine," *Journal of Chromatography A* 1364 (2014): 192-197 ("Shytuhina")
EX1007	Alqahtani, "Analysis of purified wild type and mutant adenovirus particles by SILAC based quantitative proteomics," *Journal of General Virology* 95.11 (2014): 2504-2511 ("Alqahtani")
EX1008	Anacleto and Boyd, "Calibration of ion spray mass spectra using cluster ions," *Organic Mass Spectrometry* 27.6 (1992): 660-666 ("Anacleto")
EX1009	Ansong *et al*., "Top-down proteomics reveals a unique protein S-thiolation switch in *Salmonella* Typhimurium in response to infection-like conditions," *Proceedings of the National Academy of Sciences* 110.25 (2013): 10153-10158 ("Ansong")
EX1010	Ayuso *et al*., "Manufacturing and Characterization of a Recombinant Adeno-Associated Virus Type 8 Reference Standard Material," *Human Gene Therapy* 25.11 (2014): 977-987 ("Ayuso")
EX1011	Becerra *et al*., "Direct mapping of adeno-associated virus capsid proteins B and C: a possible ACG initiation codon," *Proceedings of the National Academy of Sciences* 82.23 (1985): 7919-7923 ("Becerra")

Exhibit Number	Description
EX1012	Bondarenko *et al*., "Mass Measurement and Top-Down HPLC/MS Analysis of Intact Monoclonal Antibodies on a Hybrid Linear Quadrupole Ion Trap-Orbitrap Mass Spectrometer," *Journal of the American Society for Mass Spectrometry* 20.8 (2009): 1415-1424 ("Bondarenko")
EX1013	Burova and Ioffe, "Chromatographic purification of recombinant adenoviral and adeno-associated viral vectors: methods and implications," *Gene Therapy* 12.1 (2005): S5-S17 ("Burova")
EX1014	Byeon *et al*., "Structural Identification of a Non-Glycosylated Variant at Ser126 for O-Glycosylation Site from EPO BRP, Human Recombinant Erythropoietin by LC/MS Analysis," *Molecules and Cells* 38.6 (2015): 496-505 ("Byeon")
EX1015	Cecchini *et al*., "Toward exascale production of recombinant adeno-associated virus for gene transfer applications," *Gene Therapy* 15.11 (2008): 823-830 ("Cecchini")
EX1016	Chelius *et al*., "Analysis of the adenovirus type 5 proteome by liquid chromatography and tandem mass spectrometry methods," *Journal of Proteome Research* 1.6 (2002): 501-513 ("Chelius")
EX1017	Chen *et al*., "Molecular characterization of adeno-associated viruses infecting children," *Journal of Virology* 79.23 (2005): 14781-14792 ("Chen")
EX1018	Coon *et al*., "Tandem mass spectrometry for peptide and protein sequence analysis." *Biotechniques* 38.4 (2005): 519-523 ("Coon 2005")
EX1019	Coon, "Collisions or electrons? Protein Sequence Analysis in the 21st Century," *Anal. Chem*. (2009): 3208-3215 ("Coon 2009")

Exhibit Number	Description
EX1020	Cueto-Rojas, "Interferon-α 2b quantification in inclusion bodies using Reversed Phase-Ultra Performance Liquid Chromatography (RP-UPLC)," *Journal of Chromatography B* 878.13-14 (2010): 1019-1023 ("Cueto-Rojas")
EX1021	Davis *et al*., "Rational Design and Engineering of a Modified Adeno-Associated Virus (AAV1)-Based Vector System for Enhanced Retrograde Gene Delivery," *Neurosurgery* 76.2 (2015): 216-225 ("Davis")
EX1022	Dong *et al*., "Proteomics analysis of co-purifying cellular proteins associated with rAAV vectors," *PLoS One* 9.2 (2014): e86453 ("Dong")
EX1023	Glish and Vachet, "The Basics of Mass Spectrometry in the Twenty-First Century," *Nature Reviews Drug Discovery* 2.2 (2003): 140-150 ("Glish")
EX1024	Good and Coon, "Advancing proteomics with ion/ion chemistry," Mass Spectrometry For Proteomics Analysis Review, *Biotechniques* 40.6 (2006): 783-789 ("Good")
EX1025	Grimm and Kay, "From Virus Evolution to Vector Revolution: Use of Naturally Occurring Serotypes of Adeno-Associated Virus (AAV) as Novel Vectors for Human Gene Therapy," *Current Gene Therapy* 3.4 (2003): 281-304 ("Grimm and Kay")
EX1026	Han, *et al*. "Extending top-down mass spectrometry to proteins with masses greater than 200 kilodaltons," *Science* 314.5796 (2006): 109-112 ("Han")
EX1027	Huang *et al*., "In Vivo Deamidation Characterization of Monoclonal Antibody by LC/MS/MS," *Analytical Chemistry* 77.5 (2005): 1432-1439 ("Huang")

Exhibit Number	Description
EX1028	Loo *et al*., "Tandem Mass Spectrometry of Very Large Molecules: Serum Albumin Sequence Information from Multiply Charged Ions Formed by Electrospray Ionization," *Analytical Chemistry* 63.21 (1991): 2488-2499 ("Loo")
EX1029	Mahoney and Hermodson, "Separation of Large Denatured Peptides by Reverse Phase High Performance Liquid Chromatography; Trifluoroacetic Acid as a Peptide Solvent," *Journal of Biological Chemistry* 255.23 (1980): 11199-11203 ("Mahoney")
EX1030	Merten and Al-Rubeai, *Viral Vectors for Gene Therapy*, Vol. 737, Totowa, NJ: Humana Press, 2011 ("Merten")
EX1031	Rayaprolu *et al*. "Comparative analysis of adeno-associated virus capsid stability and dynamics," *Journal of Virology* 87.24 (2013): 13150-13160 ("Rayaprolu")
EX1032	Rhoads *et al*., "Neutron-Encoded Mass Signatures for Quantitative Top-Down Proteomics," *Analytical Chemistry* 86.5 (2014): 2314-2319 ("Rhoads")
EX1033	Richards *et al*., "Proteome sequencing goes deep," *Current Opinion in Chemical Biology* 24 (2015): 11-17 ("Richards")
EX1034	Riley *et al*., "Activated Ion Electron Transfer Dissociation for Improved Fragmentation of Intact Proteins," *Analytical Chemistry* 87.14 (2015): 7109-7116 ("Riley")
EX1035	Rouse *et al*., "Top-down characterization of protein pharmaceuticals by liquid chromatography/mass spectrometry: Application to recombinant factor IX comparability – A case study," *Therapeutic proteins: Methods and Protocols*, Totowa, NJ: Humana Press, 2005, 435-460 ("Rouse")
EX1036	Siuzdak, "Probing Viruses with Mass Spectrometry," *Journal of Mass Spectrometry* 33.3 (1998): 203-211 ("Siuzdak")

Exhibit Number	Description
EX1037	Yuan *et al*., "Reversed-phase high-performance liquid chromatography of virus-like particles," *Journal of Chromatography A* 816.1 (1998): 21-28 ("Yuan")
EX1038	Zabrouskov *et al*., "Stepwise deamidation of ribonuclease A at five sites determined by top down mass spectrometry," *Biochemistry* 45.3 (2006): 987-992 ("Zabrouskov)
EX1039	Zubarev and Makarov, "Orbitrap Mass Spectrometry," *Analytical Chemistry*, (2013): 5288-5296 ("Zubarev")
EX1040	*Genzyme Corp. v. Sarepta Therapeutics, Inc. and Sarepta Therapeutics Three, LLC*., Second Amended Complaint, C.A. No. 24-cv-00882-RGA (D. Del.)

III. LEGAL STANDARDS – OBVIOUSNESS

50. In this section, I describe my understanding of certain legal standards relating to the issue of obviousness that I have been asked to consider for claims 1-27 of the '313 patent. These legal standards have been explained to me in connection with the preparation of this Declaration. I have applied these standards in my analysis, as described in the sections below.

51. I understand that a claim is obvious when the differences between the claim and the prior art are such that the claim as a whole would have been obvious to a POSA at the relevant time. It is my understanding that four factors are applied in determining whether a claim is unpatentable as obvious under 35 U.S.C. § 103: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims at issue; (3) the level of ordinary skill in the art; and (4) objective

evidence indicating obviousness or non-obviousness – also referred to as "secondary considerations" – if present.

52. I understand that secondary considerations may include: (1) long felt but unmet need in the prior art that was satisfied by the invention of the patent; (2) commercial success or lack of commercial success of the subject matter claimed in the patent; (3) unexpected results achieved by the invention; (4) praise of the invention by others skilled in the art; (5) the taking of licenses under the patent by others; and (6) deliberate copying of the invention.

53. I understand that a claim can be obvious over a single reference in combination with the knowledge of a POSA, or based on the teachings in a combination of references. For obviousness based on a combination of prior art references, I understand that a POSA must have a motivation to combine the references. I understand that the prior art references themselves may provide a suggestion, motivation, or reason to combine. I further understand that a motivation to combine two or more prior art references need not be express, but may be based upon common sense or the knowledge available to a POSA.

54. In addition, I understand that a POSA must have a reasonable expectation of success in modifying or combining the prior art to arrive at the claimed invention. However, I understand that obviousness cannot be avoided merely because there is some degree of unpredictability in the art.

55. Finally, I understand that it is impermissible to evaluate obviousness from a hindsight perspective, using the teachings of the patent as a guide. However, I understand that a POSA is not an automaton, and is assumed to have a reasonable degree of creativity. As such, I understand that an analysis of obviousness may take account of the inferences and creative steps that a POSA would employ.

IV. SUMMARY OF INVALIDITY GROUNDS

56. The table below summarizes the invalidity grounds for claims 1-27 of the ’313 patent that I address in this declaration.

Ground	Claims	Description
1	1-27	Obvious in view of Satkunanathan and Shytuhina
2	6, 19, 27	Obvious in view of Satkunanathan, Shytuhina, and Yuan
3	7, 8, 9, 10	Obvious in view of Satkunanathan, Shytuhina, and Zabrouskov

57. I note that Satkunanathan, Shytuhina, Yuan, and Zabrouskov were not considered by the Patent Office during prosecution. EX1001 (the ’313 patent) (“References Cited”); EX1002 (the ’313 Prosecution History).

58. Satkunanathan was published in November 2014, more than one year before the earliest priority date listed on the face of the ’313 patent, August 15, 2016. EX1001 (the ’313 patent); EX1005 (Satkunanathan).

59. Shytuhina was published in June 2014, more than one year before the earliest priority date listed on the face of the ’313 patent, August 15, 2016. EX1001 (the ’313 patent); EX1006 (Shytuhina).

60. Yuan was published in August 1998, more than one year before the earliest priority date listed on the face of the ’313 patent, August 15, 2016. EX1001 (the ’313 patent); EX1037 (Yuan).

61. Zabrouskov was published in December 2005, more than one year before the earliest priority date listed on the face of the ’313 patent, August 15, 2016. EX1001 (the ’313 patent); EX1038 (Zabrouskov).

V. TECHNICAL BACKGROUND AND STATE OF THE PRIOR ART

A. Gene Therapy

62. Gene therapy involves the delivery of genetic information, specifically a therapeutic gene, to target cells in a patient. *See, e.g.*, EX1030 (Merten), p.1. A vector, such as a viral vector, is used to deliver the genetic information into the target cells. *See, e.g.*, EX1030 (Merten), p.1. The virus introduces the therapeutic gene into target cells in a process known as “transduction.” *See, e.g.*, EX1030 (Merten), p.1.

63. A variety of different viral vectors have been evaluated as gene delivery vectors for gene therapy. *See, e.g.*, EX1030 (Merten), p.1.

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B. Adeno-Associated Virus (AAV)

64. Adeno-associated viruses ("AAVs") have been extensively studied as gene therapy vectors. EX1031 (Rayaprolu), p. 13150. AAV is a replication defective virus that requires helper functions, usually provided by adenoviral genes. AAVs have a number of favorable properties as gene therapy vectors, including that they do not cause disease and can transduce a variety of different cell types. EX1031 (Rayaprolu), p. 13150; EX1025 (Grimm and Kay), p. 290, Figure (3). In general, the AAV capsid largely determines the tissues a particular AAV serotype can infect. EX1005 (Satkunanathan), p. 938; EX1030 (Merten), p. 7.

65. Modified AAV viruses, known as "recombinant" AAVs ("rAAVs") have been used as gene therapy vectors to treat diseases including Duchenne muscular dystrophy, cystic fibrosis, Parkinson's disease, hemophilia B, and Canavan disease. EX1030 (Merten), pp. 9, 20.

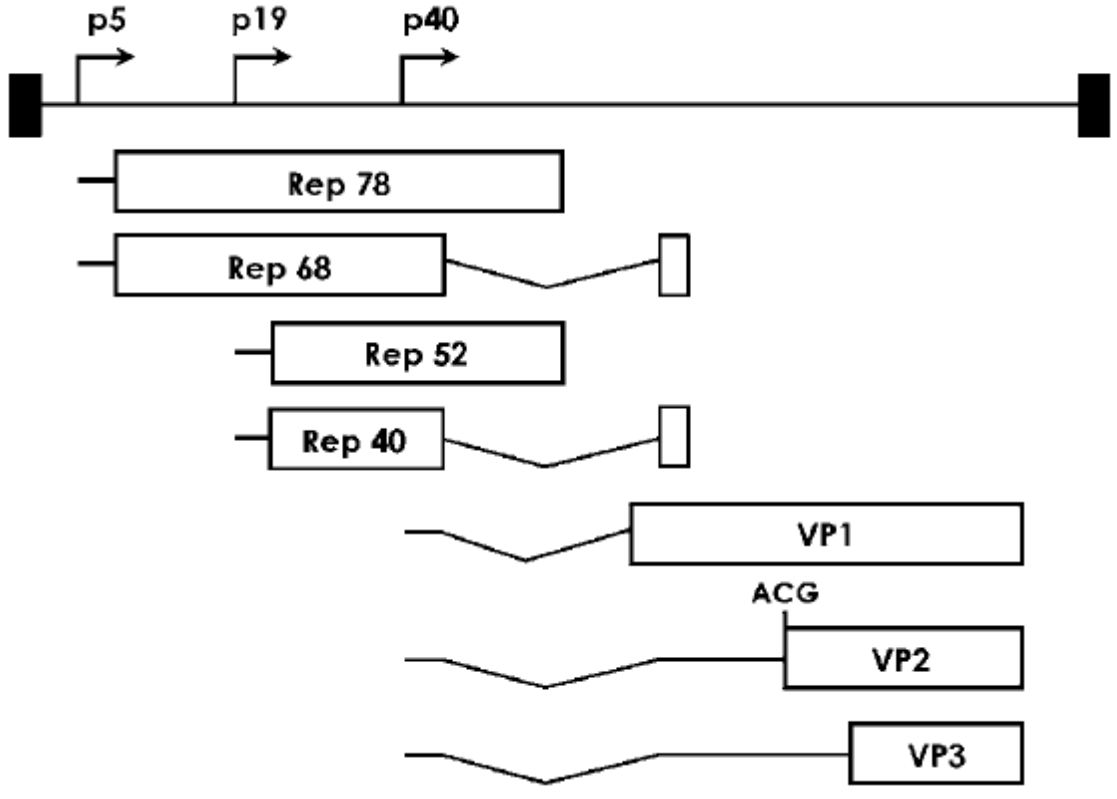
66. AAV belongs to the *Dependovirus* genus of the *Parvovirus* family and was first discovered in 1965 as a coinfecting agent of adenovirus. EX1030 (Merten), p. 7. The AAV capsid is a nonenveloped, icosahedral protein shell, approximately 22 nm in diameter. EX1030 (Merten), p. 7.

67. The genome of AAV is composed of a linear, single-stranded DNA, 4680 nucleotides long, with a 145 bp ITR sequence on each end, and two open reading frames between the ITRs. EX1030 (Merten), p. 7; EX1015 (Cecchini), pp.

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823-824, Figure 1. The 5' open reading frame contains nucleotides that code for four replication proteins, Rep 78, Rep 68, Rep 52, and Rep 40. EX1030 (Merten), p. 7. The 3' open reading frame, the *cap* gene, codes for the three AAV structural proteins, vp1, vp2 and vp3, with theoretical molecular masses of about 87, 73, and 62 kDa, respectively. EX1015 (Cecchini), pp. 823-24, Figure 1; EX1030 (Merten), p. 7; EX1031 (Rayaprolu), p. 13150-51. The vp1, vp2, and vp3 proteins overlap, sharing a common C terminus. EX1031 (Rayaprolu), p. 13151.

68. The diagram below shows the genomic structure of the AAV *rep* and *cap* transcripts:



EX1015 (Cecchini), p. 824, Figure 1.

69. As can be seen from the figure above, vp1, vp2, and vp3 are alternative transcripts from a single gene. EX1015 (Cecchini), p. 824, Figure 1, legend. The second orf (promoter p40) encodes the three capsid proteins from two transcripts. EX1015 (Cecchini), p. 824, Figure 1, legend. vp1 is initiated from the first *cap* transcript, and vp2 and vp3 are initiated at two different codon sites from the second *cap* transcript. Note that the start codon of vp2 is an ACG. EX1015 (Cecchini), p. 824, Figure 1, legend.

70. The AAV capsid contains about 60 copies of vp1, vp2, and vp3, in the ratio of approximately 1:1:10. EX1015 (Cecchini), pp. 823-24, Figure 1. This ratio reflects the stoichiometry of intracellular *cap* gene products and is regulated by splicing and utilization of the atypical ACG initiation codon. EX1015 (Cecchini), pp. 823-24, Figure 1.

71. Translational initiation from the first in-frame AUG codon produces vp1. EX1015 (Cecchini), pp. 823-24, Figure 1. Differential splicing removes the vp1 initiation codon, allowing inefficient translation of vp2 from an ACG triplet, producing vp2. EX1015 (Cecchini), pp. 823-24, Figure 1. The majority of ribosomes bypass the ACG vp2 initiation triplet and scan through to the first AUG codon, initiating vp3 translation. EX1015 (Cecchini), pp. 823-24, Figure 1.

C. Different AAV Serotypes

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72. It had been known in the art for over a decade, at least as of 2003, that identification and characterization of different AAV serotypes was useful and important for developing gene therapies based on rAAV vectors. EX1025 (Grimm and Kay), Abstract.

73. Researchers for decades had been studying different serotypes of AAV, mutated versions of AAV, and genetically engineered versions of AAV. EX1017 (Chen), EX1021 (Davis).

74. For example, Chen disclosed a study of genetic diversity of AAV circulating in humans and monkeys. EX1017 (Chen), p. 14781. In particular, Chen studied nine different isolates of AAV. Chen sequenced cap genes from these isolates, finding that eight of them showed about 98% amino acid identity to AAV2, while the ninth was intermediate between AAV2 and AAV3. EX1017 (Chen), Abstract.

75. Chen found that the eight AAV2-like isolates had point mutations at positions 585 and 588 of the capsid protein, specifically, R585S and R588T mutations. EX1017 (Chen), Abstract, p. 14784. Chen tested two of these mutants and found that they were unable to bind heparin *in vitro*. EX1017 (Chen), pp. 14784-87.

76. Davis disclosed engineering a novel AAV vector by inserting a neuronal targeting peptide into the AAV1 capsid. EX1021 (Davis), Abstract. The

goal of the study was to enhance AAV transduction of motor neurons. EX1021 (Davis), Abstract.

77. Davis used computational modeling to analyze capsid structure and conformation and to visualize and determine the optimal structural context for peptide display. EX1021 (Davis), p. 217.

78. Davis noted that the "convergence of high-speed computing, a tremendous increase in capsid structural information, and a growing understanding of the forces that control protein structure and maintain essential viral functions has resulted in dramatic advances in our ability to engineer protein function and structure and to create novel, rationally designed virus-based gene transfer vectors." EX1021 (Davis), p. 217.

79. Grimm and Kay discussed the need for AAV serotypes other than AAV2, the most well-characterized serotype at the time. EX1025 (Grimm and Kay), Abstract. Grimm and Kay noted that vectors based on AAV2 were found to be inefficient at transducing some cells of therapeutic interest, such as liver and muscle cells. EX1025 (Grimm and Kay), Abstract.

80. In addition, Grimm and Kay stated that gene transfer using an AAV2-based vector might be hampered by neutralizing anti-AAV-2 antibodies, which are highly prevalent in the human population. EX1025 (Grimm and Kay), Abstract.

81. As a result, researchers focused on the seven other naturally occurring serotypes of AAV known at the time (AAV1 and AAV3 to AAV8), which are structurally and functionally different from AAV2. EX1025 (Grimm and Kay), Abstract.

82. One strategy for designing rAAV gene therapy vectors with new properties involved cross-packaging an AAV2 vector genome into the capsids of other AAV serotypes, resulting in "pseudotyped" AAV vectors. EX1025 (Grimm and Kay), Abstract. Both *in vitro* and *in vivo*, these rAAV vectors were shown to have different tropism from (ability to infect different cell types than) AAV2, and to escape the anti-AAV2 immune response. EX1025 (Grimm and Kay), Abstract.

83. About a decade later, in 2014, researchers continued to investigate and compare the properties of different AAV serotypes. EX1031 (Rayaprolu), Abstract. Rayaprolu used limited proteolysis and peptide mass mapping of intact particles of AAV1, AAV2, AAV5, and AAV8, to investigate the properties of these serotypes. EX1031 (Rayaprolu), Abstract.

84. Rayaprolu explained that investigating the biophysical properties of the different AAV serotypes is important for understanding viral function inside cells and is critical for viral "production, storage, and use for gene therapy." EX1031 (Rayaprolu), Abstract.

85. Rayaprolu noted that interest in the use of serotypes other than AAV2 – for example, AAV1, AAV5, AAV6, and AAV8 – was "growing because of their different tissue specificities, cell transduction efficiencies, and antigenicities." EX1031 (Rayaprolu), p. 13150.

D. Purification of rAAV

86. The process for creating vectors from AAVs begins with the deletion of genes coding for the Rep and Cap proteins. EX1030 (Merten), p. 8. This deletion provides approximately 5 kb of packing space for foreign DNA, such as a therapeutic transgene. EX1030 (Merten), p. 8.

87. The new DNA is inserted into an AAV viral vector that contains only the ITRs. EX1030 (Merten), p. 8. The ITRs contain all cis-acting elements necessary for replication and packaging of the therapeutic transgene in the presence of a helper virus, such as adenovirus. EX1030 (Merten), p. 8. The Rep and Cap proteins and all necessary adenoviral helper genes are expressed on one or two plasmids. EX1030 (Merten), p. 8.

88. A variety of different purification methods for rAAV have been used over the years, including methods relying on density gradient ultracentrifugation and column chromatography. *See, e.g.*, EX1010 (Ayuso); EX1013 (Burova).

89. A common approach to AAV purification involves cesium chloride (CsCl) density gradient centrifugation. EX1013 (Burova), p. S8. One benefit of

CsCl density gradient centrifugation is that it can separate empty capsids, lacking the DNA genome, from filled capsids. EX1013 (Burova), p. S8.

90. Different types of chromatography, including anion exchange chromatography, affinity chromatography, size exclusion chromatography, and hydrophobic interaction chromatography, have also been used for AAV purification. EX1013 (Burova), p. S9-10, Table 1.

E. Analytical Characterization of Viral Particles

91. Developing purification methods for rAAV requires efficient and accurate analytical methods to assess the quantity and purity of the rAAV particles at various stages of the purification. EX1013 (Burova), p. S13.

92. High performance liquid chromatography (HPLC) and mass spectrometry (MS) methods have proven to be powerful analytical techniques to assess purified viral particles such as AAV for process development and process control. EX1013 (Burova), p. S13; EX1006 (Shytuhina); EX1005 (Satkunanathan); EX1037 (Yuan).

93. In particular, reversed phase HPLC (RP-HPLC) and combined liquid chromatography/mass spectrometry (LC-MS) methods have proven extremely useful analytical methods for rAAV and other viruses. EX1013 (Burova), p. S13; EX1006 (Shytuhina); EX1005 (Satkunanathan); EX1037 (Yuan).

94. I discuss some of these techniques in more detail below.

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1. Liquid Chromatography (LC)

95. In RP-HPLC, the viral capsid is irreversibly denatured by an organic solvent such as acetonitrile. EX1013 (Burova), p. S13. Hydrophobic interactions of the exposed regions of the denatured viral proteins with the hydrophobic surface of the resin promote binding to the reversed phase column. EX1013 (Burova), p. S13. The chromatogram resulting from protein elution with increasing concentrations of acetonitrile allows evaluation of the viral capsid proteins. EX1013 (Burova), p. S13.

96. Ultra-high-pressure liquid chromatography (UPLC), also known as "ultra-performance liquid chromatography," is a chromatographic technique that, for example, can use sub-2 μm particles, mobile phases at high linear velocities, and instrumentation that operates at high pressure.[1] EX1020 (Cueto-Rojas), p. 1019.

97. A POSA would have been aware that UPLC could offer certain benefits as compared with HPLC, including increased resolution, sensitivity and speed of the chromatographic analysis, which are desirable for a chromatographic method to

[1] The '313 patent states, "In some embodiments, liquid chromatography (e.g., used in LC/MS as described herein) is ultra-performance liquid chromatography (UPLC; the term 'ultra high performance liquid chromatography' or UHPLC may be used interchangeably herein)." EX1001 ('313 patent), 20:65-21:2. Accordingly, I have used the terms UPLC and UHPLC interchangeably throughout my declaration.

analyze proteins for in-process control. EX1020 (Cueto-Rojas), p. 1019; EX1009 (Ansong), p. 10154.

2. Mass Spectrometry

98. Mass spectrometry determines the mass-to-charge ratio (m/z) of an ionized particle. EX1023 (Glish), p. 140. A mass spectrum is a plot of ion abundance versus m/z. EX1023 (Glish), p. 140.

99. Isotopes are important in mass spectrometry. EX1023 (Glish), p. 141. For example, chlorobenzene, which has a molecular weight of 112.56, does not form ions of m/z 112.56. EX1023 (Glish), p. 141. The 112.56 Da molar mass is actually a weighted average of all the stable isotopes, and their relative abundances, of the elements comprising the molecule. In the case of chlorobenzene, the two chlorine isotopes (^{35}Cl, 75.4% and ^{37}Cl, 24.6%) have the largest impact. EX1023 (Glish), p. 141. Individual chlorobenzene ions measured in the mass spectrometer will have either a ^{35}Cl or a ^{37}Cl, resulting in ions that are detected at m/z 112.01 and 114.01. EX1023 (Glish), p. 141. Also, on account of the isotopic distribution of Cl, approximately one ion at m/z 114 will be detected for every three ions detected at m/z 112 (24.6/75.4). EX1023 (Glish), p. 141.

100. There are three main components of a mass spectrometer: an ionization source, a mass analyzer and a detector (FIG. 1). EX1023 (Glish), p. 141. I discuss each of these components below.

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(a) Ionization Techniques

101. MS requires the conversion of condensed-phase atoms and molecules into gas phase ions. EX1023 (Glish), p. 141. Historical ionization techniques, involving volatizing and then ionizing samples, limited MS to relatively low-molecular weight, thermally stable compounds. EX1023 (Glish), p. 141.

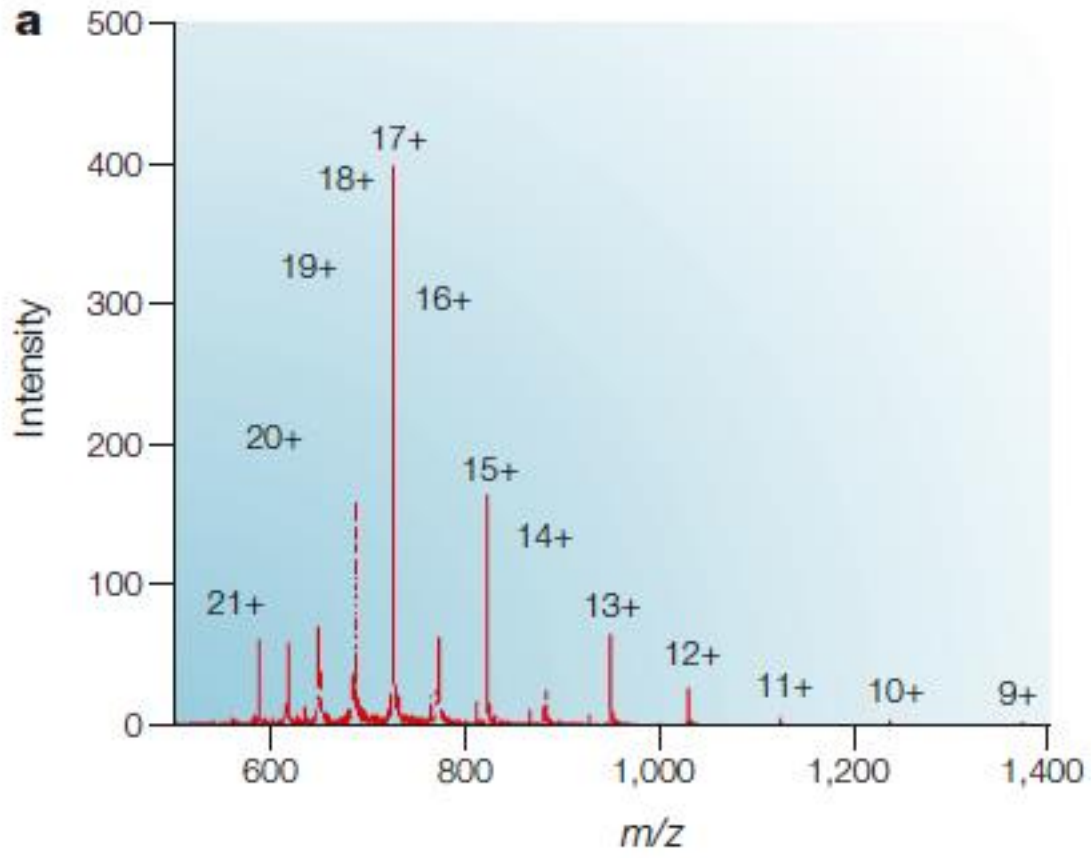
102. Electrospray ionization (ESI) is a technique used to convert condensed phase ions, such as those eluting from an HPLC column, to multiply protonated molecules in the gas phase. EX1024 (Good), p. 783.

103. Electrostatic spraying of a sample solution involves passing a sample through a capillary at a potential difference relative to a counter electrode. EX1023 (Glish), p. 141. This process initially generates an aerosol of charged droplets, consisting of both solvent and analyte molecules with a net positive or negative charge, depending on the polarity of the applied voltage. EX1023 (Glish), p. 141. Eventually, ions become free of the solvent that surrounds them and make their way into the mass analyzer of the spectrometer. EX1023 (Glish), p. 141.

104. The creation of biologically relevant ions in ESI generally occurs through protonation and deprotonation. EX1023 (Glish), p. 141. Proteins, peptides, oligonucleotides, and other molecules with acid/base functionality often have several sites of protonation or deprotonation, leading to multiply charged ions. EX1023 (Glish), p. 141.

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105. The figure below shows an example of the positive ESI mass spectrum of cytochrome c:



EX1023 (Glish), p. 143, Figure 2a.

106. Although multiple charging might be expected to make molecular weights more difficult to determine, it can actually increase the precision of the measurement. EX1023 (Glish), pp. 141, 143, Figure 2a. As can be seen in the figure above, each different charged species gives rise to a separate peak. EX1023 (Glish), pp. 141, 143, Figure 2a.

107. Multiple charging also enables mass spectrometers with limited m/z ranges to analyze higher-molecular weight molecules, because increasing the charge decreases the m/z ratio. EX1023 (Glish), pp. 141, 143, Figure 2a. For example, cytochrome c has a molecular weight of 12,360 Da, but the addition of between 10 and 20 protons brings the m/z ratio of the protein below 2,000, which is within the working range of most mass analysers. EX1023 (Glish), pp. 141, 143, Figure 2a.

108. ESI has been used successfully to ionize extremely large biomolecules such as Coliphage T4 DNA, which has a nominal molecular weight around 1.1×10^8 Da. EX1023 (Glish), p. 141. ESI has also been used to analyze intact, non-covalent biological macromolecular complexes, including tobacco mosaic virus. EX1023 (Glish), p. 142. In fact, after ionization, mass analysis and deposition, the tobacco mosaic virus retained both its rod-like structure and its viability. EX1023 (Glish), p. 142.

109. Importantly, ESI allows the coupling of MS and liquid chromatography techniques. EX1023 (Glish), p. 142; EX1024 (Good), p. 783.

110. ESI has two shortcomings that are relevant for biological samples. First, ESI is susceptible to ion suppression effects. EX1023 (Glish), p. 142. When solutions contain high salt concentrations (that is, > ~1 mM), analyte ion formation is usually hindered, so that most biological samples need to be desalted before analysis. EX1023 (Glish), p. 142. Second, when complex mixtures of compounds

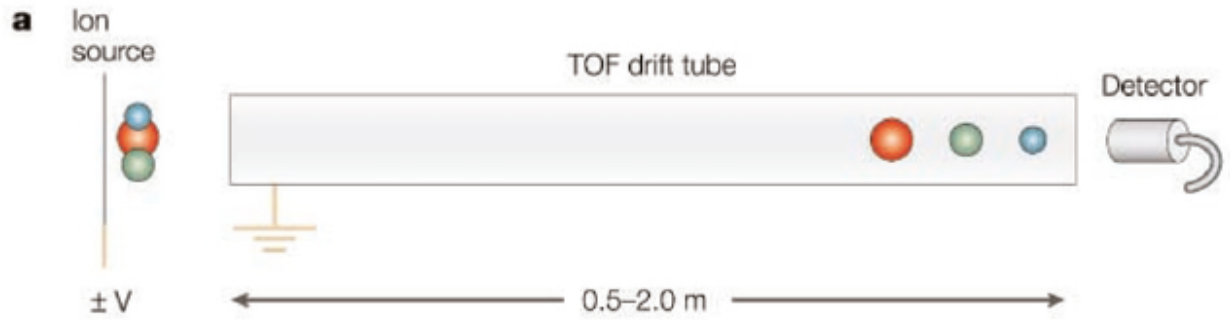
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are present, higher-concentration analytes can suppress ion formation by lower-concentration analytes. EX1023 (Glish), p. 142.

(b) Mass Analyzers

111. Ions generated by a method such as ESI are then measured in mass analyzers. EX1023 (Glish), p. 143. In MS, the precision of the measurement is related to the resolution, *i.e.*, the ability to resolve two adjacent peaks. EX1023 (Glish), p. 143. In general, resolution is defined as m/Δm, where m is the integer mass of the peaks being resolved and Δm is the mass difference between the two peaks. EX1023 (Glish), p. 143.

112. Analyzers can be divided into two groups: beam analyzers and trapping analyzers. EX1023 (Glish), p. 143. In beam analyzers, ions leave the ion source in a beam and pass through the analyzing field to the detector, as shown below:



EX1023 (Glish), p. 144, Figure 3(a).

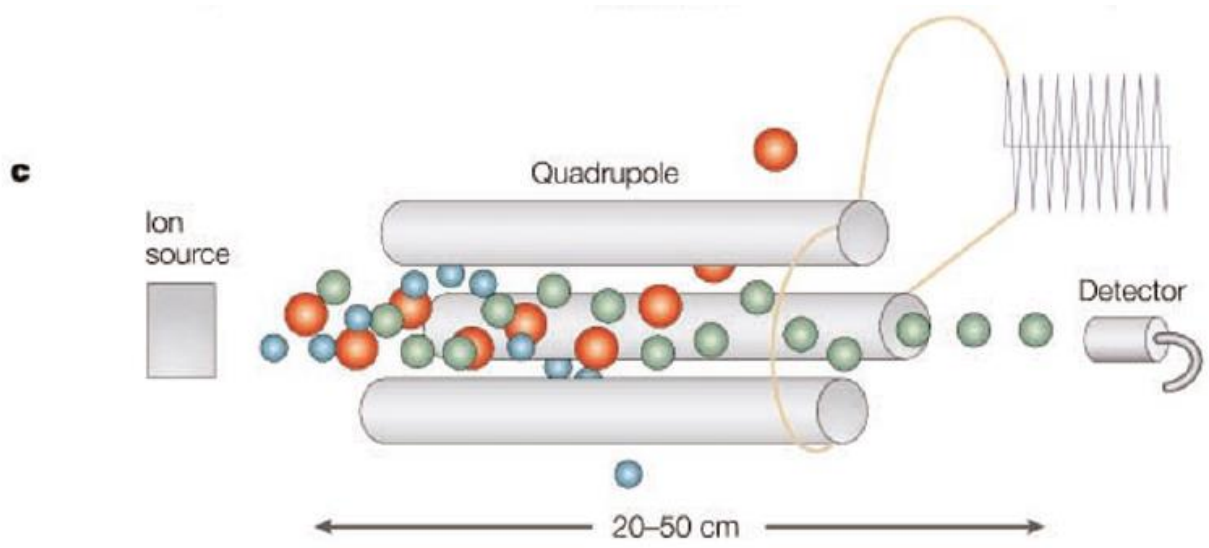
113. The time-of-flight (TOF) mass spectrometer is a relatively simple mass analyzer. EX1023 (Glish), p. 143. Ions are formed at the same time and place in the ion source and then accelerated through a fixed potential (for example, 1-20 kV)

into the TOF drift tube. EX1023 (Glish), p. 143. The TOF spectrometer separates ions based on their velocity and measures the time it takes each ion to reach the detector. EX1023 (Glish), p. 143.

114. Because ion velocities are inversely related to the square root of *m*/*z*, ions with same charge will have the same kinetic energy after acceleration, and ions with smaller *m*/*z* ratios will achieve higher velocities than ions with larger *m*/*z* ratios. EX1023 (Glish), pp. 143. After the ions are accelerated, they travel through a fixed distance, typically 0.5-2.0 meters, before striking the detector. EX1023 (Glish), pp. 143-44. Thus, the *m*/*z* of each ion can be determined by measuring the time it takes each ion to reach the detector. EX1023 (Glish), p. 144.

(c) Quadrupole Mass Analyzer

115. Over the years, the quadrupole has probably been the most widely used mass analyser, and was typically the choice for gas chromatography MS (GC/MS) and liquid chromatography MS (LC-MS) instruments in the 1970s, 1980s and 1990s. EX1023 (Glish), p. 145. A diagram of a quadrupole mass analyzer is shown below:



EX1023 (Glish), p. 144, Figure 3.

116. As the figure above shows, a quadrupole mass analyzer (top rod not shown), selects ions based on a particular *m*/*z* ratio. EX1023 (Glish), p. 144, Figure 3, Legend. The application of particular radio frequency (RF) and direct current (DC) voltages to the rods allows ions of a single *m*/*z* to (green ions in the figure above) maintain stable trajectories from the ion source to the detector, while ions with different *m*/*z* values are unable to maintain stable trajectories. EX1023 (Glish), p. 144, Figure 3, Legend.

(d) Orbitrap Mass Analyzer

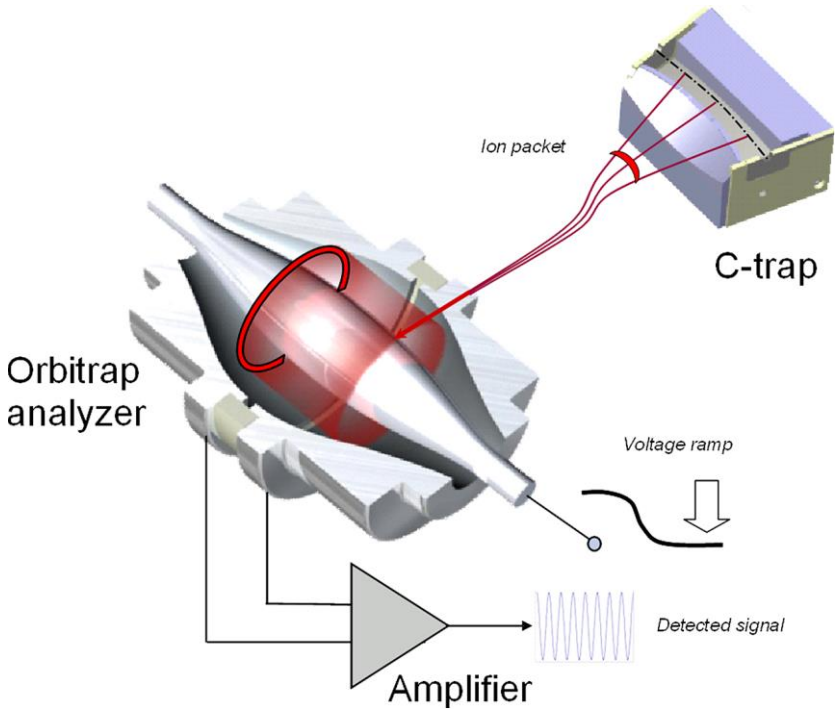
117. The Orbitrap mass analyzer consists essentially of three electrodes, a central electrode and two outer electrodes. EX1039 (Zubarev), p. 5289. Ions are injected into the volume between the central and outer electrodes essentially along a

tangent through a specially machined slot with a compensation electrode (a "deflector") in one of the outer electrodes. EX1039 (Zubarev), p. 5289.

118. When voltage is applied between the central and outer electrodes, a radial electric field bends the ions toward the central electrode while tangential velocity creates an opposing centrifugal force. EX1039 (Zubarev), p. 5289. If the parameters are chosen correctly, the ions remain on a nearly circular spiral inside the trap, much like a planet in the solar system. EX1039 (Zubarev), p. 5289.

119. At the same time, the axial electric field caused by the conical shape of electrodes pushes ions toward the widest part of the trap initiating harmonic axial oscillations. EX1039 (Zubarev), p. 5289. Outer electrodes are then used as receiver plates for image current detection of these axial oscillations. EX1039 (Zubarev), p. 5289.

120. As shown in the diagram below, the Orbitrap has an external ion storage device that effectively decouples the Orbitrap analyzer from any preceding ion source, ion transmission device, or analyzer. EX1039 (Zubarev), pp. 5289-90. Therefore, any device capable of selecting or transmitting precursor ions as well as any fragmentation technique could be interfaced to the Orbitrap:



EX1039 (Zubarev), p. 5290, Figure 2, legend.

(e) Calibration

121. A POSA at the time understood that mass spectrometers required calibration. EX1008 (Anacleto).

122. An ideal calibrant gives a mass spectrum in which the peaks are sufficiently closely spaced that interpolation errors are negligible, but not so closely spaced as to render the spectrum so confusing that it would be difficult to identify individual peaks. EX1008 (Anacleto), p. 660. It will also have *m*/*z* peaks across the entire mass range for which subsequent experimental masses will be determined. EX1008 (Anacleto), p. 660.

123. A POSA would further have understood that different calibrants were best suited to different applications. EX1008 (Anacleto), Abstract. For example,

metal salt calibrants such as sodium iodide (NaI) were known to be excellent for calibrating mass spectrometers. EX1008 (Anacleto), p. 665. In particular, NaI was known to provide excellent results up to m/z 2300, with inter-peak spacings of about 150 μ, in both positive- and negative-ion modes. EX1008 (Anacleto), p. 665.

124. As a result, metal salt calibrants such as NaI were known to be equally applicable to both small (relative molecular mass <1000) or large (*e.g.*, protein) analytes. EX1008 (Anacleto), p. 665.

125. As of the priority date, researchers were actively using Q-TOF MS to analyze post-translational modifications of proteins such as human erythropoietin. EX1014 (Byeon), p. 497. Notably, NaI was used to calibrate the Q-TOF mass spectrometer in this study. EX1014 (Byeon), p. 497.

126. A POSA would further have understood that software available at the time could carry out the calibration – a function referred to in the specification of the '313 patent as "assisted calibration": "In some embodiments, mass spectrometry (e.g., used in LC/MS as described herein) uses assisted calibration. Calibration when used in reference to mass spectrometry, may include the introduction of one or more compounds having a known mass (e.g., a standard) for the purpose of calibrating the instrument with respect to mass detection (e.g., m/z measurements). In some embodiments, assisted calibration may refer to using software to correlate a peak

and/or position of a known standard (e.g., a calibrant) to a specific mass-to-charge (m/z) ratio." EX1001 ('313 patent), 21:47-56.

127. A POSA would have understood that calibration could be carried out with the "assistance" of software. EX1014 (Byeon), p. 497 (disclosing external calibration with NaI carried out with the use of a Maximum Entropy program (MaxEnt)).

3. Liquid Chromatography/Mass Spectrometry

128. Over the past few decades many MS-based protein identification strategies have emerged. EX1018 (Coon 2005), p. 519. These strategies have been applied to complex mixtures of proteins, for example in proteomics studies.

(a) LC-MS Using Enzymatic Digestion

129. Peptide mass fingerprinting was among the first to gain widespread popularity. EX1018 (Coon 2005), p. 519. In this method, an isolated protein is digested enzymatically, and the resulting peptide molecular weights are measured. EX1018 (Coon 2005), p. 519. The measured peptide masses are then searched against a database of proteins that have been digested *in silico*. EX1018 (Coon 2005), p. 519.

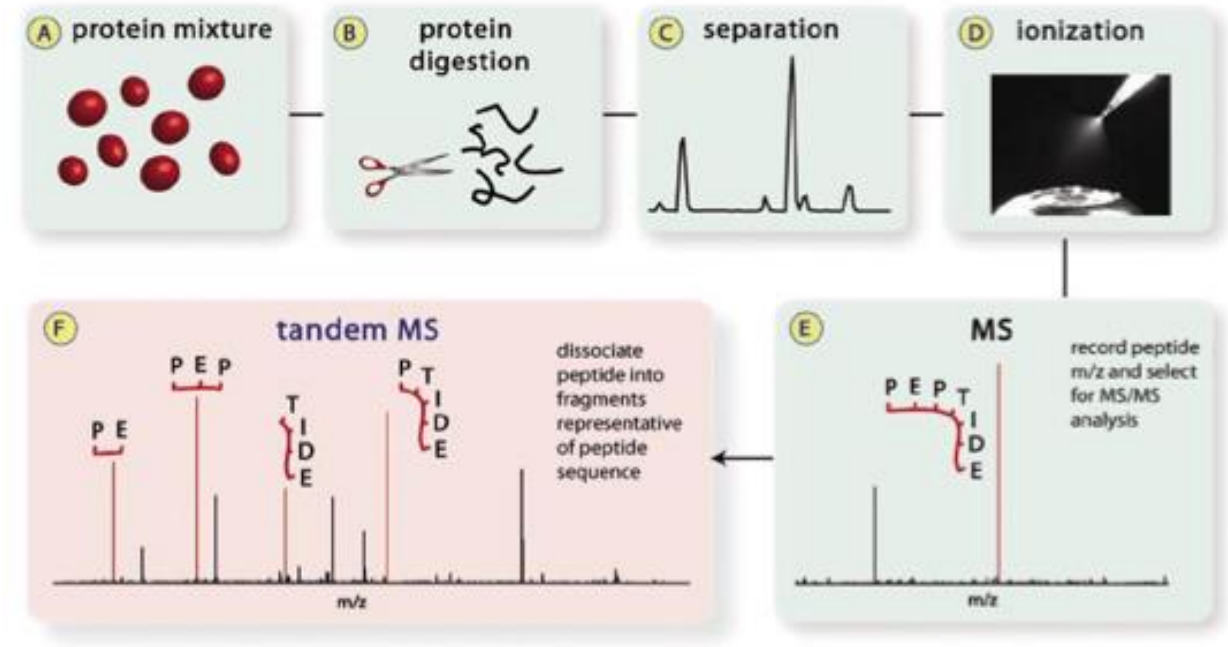
130. Technical developments in chromatography and MS instrumentation led to another strategy, known as "shotgun proteomics." EX1018 (Coon 2005), p. 519. Here an entire proteome is enzymatically digested, chromatographically

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separated, and interrogated with tandem mass spectrometry (MS/MS). EX1018 (Coon 2005), p. 519. This type of analysis was commonly known as "bottom-up" analysis. *See, e.g.*, EX1033 (Richards), p. 12, Figure 1; EX1024 (Good), p. 783.

131. Another MS strategy that seeks to analyze complicated mixtures of intact proteins directly is "top-down proteomics," where the proteins being analyzed have not been enymatically digested, a technique that I also discuss further below. EX1018 (Coon 2005), p. 519; EX1024 (Good), p. 784.

132. In a typical shotgun proteomics experiment, for example, a cell lysate, containing as many as several thousand proteins, is analyzed. The figure below shows the process of shotgun proteomics using the technique of liquid chromatography combined with tandem mass spectrometry (LC-MS/MS):



EX1018 (Coon 2005), p. 519, Figure 1.

133. As the diagram above shows, the sample is digested with a proteolytic enzyme resulting in a complex mixture of peptides (approximately 40 peptides/protein; Figure 1B).

134. Next, the digested sample is chromatographically separated (in one or multiple dimensions) and introduced to the mass spectrometer by means of a nanoflow high-performance liquid chromatography (nHPLC; approximately 50 nL/min) column integrated directly to an ESI source on the mass spectrometer (Figure 1, C and D, above). EX1018 (Coon 2005), p. 519. Figure 1.

135. The ESI source converts condensed phase ions, eluting from the HPLC column, to multiply protonated molecules (cations; the number of attached protons is proportional to the number of basic residues contained in the peptide) in the gas phase – a requirement for MS analysis. EX1018 (Coon 2005), p. 519.

136. The mass spectrometer first records the mass/charge (m/z) of each peptide ion (Figure 1E above) and then selects the peptide ions individually to obtain sequence information via MS/MS (Figure 1F). EX1018 (Coon 2005), p. 519, Figure 1.

137. Two methods for fragmenting peptide ions in MS/MS are collision-activated dissociation (CAD) and electron capture dissociation (ECD). EX1018 (Coon 2005), pp. 519-20.

138. CAD involves isolating the protonated peptide m/z of interest and subjecting it to collisions with rare gas atoms. EX1018 (Coon 2005), p. 519. CAD supplies sufficient internal energy to induce covalent bond breakage. EX1018 (Coon 2005), p. 519. In the gas phase, the amide bonds of the peptide backbone are typically the preferred sites of protonation. EX1018 (Coon 2005), p. 519. These protonated amide linkages are weakened and, upon collisional-activation, are favored for cleavage to create a series of homologous product ions. EX1018 (Coon 2005), p. 519. For example, the EX1018 (Coon 2005), p. 519.

139. MS/MS spectrum in Figure 1F above contains the consecutive peptide fragment ions PE, PEP, etc. EX1018 (Coon 2005), p. 519, Figure 1. Thus, the process aims to produce a collection of peptide fragment ions that differ in mass by a single amino acid, allowing one to read the amino acid sequence of the precursor peptide. EX1018 (Coon 2005), p. 519; EX1019 (Coon 2009), p. 3208.

140. I note, however, that production of this series relies on a random distribution of protonated amide bonds within the starting precursor peptide ion population (e.g., in an ion trap mass spectrometer the MS/MS experiment starts with approximately 10,000 peptide precursor ions). EX1018 (Coon 2005), p. 520.

141. During the collisional-activation of a selected peptide precursor ion, the energy is deposited on a relatively slow time scale (picoseconds). EX1018 (Coon 2005), p. 520. This imparted energy is redistributed throughout the peptide

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precursor ion and ultimately induces cleavage of the weakest bond(s). EX1018 (Coon 2005), p. 520.

142. When peptides contain important posttranslational modifications (PTMs) (e.g., phosphorylation, glycosylation, sulfonation, etc.), the preferred dissociation pathways described above can change. EX1018 (Coon 2005), p. 520.

143. In phosphorylated peptides, for example, the phosphate group competes with the amide bonds of the backbone as the preferred site of protonation (in the gas phase). EX1018 (Coon 2005), p. 520. Consequently, collisional-activation of these peptides readily displaces phosphoric acid from the peptide following nucleophilic attack of the protonated sidechain. EX1018 (Coon 2005), p. 520.

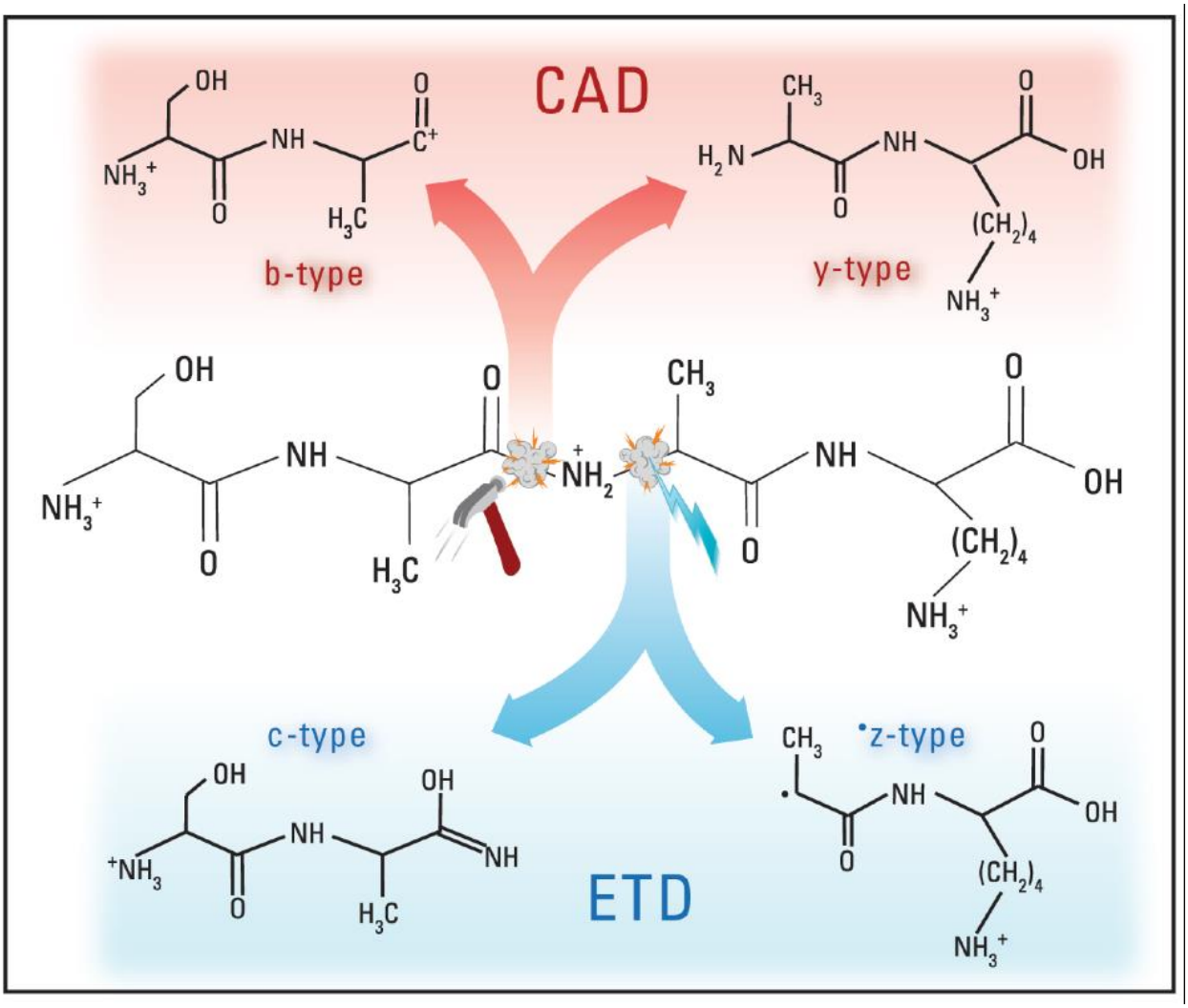
144. Regardless of the mechanisms defining these preferential cleavages, the net result is often the same: CAD MS/MS spectra of PTM-containing peptides are frequently devoid of the peptide ion fragment m/z (those corresponding to the consecutive backbone cleavages), which are necessary for sequence identification. EX1018 (Coon 2005), p. 520.

145. In ECD, low energy electrons are reacted with peptide cations in the magnetic field of a Fourier transform ion cyclotron resonance MS (FT-ICR-MS). EX1018 (Coon 2005), p. 520. The reaction results in the attachment of electrons to the protonated peptides producing peptide cations containing an additional electron. EX1018 (Coon 2005), p. 520. The odd electron peptide then undergoes very rapid

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(femtoseconds) rearrangement with subsequent dissociation. EX1018 (Coon 2005), p. 520.

146. Unlike the collision-activated process, ECD does not cleave chemical modifications from the peptide, but rather induces random breakage of the peptide backbone – cleavage that is indifferent to either peptide sequence or length. EX1018 (Coon 2005), p. 520; EX1019 (Coon 2009), p. 3209, Figure 1. The figure below shows the difference between CAD and ECD:



EX1019 (Coon 2009), p. 3209, Figure 1.

147. The more widely accessible instruments, those that use radio frequency (RF) fields to confine peptide ions (e.g., ion trap or quadrupole TOF), however, are not capable of simultaneously storing peptide cations and electrons.

148. Our laboratory developed a new ion dissociation method to translate the unique features of ECD to the common benchtop ion trap mass spectrometers routinely used in proteomics research. EX1018 (Coon 2005), p. 520.

149. Our plan, however, did not involve reacting electrons with peptide cations as in ECD. EX1018 (Coon 2005), p. 520. Instead, we chose to use negatively charged ions (anions) as vehicles for electron delivery, as shown in the equation below:

$$(\text{Peptide} + 3\text{H})^{3+} + \text{Anion}^{-\bullet} \rightarrow (\text{Peptide} + 3\text{H})^{2+\bullet} + \text{Anion}$$

EX1018 (Coon 2005), p. 520.

150. Though the RF fields used by the ion trap cannot confine both cations and electrons, the device can simultaneously contain cations and anions. EX1018 (Coon 2005), p. 520. Given the appropriate anion, the reaction should proceed as described in the equation above to donate an electron to the peptide. EX1018 (Coon 2005), p. 520. Subsequently, the peptide would contain an extra electron, and that should induce peptide backbone fragmentation, just as in ECD. EX1018 (Coon 2005), p. 520.

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151. Further, gas phase peptide cations and small organic anions react rapidly (ion-ion reactions, milliseconds time scale) – reactions whose duration and timing are easily controlled. EX1018 (Coon 2005), p. 520.

152. Ion-ion reactions had been studied for nearly a decade in 3-D ion traps, but the electron transfer reaction described in the equation above remained elusive. EX1018 (Coon 2005), p. 520. Due to a number of limitations with conventional 3-D ion traps, our efforts centered around construction of a novel ion-ion device: the quadrupole linear ion trap (QLT). EX1018 (Coon 2005), p. 520.

153. This mass spectrometer consists of a set of four hyperbolic rods (electrodes) in which RF fields are used to confine peptide cations along the central axis. EX1018 (Coon 2005), p. 520. The commercial version of this instrument (Finnigan LTQ™; Thermo Electron, San Jose, CA, USA) places an ESI ion source at one end of the device (for peptide ion generation) and leaves the other end unoccupied.

154. For our experiments we placed an additional ion source on the vacant end of the QLT. EX1018 (Coon 2005), p. 520. This way peptide cations were generated from the front, as normal, and anions from various compounds could be created and injected into the QLT from the back. EX1018 (Coon 2005), p. 520.

155. LC-MS/MS had been used for about a decade before the earliest possible priority date for the ’313 patent to study deamidation (conversion of

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asparagine and glutamine to, for example, aspartic acid and glutamic acid) of proteins. EX1027 (Huang), Abstract.

156. For example, LC-MS/MS had been used to identify deamidation of monoclonal antibodies. EX1027 (Huang), Abstract. Huang studied a humanized IgG1 antibody that was known to have certain "hotspots" for spontaneous deamidation. EX1027 (Huang), Abstract.

157. Huang digested the monoclonal antibody with trypsin, separated the fragments on RP-HPLC, and then analyzed them by MS/MS. Using this method, they determined the deamidation half-life of amino acid residue Asn55 *in vivo*, and the ratio of the deamidated derivatives, *i.e.*, isoAsp55 and Asp55. EX1027 (Huang), Abstract.

158. It was known in the art, however, that enzymatic digestion is a laborious, time-consuming process, which can introduce artificial modifications, such as cyclization of N-terminal glutamine, and deamidation. EX1012 (Bondarenko), p. 1415.

159. In addition, a POSA at the time would have understood that another limitation of MS analysis that involves enzymatic digestion of proteins into peptides is the ability to identify the origin of certain protein fragments.

160. For example, in an analysis of AAV that I discuss in more detail below, the researchers identified capsid proteins from three different AAV serotypes, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table 1.

161. A POSA would have understood that, given the structure of the AAV capsid proteins, vp1, vp2, and vp3, it would have been difficult, perhaps impossible, to determine whether a fragment towards the C terminus of a given capsid protein originated from vp1, vp2, or vp3.

162. Specifically, as I discussed above, vp1, vp2, and vp3 are alternate transcripts from a single gene that differ at their N termini but share a common C terminus. EX1015 (Cecchini), p. 824, Figure 1.

163. As a result, as a POSA at the time would have understood, LC-MS/MS using proteolysis to generate peptide fragments of the AAV capsid proteins before MS analysis could not have allowed characterization of vp3, for example, separate from vp1 or vp2.

164. A POSA would similarly have understood that LC-MS/MS involving enzymatic digestion of AAV capsid proteins (vp1, vp2, vp3) could not have allowed characterization of vp2 separate from vp1.

165. As I discuss below, a POSA would have understood that a different technique, LC-MS of intact proteins, would allow identification of each of the AAV capsid proteins vp1, vp2, and vp3 individually. Moreover, a POSA would have

understood that LC-MS of intact proteins, unlike LC-MS of enzymatically digested proteins, would have further enabled analysis of PTMs of each of the AAV capsid proteins individually.

(b) Intact LC-MS Analysis

166. Mass spectrometry of intact molecules had been known since at least 1991. *See, e.g.*, EX1028 (Loo), p. 2488. By at least as early as 2004, a different LC-MS technique had been developed involving LC-MS analysis of intact proteins that have not been enzymatically digested before MS analysis. EX1018 (Coon 2005), p. 519; EX1035 (Rouse), p. 436; EX1012 (Bondarenko), p. 1415.

167. The term "top-down" proteomics or protein analysis is sometimes applied to this technique, which involves accurate mass determination of intact (undigested) proteins, protein subunits, or large proteolytic fragments, with the option of gas-phase ion fragmentation of the intact species. EX1018 (Coon 2005), p. 519; EX1035 (Rouse), p. 436; EX1012 (Bondarenko), p. 1415.

168. The intact protein or top-down strategy also has the potential to reduce the number of upstream sample handling and separation steps. EX1035 (Rouse), p. 436.

169. Intact protein or top-down MS offers many advantages, including the ability to characterize the entire primary sequence of a given protein and identify

combinatorial patterns of PTMs. EX1032 (Rhoads), p. 2314; EX1012 (Bondarenko), p. 1415; EX1019 (Coon 2009), p. 3213.

170. Using ETD, high-resolution mass analysis is not required to identify intact proteins. EX1019 (Coon 2009), p. 3213. The high sensitivity of the ion trap mass analyzer can allow for the detection of proteins as large as 70 kDa in just a few seconds. EX1019 (Coon 2009), p. 3213. The lower resolving power of the ion trap, however, can make spectral identification challenging. EX1019 (Coon 2009), p. 3213. To settle this, the highly charged c- and z•-type fragments generated from whole-protein dissociation via ETD can be deprotonated by a secondary reaction with a proton transfer anion. EX1019 (Coon 2009), p. 3213.

171. These sequential ion-ion reactions can be accomplished in ~100 ms and produce a very simple and straightforward ladder of fragment ions to interpret. EX1019 (Coon 2009), p. 3213. Some very nice reports on using low-resolution mass spectrometers with ETD for whole-protein analysis have been published as early as 2007 and 2008. EX1019 (Coon 2009), p. 3213.

172. The combination of intact protein mass measurement and top-down fragmentation analysis also provided useful information about sites of posttranslational modifications (PTMs) in studies including those of the oxidation of viral prolyl-4-hydroxylase and deamidation of ribonuclease A. EX1012 (Bondarenko), p. 1415; EX1038 (Zabrouskov), Abstract.

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173. To avoid time-consuming and costly fractionation and fraction collection and subsequent peptide mapping of the separated isoforms of a protein, researchers understood that it was very attractive to separate the intact protein isoforms by liquid chromatography and then perform on-line (automated) mass and top-down analyses to determine the sites of modifications and their abundances in one short assay. EX1012 (Bondarenko), p. 1416.

174. Moreover, because intact proteins are more resistant than peptides to the collisional activation processes that occur during MS analysis, labile PTMs (*e.g.*, sulfate and carboxylate on tyrosine-O-sulfate and γ-carboxyglutamate, respectively) are largely retained during intact LC-MS. EX1035 (Rouse), p. 436. This phenomenon results in a representative distribution of the protein isoform heterogeneity in the mass spectrum. EX1035 (Rouse), p. 436.

175. In addition, other methods of dissociation such as ETD/ECD can be used with intact protein analysis that preserve labile post-translational modifications. EX1034 (Riley), p. 7109 ("ECD and ETD facilitate fragmentation of peptide and protein backbone bonds while retaining PTMs, providing more extensive and informative cleavage of proteins than CAD and IRMPD").

176. Bondarenko used top-down, intact LC-MS to analyze monoclonal antibodies after reduction and separation using RP-HPLC. EX1012 (Bondarenko), p. 1416. Bondarenko described analysis of human monoclonal antibodies and their

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large subunits (light and heavy chains after reduction of disulfide bonds) using the LTQ Orbitrap. EX1012 (Bondarenko), p. 1416.

177. Bondarenko reported mass measurements and top-down fragmentation analyses of intact and reduced IgG antibodies on the LTQ Orbitrap connected to reversed-phase HPLC.

178. Bondarenko stated that "it is very attractive to separate the intact protein isoforms by liquid chromatography and then perform on-line mass and top-down analyses to determine the sites of modifications and their abundances in one short assay." EX1012 (Bondarenko), p. 1416.

179. Zabrouskov studied deamidation of ribonuclease A using top-down (intact) MS/MS analysis. EX1038 (Zabrouskov), pp. 987-88. Zabrouskov found five stepwise deamidation sites on ribonuclease A, only one of which had been previously identified. EX1038 (Zabrouskov), pp. 987-88.

180. Zabrouskov noted that top-down tandem mass spectrometry (MS/MS) was known to be uniquely useful for kinetic studies of multicomponent protein systems. EX1038 (Zabrouskov), pp. 987-88. A POSA would have understood that a viral particle is a multi-component protein system, comprised of several different structural proteins and/or several different copies of a single structural protein.

181. Zabrouskov further noted that after MS separation of the target protein's molecular ions, further MS dissociation yields fragment ions whose mass

shifts show which amino acids have been modified. EX1038 (Zabrouskov), pp. 987-88.

182. However, Zabrouskov stated, deamidation was known to pose the special challenge that its covalent modification $–NH_2 \rightarrow {}^{-}OH$ causes only a 0.984 Da mass increase, closely matching the 1.002 Da spacing of the molecular ion isotope peaks. EX1038 (Zabrouskov), pp. 987-88.

183. This small mass difference renders the usual MS/MS isolation of product ions for their separate characterization difficult. EX1038 (Zabrouskov). But using top-down MS/MS, Zabrouskov was able to identify multiple deamidated forms of Ribonuclease A:

> In "bottom up" MS proteomics, initial digestion of the protein gives peptides whose mass spectra often provide a fast, reliable identification of the protein, but are of far less value for characterizing posttranslational modifications. In top down MS/MS, electrospray ionization (ESI) of a protein mixture introduces their gaseous molecular ions into a Fourier-transform MS. For a specific protein, an accurate molecular weight differing from that of the gene-model predicted value indicates sequence errors, alternative splicing, protein or RNA editing, and/or posttranslational modifications. These discrepancies can be identified and located by MS/MS separation and dissociation of the protein molecular ions, using methods such as collisionally activated dissociation (CAD), infrared multiphoton dissociation (IRMPD), or electron capture dissociation (ECD). CAD and IRMPD cleave –CO—NH– bonds to produce *b*, *y* fragment ions, and ECD cleaves –NH—CHR– bonds to produce mainly *c*, *z*• ions. Here for reduced RNase A, these techniques establish five deamidation sites, as well their kinetic order of deamidation, that indicate extensive conformational selectivity despite the strong denaturing conditions employed.

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EX1038 (Zabrouskov), p. 988.

184. A POSA would have understood that analysis of larger proteins (*e.g.*, 100 to 200 kDa and larger) using top-down LC-MS, including LC-MS/MS, could require adjusting certain conditions of the MS analysis to ensure sufficient cleavage of the protein. EX1026 (Han), Abstract.

185. To improve dissocation of proteins larger than about 500 residues and 50 kDa, Han used electrospray additives, heated vaporization, and separate noncovalent and covalent bond dissociation. EX1026 (Han), Abstract.

186. They refer to their technique as "prefolding dissociation" or "PFD," explaining that it prevents stabilization of ions formed in the mass spectrometer when solvent is removed from a denatured protein during ESI. EX1026 (Han), p. 110.

187. Han concludes that their study "indicates the further applicability of PFD to characterize stable posttranslational modifications such as methylation, acetylation, oxidation, and deamidation in large proteins." EX1026 (Han), p. 112.

4. Analysis of Viral Particle Composition Using LC-MS Techniques

188. Well over a decade before the earliest possible priority date for the '313 patent, researchers were using liquid chromatography, RP-HPLC in particular, to detect and quantify viral capsid proteins for purification process development. EX1037 (Yuan), Abstract.

189. MS had also been used to explore the properties of viruses for more than a decade before the earliest priority date for the '313 patent. EX1036 (Siuzdak), Abstract. A 1998 review, "Probing Viruses with Mass Spectrometry," stated, "[m]ass measuring viral proteins is now routine and since viruses are typically well characterized, in that the capsid proteins and DNA (or RNA) sequences are known, identifying a virus based on the mass of the protein and enzymatic digestion fragments is relatively straightforward." EX1036 (Siuzdak), Abstract, p. 205; *see also* EX1033 (Richards), p. 11.

190. This review (Siuzdak), discusses the use of MS to identify capsid proteins, including in cases in which "more than one type of protein inhabits the capsid." EX1036 (Siuzdak), p. 205.

191. Siuzdak also describes the use of MS to identify viral protein PTMs such as myristoylation, phosphorylation, and disulfide bridging, including the use of MS to identify a previously uncharacterized site of a particular PTM in a viral capsid protein. EX1036 (Siuzdak), Abstract, pp. 205-206.

192. I note that it also had been known since at least 1985 that AAV vp3 is acetylated *in vivo*. EX1011 (Becerra), p. 7920.

193. At least as early as 2002, LC-MS had been used to analyze viral composition. *See, e.g.*, EX1016 (Chelius). Chelius disclosed the use of LC-MS/MS to analyze the composition of adenovirus particles. EX1016 (Chelius), Abstract.

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Chelius separated adenoviral proteins by RP-HPLC, and then carried out tryptic digestion and analysis by LC-MS/MS. EX1016 (Chelius), Abstract. All of the major adenoviral proteins were found through this method. EX1016 (Chelius), Abstract.

194. LC-MS/MS was also used before the priority date for process development for rAAV. EX1022 (Dong), Abstract; EX1005 (Satkunanathan), Abstract.

195. As I discuss in more detail below, Satkunanathan used LC-MS/MS to explore cellular components associated with three different serotypes of rAAV, specifically, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Abstract.

196. Satkunanathan identified capsid proteins unique to each serotype, and cellular components associated with one or more of the serotypes tested. EX1005 (Satkunanathan), Supplementary Table S1.

197. Researchers at the time understood that co-purifying cellular proteins could raise concerns about the safety of gene therapy, and therefore that the identification and characterization of such co-purifying proteins, including their PTMs, was important for process development. *See, e.g.*, EX1022 (Dong), Abstract. Satkunanathan cites Dong, an earlier proteomics analysis of co-purifying cellular proteins associated with rAAV2 vectors. EX1022 (Dong).

198. Using LC-MS, Dong identified two PTMs, carbamidomethylation of cysteine and oxidation of methionine, in AAV capsid proteins. EX1022 (Dong),

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Abstract, p. 2, Figure S1. Also using LC-MS, Dong identified AAV capsid protein fragments that had a high affinity for capsid proteins. EX1022 (Dong), p. 2.

199. Dong stated that "[c]ontaminants in the vector preparations, such as plasmid DNA, replication-competent AAV, and co-purifying cellular proteins, could decrease the efficiency of gene therapy and raise concerns about its safety." EX1022 (Dong), p. 1. Dong further noted that "knowledge of the co-purifying proteins can also improve our knowledge of cellular factors involved in viral biology and potentially lead to better vectors and production systems." EX1022 (Dong), p. 1.

200. Dong further commented that "[p]rotein identification has benefited from advancements in sensitivity and accuracy of mass spectrometry (MS) that led to its broad use in proteomics." EX1022 (Dong), p. 1.

201. Unlike Satkunanathan, Dong studied only AAV2-based vectors and did not explore differences in co-purifying proteins among different serotypes. EX1022 (Dong); EX1005 (Satkunathan). Also unlike Satkunanathan, Dong used gel electrophoresis as part of its LC-MS method. EX1022 (Dong); EX1005 (Satkunathan).

202. Before the priority date, researchers had also used MS techniques to study mutant viral proteins in proteomics studies. EX1007 (Alqahtani), Abstract, p. 2509. Alqahtani used SILAC (stable isotope labeling of amino acids in cell culture) and high-throughput quantitative MS mass spectrometry to analyze the protein

composition of highly purified wild-type (WT) adenoviruses, mutant adenoviruses lacking an internal protein component (protein V), and recombinant adenoviruses of the type commonly used in gene therapy, including one virus that had been used in a clinical trial. EX1007 (Alqahtani), Abstract.

203. Alqahtani concluded that their approach would be a valuable way to analyze the composition and batch-to-batch variation of WT, mutant, and genetically engineered viruses, VLPs, or nanoparticles. EX1007 (Alqahtani), p. 2510.

204. Also before the priority date, researchers were using intact LC-MS to analyze viral structural proteins for process development. *See, e.g.*, EX1006 (Shytuhina), Abstract. Shytuhina applied intact LC-MS to analyze undigested viral structural proteins, including to evaluate post-translational modifications. EX1006 (Shytuhina), Abstract, pp. 193-94.

VI. THE '313 PATENT

205. The '313 patent is titled "Methods for Detecting AAV." EX1001 ('313 patent). The patent names Xiaoying Jin, Catherine O'Riordan, Lin Liu, and Kate Zhang as inventors. EX1001 ('313 patent). The '313 patent issued on May 13, 2025. EX1001 ('313 patent).

206. The '313 patent is assigned to Genzyme Corporation. EX1001 ('313 patent).

A. The Claims

207. The challenged claims of the ’313 patent are directed to methods of using intact LC-MS to analyze preparations of AAV particles. EX1001 (’313 patent), 83:1-85:7. Each claim requires “denaturing the AAV particles” and then “subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis.”

208. I have reproduced the challenged claims in the table below.

Claim	Element
1 [pre]	A method of detecting post-translational modifications of one or more viral proteins (VPs) in a preparation of adeno-associated virus (AAV) particles, the method comprising
1[a]	a) denaturing the AAV particles;
1[b]	b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;
1[c]	c) determining the masses of the one or more VPs; and
1[d]	d) determining any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs that have not undergone post-translational modifications to detect a deviation in the compared masses,
1[e]	Wherein the VPs comprise VP1, VP2 and VP3 capsid proteins, and wherein the method is performed in the absence of a gel separation step.
2	The method of claim 1, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.

Claim	Element
3	The method of claim 2, wherein the post-translational modification is N-terminal acetylation.
4	The method of claim 1, wherein the AAV particles are denatured using detergent, heat, high salt or buffer with low or high pHs.
5	The method of claim 1, wherein the liquid chromatography is reverse phase chromatography.
6	The method of claim 5, wherein the reverse phase chromatography is C8 reverse phase chromatography.
7	The method of claim 1, further comprising determining the sequence of one or more VPs that has undergone post-translational modifications.
8	The method of claim 7, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.
9	The method of claim 8, wherein the post-translational modification is N-terminal acetylation.
10	The method of claim 7, wherein the sequences of VP1, VP2 and VP3 are determined.
11 [pre]	A method of determining the heterogeneity of viral particles in a preparation of adeno-associated virus (AAV) particles comprising VP1, VP2 and VP3 capsid proteins, the method comprising
11[a]	a) denaturing the AAV particles;
11[b]	b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein

Claim	Element
	analysis, thereby separating the peaks of the VP1, VP2 and VP3 capsid proteins;
11[c]	c) deconvoluting the peaks of the VP1, VP2 and VP3 capsid proteins; and
11[d]	d) determining the masses of one or more of the VP1, VP2 and VP3 capsid proteins and additional capsid proteins within one or more of the deconvoluted peaks,
11[e]	wherein the method is performed in the absence of a gel separation step.
12	The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are variant capsids.
13	The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are capsid amino acid substitutions.
14	The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are truncated capsids.
15	The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are modified capsids.
16	The method of claim 15, wherein the modifications of the modified capsids are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.
17	The method of claim 16, wherein the modification is N-terminal acetylation.

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Claim	Element
18	The method of claim 11, wherein the liquid chromatography is reverse phase chromatography.
19	The method of claim 18, wherein the reverse phase chromatography is C8 reverse phase chromatography.
20 [pre]	A method of preparing a pharmaceutical composition of adeno-associated virus (AAV) particles, the method comprising:
20[a]	monitoring AAV particles for consistency and/or identity;
20[b]	wherein the AAV particles comprise viral proteins (VPs) comprising VP1, VP2 and VP3 capsid proteins of an AAV particle capsid,
20[c]	wherein the AAV particle is monitored for consistency and/or identity by:
20[d]	a) extracting an aliquot of an AAV particle preparation;
20[e]	b) denaturing the AAV particles;
20[f]	c) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;
20[g]	d) determining the masses of one or more VPs of the AAV particles; and
20[h]	e) comparing the determined masses of the one or more VPs to theoretical masses of corresponding VPs, wherein the theoretical masses of corresponding VPs are those VPs of known AAV serotypes and/or those that have not undergone undesired post-translational modifications; and
20[i]	f) determining if there is any deviation of the determined masses of the one or more VPs from the theoretical masses of the corresponding VPs;

Claim	Element
20[j]	wherein the determination of any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs thereby monitors the AAV particles for consistency and/or identity;
20[h]	wherein the monitoring for consistency and/or identity is performed in the absence of a gel separation step; and
20[i]	wherein if less than an undesirable amount of deviation is determined during the monitoring for consistency and/or identity, the AAV particles are combined with one or more pharmaceutically acceptable excipients to form the pharmaceutical composition.
21	The method of claim 20, wherein the monitoring of the AAV particles for consistency and/or identity includes determining the serotype of the AAV particles based on the comparison of the determined masses of the VPs to the theoretical masses of the corresponding VPs.
22	The method of claim 20, wherein a determination of any actual deviation in masses reflects heterogeneity in the AAV particle preparation.
23	The method of claim 22, wherein the heterogeneity in the AAV particle preparation is due to mixed AAV capsid serotypes, variant AAV capsid proteins, AAV capsid protein amino acid substitutions, truncated AAV capsid proteins or modified AAV capsid proteins.
24	The method of claim 21, wherein the undesired post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination.

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Claim	Element
25	The method of claim 21, wherein the AAV particles are denatured using detergent, heat, high salt or buffer with low or high pHs.
26	The method of claim 21, wherein the liquid chromatography is reverse phase chromatography.
27	The method of claim 26, wherein the reverse phase chromatography is C8 reverse phase chromatography.

209. As the table shows, claims 1, 11, and 20 are independent claims. EX1001 (’313 patent), 83:1-18, 43-58, 84:15-50. The remaining claims are dependent claims, which recite additional elements. EX1001 (’313 patent), 83:19-42, 83:59-84:14, 84:51-85:7.

B. The Specification

210. The specification of the ’313 patent discusses using LC-MS[2] as an analytical tool to evaluate viral preparations. EX1001 (’313 patent), 2:10-24. The specification discusses using LC-MS to discern characteristics of the viral

[2] In my experience, it is customary to abbreviate “liquid chromatography mass spectrometry” as “LC-MS.” I note that the patent claims and the specification use the abbreviation “LC/MS.” A POSA would understand these two abbreviations to refer to the same technology. I have therefore used “LC-MS” in my declaration, other than when referring to the specific language of the challenged claims.

preparation including the identity of the capsid serotype. EX1001 ('313 patent), 2:14-17. The specification states that the method can be used as an AAV serotype identity test or to monitor viral capsid protein heterogeneity in rAAV gene therapy development. EX1001 ('313 patent), 2:17-20.

211. The specification describes "Examples" of LC-MS and LC-MS/MS for characterization of rAAV viral capsid protein. EX1001 ('313 patent), 51:24-56:45.

212. The specification discusses methods of denaturing rAAV samples before LC-MS analysis. EX1001 ('313 patent), 54:24-34.

213. The specification discusses LC-MS intact protein analysis. EX1001 ('313 patent), 53:34-51; 54:35-55:7.

214. The specification also discusses LC-MS/MS peptide mapping. EX1001 ('313 patent), 53:63-54:20; 55:8-50; 62:57-63:9. The specification does not disclose LC-MS/MS analysis of intact proteins.

C. The Prosecution History

215. The applicants filed a preliminary amendment, and a terminal disclaimer. EX1002 ('313 file history), pp. 147-54, 229-31. The applicants also filed an Amendment after Allowance, which was entered by the Examiner. EX1002 ('313 file history), pp. 272-79, 283-85.

D. The Priority Date

216. The ’313 patent claims priority to U.S. Provisional Application No. 62/375,314, filed August 15, 2016 (“the ’314 provisional”).

217. The ’313 patent issued from U.S. Patent Application 19/013,863, filed January 8, 2025, and is a continuation of U.S. Patent Application No. 18/801,293, filed August 12, 2024, which is a divisional of U.S. Patent Application No. 18/321,542 (U.S. Pat. No. 12,123,880), filed May 22, 2023, which is a divisional of U.S. Patent Application No. 16/325,653 (U.S. Pat. No. 11,698,377), which adopts the international filing date of August 14, 2017, which is a National Phase application under 35 U.S.C. § 371 of International Application No. PCT/US2017/046814, filed August 14, 2017, which claims the priority benefit of the ’314 provisional.

218. The earliest possible priority date for the ’313 patent claims is therefore the filing date of the ’314 provisional, August 15, 2016.

VII. THE PERSON OF ORDINARY SKILL IN THE ART (“POSA”)

219. I understand that a POSA is a hypothetical person who is presumed to be aware of all pertinent art, understands conventional wisdom in the art, and is a person of ordinary creativity.

220. I have carried out my analysis of obviousness from the perspective of a POSA as of August 2016, given my understanding that the earliest possible priority date for the ’313 patent is August 15, 2016.

221. I have been asked to consider the level of education and experience of a POSA for the ’313 patent. In evaluating the qualifications of a POSA, I have considered the following factors: (i) the types of problems encountered in the art, (ii) prior art solutions to those problems, (iii) the rapidity with which innovations are made, (iv) the sophistication of the technology, and (v) the educational level of active workers in the field. I have also relied on my experience working with and supervising others in the fields of chemistry and biochemistry and the area of biological mass spectrometry.

222. In my opinion, a POSA in the technical field of the ’313 patent would have had at least a Ph.D. in biochemistry, chemistry, pharmaceutical sciences, or a related field, and between one and four years of post-doctoral experience in the field of chemistry or pharmaceutical sciences, including analytical techniques such as chromatography and mass spectrometry. Alternatively, a POSA would have had at least a Master’s or Bachelor’s Degree in biochemistry, chemistry, pharmaceutical sciences, or a related field, with a corresponding number of additional years of experience in the field of chemistry or pharmaceutical sciences.

VIII. THE PRIOR ART

223. As I discuss in detail below, before the earliest possible priority date for the ’313 patent, researchers had been using LC-MS, including intact LC-MS, to

analyze viral capsid proteins and other associated viral proteins, for process development. *See, e.g.*, EX1006 (Shytuhina); EX1005 (Satkunanathan).

224. Researchers were using intact LC-MS to study post-translational modifications of viral proteins for process development. EX1006 (Shytuhina).

225. Researchers in the AAV field specifically were also using LC-MS techniques to identify and analyze AAV capsid proteins and their associated cellular co-purifying proteins, for research and for process development. EX1066 (Satkunanathan); EX1022 (Dong).

226. AAV researchers were well aware of the importance of studying and comparing different AAV serotypes for process development and for understanding the different functions and capabilities of the different serotypes, which influence their suitability for use as therapeutic gene delivery vectors in different kinds of gene therapies. EX1005 (Satkunanathan).

227. LC-MS techniques, moreover, were well known to be a powerful tool for analyzing differences among various AAV serotypes. EX1005 (Satkunanathan).

A. Satkunanathan

228. Satkunanathan was published in November 2014, more than one year before the earliest possible filing date for the ’313 patent. EX1005 (Satkunanathan). Therefore, Satkunanathan is AIA §102(a)(1) prior art. EX1005 (Satkunanathan).

229. Satkunanathan studied three different AAV serotypes (AAV2, AAV5, and AAV8), focusing in particular on analyzing the protein composition present in purified AAV vectors of each serotype. EX1005 (Satkunanathan), Abstract.

230. Satkunanathan used LC-MS/MS to identify the proteins present in preparations of these purified AAV vectors. EX1005 (Satkunanathan), Abstract.

231. Satkunanathan states that they sought to identify differences in protein composition among the three AAV serotypes AAV2, AAV5, and AAV8 by analyzing host cellular proteins co-produced and co-purified with AAV vectors. EX1005 (Satkunanathan), p. 930.

232. Satkunanathan states that their work is directed towards improving the production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract, p. 930. Satkunanathan discusses the problem of pre-existing immune responses in patients, requiring administration of higher titers and, presumably, the design of vectors based on different AAV serotypes. EX1005 (Satkunanathan), Abstract.

1. Methods

233. Satkunanathan digested purified and concentrated vector samples with trypsin before LC-MS/MS. EX1005 (Satkunanathan), pp. 930-31.

234. The LC-MS/MS was carried out using a hybrid mass spectrometer (Thermo Fisher) equipped with a nano-electrospray ion source and two mass analyzers, that is, linear trap (LTQ) and orbitrap, coupled with an Ultimate 3000

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nano-LC system, comprising a solvent degasser, a loading pump, a nanopump, and a thermostated autosampler. EX1005 (Satkunanathan), p. 931.

235. After an automated injection, the peptides resulting from each digestion were desalted in a trapping cartridge (PepMap reversed phase C18, 5 µm 100 Å, 300µ id x 5mm length) (Thermo Fisher) and eluted onto a C18 reversed phase nano-column (3 µm, 100Å, 5 cm length) (Thermo Fisher) and followed by a 60 minute separation under a column flow rate of 0.3 µL/min using a linear gradient from 5-70% acetonitrile and 0.1% formic acid. EX1005 (Satkunanathan), p. 931.

236. Data analysis including mass spectra processing and database searching was carried out using Thermo Proteome Discoverer 1.2 with built-in Sequest. EX1005 (Satkunanathan), p. 931. Up to two missed tryptic cleavages were considered and methionine oxidation was set as a dynamic modification. EX1005 (Satkunanathan), p. 931. I note that, as I discussed above, Dong had identified oxidation of methionine as a PTM found in the AAV2 capsid proteins. EX1022 (Dong), Abstract, p. 2, Figure S1.

237. Peptide sequences by MS/MS were only included when Xcorrelation scores were greater than 1.5, 2, or 2.2 for charge states 1, 2, and 3, respectively. EX1005 (Satkunanathan), p. 931.

238. An unambiguous identification was considered when at least two peptides matched to the protein. EX1005 (Satkunanathan), p. 931. The protein

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FASTA databases were downloaded from www.uniprot.org, release 2012-07, including the complete entries from homo sapiens (taxon identifier 9606) bos taurus (9913); complete genome of AAV2, AAV5, and AAV8; and green fluorescent protein (GFP; P42212). EX1005 (Satkunanathan), p. 931.

2. Results

239. Equal amounts of total proteins from three different types of purified AAV vector samples, that is, AAV2-GFP, AAV5-GFP, and AAV8-GFP were subjected to LC-MS/MS analysis. EX1005 (Satkunanathan), p. 932.

240. To minimize data variation, three batches of samples were prepared for each type of vector, with each batch pooled from 40 tissue culture plates (150mm diameter). EX1005 (Satkunanathan), p. 932. Three MS runs were performed for each batch of samples. EX1005 (Satkunanathan), p. 932.

241. Satkunanathan's results showed that 44 proteins were detected in at least two out of three runs of three batches of samples. EX1005 (Satkunanathan), pp. 932, Supplementary Table S1. These proteins were considered to be significant components and further studied. EX1005 (Satkunanathan), pp. 932, Supplementary Table S1.

242. Among the significant proteins, ten were common to three of the AAV serotypes, including 70 kDa protein 1A/1B, alpha-enolase, GapDH, heat shock,

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histotone H2Atype 1H, histone H2B, nucleolin, nucleophosmin, RuvB2, and YB1. EX1005 (Satkunanathan), pp. 932, Supplementary Table S1.

243. Out of eight proteins shared by two serotypes, five were shared by AAV2 and AAV5, indicating a relative similarity between AAV2 and AAV5 vectors. EX1005 (Satkunanathan), pp. 932, Supplementary Table S1. Twenty-six were unique to individual serotypes of vectors. EX1005 (Satkunanathan), pp. 932, Supplementary Table S1.

244. As shown in the excerpt from Supplementary Table S1 below, among the unique proteins for each serotype, Satkunanathan identified AAV2, AAV5, and AAV8 capsid proteins:

SUPPLEMENTARY TABLE S1. (CONTINUED)

Protein ID	*AAV2*	*AAV5*	*AAV8*
Actin, gamma 1 OS=Homo sapiens GN=ACTG1 PE=3 SV=1 - [F5H0N0_HUMAN]		+	
Annexin A2 (Fragment) OS=Homo sapiens GN=ANXA2 PE=4 SV=1 - [H0YKZ7_HUMAN]		+	
ATP synthase subunit alpha OS=Homo sapiens PE=2 SV=1 - [B4DY56_HUMAN]		+	
ATP synthase-coupling factor 6, mitochondrial OS=Homo sapiens GN=ATP5J PE=1 SV=1 - [ATP5J_HUMAN]		+	
Capsid protein VP1 OS=Adeno-associated virus 2 (isolate Srivastava/1982) PE=1 SV=2 - [CAPSD_AAV2S]	+		
Capsid protein OS=Adeno-associated virus - 5 GN=cap PE=1 SV=1 - [Q9YIJ1_9VIRU]		+	
Capsid protein OS=Adeno-associated virus - 8 PE=1 SV=1 - [Q8JQF8_9VIRU]			+

EX1005 (Satkunanathan), Supplementary Table S1 (excerpt) (yellow highlights added).

245. In the table above, a POSA would have understood OS to indicate the name of the organism or species. A POSA would also have understood that PE,

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which stands for "protein existence," is a metric of certainty in the existence of a protein. Where PE=1, there is credible experimental evidence for the existence of the protein. A POSA would also have understood that SV indicates the version of the sequence in the UniProt database of protein sequences. Lastly a POSA would have understood "GN" to stand for "gene name."

246. Satkunanathan found a serotype-specific role for an AAV-associated cellular protein, YB1. EX1005 (Satkunathan), Abstract, p. 938. Satkunathan found that knockdown of YB1 improved AAV2 and AAV8 production by 45- and 9-fold, respectively, but had no significant effect on AAV5 production. EX1005 (Satkunathan), p. 938.

247. In considering differences that could account for this result, Satkunanathan notes that the *cap* gene sequences are clearly different among the three serotypes tested (AAV2, AAV5, and AAV8). EX1005 (Satkunathan), p. 938.

248. Satkunanathan points out that AAV5 is one of the most divergent AAV serotypes, sharing only about 55% sequence homology with AAV2 and AAV8, which, in contrast, share about 82% homology with one another. EX1005 (Satkunathan), p. 938.

249. Satkunanathan therefore teaches the importance of identifying and characterizing different AAV serotypes accurately for rAAV vector purification and production. Satkunanathan's discussion of the problem of pre-existing immunity

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among patients to various AAV serotypes further underscores the need to ensure the serotypic purity of any preparation of rAAV for possible clinical use. EX1005 (Satkunanathan), p. 929.

250. Satkunanathan also teaches that as of 2014, it was routine to identify capsid proteins of different AAV serotypes using LC-MS/MS. EX1005 (Satkunanathan), Supplementary Table S1.

251. Using LC-MS/MS, Satkunanathan identified a cellular Y-box binding protein (YB1) that co-purified with all three serotypes of AAV vectors tested. EX1005 (Satkunanathan), p. 930, Supplementary Table S1. They investigated the role of YB1 in in AAV vector production and found that down regulating YB1 in AAV producer cells resulted in up to a 45-fold increase in physical vector genome titers of AAV2, up to a 9-fold increase in physical vector genome titers of AAV8, and up to a 7-fold increase in AAV2 infectious vector genome titers. EX1005 (Satkunanathan), p. 930.

252. Satkunanathan concluded that their results "show a serotype-specific role of YB1 in AAV production; in particular, knockdown of YB1 improved AAV2 and AAV8 production by 45- and 9-fold, respectively, but had no significant effect on AAV5 production." EX1005 (Satkunanathan), p. 938.

253. Satkunanathan attributed this serotype-specific effect to differences in the capsid genes of the three serotypes:

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> In terms of differences among the three serotypes of AAV2, AAV5, and AAV8 vectors, the serotype-specific *cap* gene sequences are clearly one of them. AAV2 and AAV8 capsid proteins share more than 82% homology in their primary sequence and a much similar overall topology in the structure of capsid proteins. The notable structural differences between AAV2 and AAV8 capsid proteins are located on the capsid surface and are known to be associated with the binding property of AAV2 and AAV8 to target cells rather than being involved in capsid assembly and genome packaging, further demonstrating the similarity between AAV2 and AAV8 in terms of capsid assembly. In contrast, AAV5 is one of the most divergent AAV serotypes, sharing only ~55% sequence homology to other serotypes, including AAV2 and AAV8. Unique structural features of AAV5 capsid proteins, including a smaller HI and VR-IV loop and larger VR-VII, are located in the VP region that controls the specificity of capsid assembly, genome packaging, and antigenic determinants, and may explain the difference in AAV2 and AAV8 vector production that we observed. Our results also show that YB1 gene knockdown resulted in up to 12- and 13-fold increases in *rep* gene expression and vector DNA production, respectively, and an ~7-fold decrease in cap gene expression, underlying the molecular mechanism of YB1 influence on AAV vector production.

EX1005 (Satkunanathan), p. 938 (internal citations omitted).

254. Satkunanathan states that YB1 may compete with AAV capsids for binding to the AAV ITRs, compromising encapsidation of the AAV genome. EX1005 (Satkunanathan), p. 938.

255. Satkunanathan concludes that the precise mechanism of TB1 effects in AAV production required further investigation and that "[u]nderstanding the role of YB1 in the AAV life cycle greatly facilitates future production of high titer AAV vectors and, ultimately, improves the quality and safety of AAV vectors for clinical use." EX1005 (Satkunanathan), p. 939.

B. Shytuhina

256. Shytuhina was published in 2014, more than a year before the earliest possible priority date for the ’313 patent (August 15, 2016), and is therefore AIA §102(a)(1) prior art. EX1006 (Shytuhina).

257. Shytuhina discloses the development and application of an RP-HPLC-MS method for analysis of intact viral structural proteins for process development for a vaccine. EX1006 (Shytuhina), Abstract. In particular, Shytuhina disclosed using their HPLC-MS method to identify post-translational modifications on intact viral structural proteins. EX1006 (Shytuhina), Abstract.

258. Shytuhina is directed towards development of a vaccine against Chikungunya virus, a mosquito-borne virus that causes acute illness including fever, rash, and severe arthralgia. EX1006 (Shytuhina), Abstract, p. 192. Chikungunya virus causes incapacitating and prolonged joint pain. EX1006 (Shytuhina), p. 192.

259. Proof-of-concept experiments had shown that Chikungunya virus like particles (VLPs) were sufficient to elicit a protective humoral response against Chikungunya infection. EX1006 (Shytuhina), p. 192. Researchers therefore sought further characterization of Chikungunya VLPs. EX1006 (Shytuhina), p. 192.

260. Shytuhina explains that, traditionally, vaccines composed of VLPs are characterized by SDS-PAGE for purity and quantified by colorimetric protein assays

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such as Bradford, bicinchoninic acid (BCA) or Lowry assay. EX1006 (Shytuhina), p. 192.

261. Shytuhina describes the disadvantages of these approaches. EX1006 (Shytuhina), p. 192. Specifically, Shytuhina states that SDS-PAGE is labor and time intensive. EX1006 (Shytuhina), p. 192. Colorimetric protein assays, according to Shytuhina, can be sensitive to detergents, reducing agents or certain salts. EX1006 (Shytuhina), p. 192.

262. Moreover, Shytuhina explains that the colorimetric protein assays measure total protein concentration, and are therefore not specific for the antigenic components of the vaccine product. EX1006 (Shytuhina), p. 192.

263. Shytuhina states that to support process and formulation development effectively, it is highly desirable to have a sensitive and robust method available that can be automated to measure both vaccine purity and antigen specific vaccine mass. EX1006 (Shytuhina), p. 192.

264. Shytuhina discloses that HPLC was an attractive analytical tool, in light of its high sensitivity and reproducibility. EX1006 (Shytuhina), p. 192. Shytuhina notes that HPLC had been applied for the identification and quantitation of viral proteins and VLPs from a variety of other viruses, including serotypes of adenovirus (types 3 and 5), influenza, lentivirus, Sendai virus, poliovirus, human papillomavirus VLP, and Hepatitis B VLP. EX1006 (Shytuhina), pp. 192-93.

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265. Shytuhina explains that as a result of the hydrophobic nature of most viral glycoproteins and the presence of lipids with enveloped virus (such as Sendai virus and lentivirus), it had been challenging to achieve good resolution and recovery for all the viral components. EX1006 (Shytuhina), p. 193.

266. The Chikungunya VLP has three structural proteins – E1 (envelope protein 1), E2 (envelope protein 2), and a capsid protein. EX1006 (Shytuhina), p. 193. The capsid and envelope are organized as follows: an outer surface composed of 240 copies of glycoproteins E1 and E2 embedded in a lipid bilayer surrounding a nucleocapsid made of 240 copies of capsid protein. EX1006 (Shytuhina), p. 193.

267. Shytuhina states that their goal was to develop a RP-HPLC assay that would separate E1, E2, and capsid proteins of Chikungunya VLPs. EX1006 (Shytuhina), p. 193. This assay would evaluate and quantitate the mass and purity of the vaccine product. EX1006 (Shytuhina), p. 193. This method would be a tool to assess both protein degradation and post-translational modifications for formulation and process development. EX1006 (Shytuhina), p. 193.

268. Shytuhina used LC-MS intact protein analysis to validate their RP-HPLC method, and to identify specific PTMs on the E1 and E2 proteins. EX1006 (Shytuhina), pp. 193-96. Shytuhina discusses monitoring PTMs as a key element of process development. EX1006 (Shytuhina), pp. 196-97.

1. Methods

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(a) RP-HPLC

269. Shytuhina describes the RP-HPLC method they used to separate the Chikungunya capsid and envelope proteins. EX1006 (Shytuhina), p. 193.

270. They analyzed Chikungunya VLPs on an XBridge BEH300 C4 column (3.5 µm, 4.6 × 150 mm, 300Å, Part # 186004504, from Waters), held at 60°C, with a linear AB gradient elution. EX1006 (Shytuhina), p. 193.

271. Their mobile phases were: mobile phase A contained 0.1% TFA in water; mobile phase B contained 30% ACN, 70% 2-propanol and 0.1% TFA. EX1006 (Shytuhina), p. 193.

272. The separation was carried out with a 60-min gradient ranging from 0% to 100% mobile phase B followed by a 7-min re-equilibration with mobile phase A at a flow rate of 1 mL/min. EX1006 (Shytuhina), p. 193. Eluted proteins were detected by fluorescence at excitation at 280 nm and emission at 350 nm. EX1006 (Shytuhina), p. 193.

273. To improve recovery, Shytuhina pretreated samples with Zwittergent. EX1006 (Shytuhina), p. 193. Samples were incubated with 5% Zwittergent 3-12 detergent and injected at a volume of 100 L. EX1006 (Shytuhina), p. 193.

274. It appears that Shytuhina may not have carried out this pretreatment with Zwittergent for the samples that were ultimately analyzed by intact LC-MS (which I describe below). Shytuhina states: "It was noted that in the early

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applications, for example studies that monitored the degradation and post-translational modifications, sample pre-treatment was not yet implemented. However, since Zwittergent was used to improve recovery, the lack of Zwittergent should not impact the characteristics of eluted E1 and E2." EX1006 (Shytuhina), p. 193.

275. Nonetheless, it is my opinion that Shytuhina would have been understood by a POSA to teach that it is advantageous to carry out sample pre-treatment with Zwittergent.

(b) Intact Protein Mass Analysis by LC-MS

276. Shytuhina used a Waters Acquity LC system coupled to a Synapt G2 mass spectrometer (Waters, Milford, MA) to separate and characterize the proteins in the VLP samples by LC-MS. EX1006 (Shytuhina), p. 193.

277. The mobile phases were as follows: mobile phase A was 0.1% formic acid (v/v) in water, and mobile phase B was 0.1% formic acid (v/v) in 30% ACN/70% isopropanol. EX1006 (Shytuhina), p. 193. Shytuhina explains that formic acid was used as the mobile phase modifier because TFA caused ion suppression. EX1006 (Shytuhina), p. 193.

278. Shytuhina used 10 consecutive injections of 25 µL of the sample to increase the signal intensity for intact protein accurate mass measurement. EX1006

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(Shytuhina), p. 193. These injections were made with a short 2 min isocratic flow of 5% mobile B. EX1006 (Shytuhina), p. 193.

279. Following the last injection, the proteins were eluted using a linear gradient of 5-80% of mobile phase B in 18 min at a flow rate of 0.2 mL/min. EX1006 (Shytuhina), p. 193.

280. Mass spectra were obtained in positive mode by spraying the eluent into the mass spectrometer using an ESI source. EX1006 (Shytuhina), p. 193. The capillary, source cone, and extraction cone voltages were set at 3 kV, 20 V, and 4 V, respectively. EX1006 (Shytuhina), p. 193. Nitrogen was used as a desolvation gas at a flow rate of 800 L/h. EX1006 (Shytuhina), p. 193. The source and desolvation temperatures were set at 110 and 450 ◦C, respectively. EX1006 (Shytuhina), p. 193. The instrument was operated in Sensitivity mode and spectra were acquired in an m/z range of 1000–2500. EX1006 (Shytuhina), p. 193.

281. Data acquisition and analysis (deconvolution) were performed with Waters MassLynx 4.1 software. EX1006 (Shytuhina), p. 193. Protein spectra were deconvoluted to obtain the observed intact protein masses. EX1006 (Shytuhina), p. 193. MaxEnt deconvolution parameters were set with output mass range of 40,000-60,000 and resolution of 0.1 Da/channel. EX1006 (Shytuhina), p. 193. Minimum intensity ratios were 33% for both the left and right parameters. EX1006 (Shytuhina), p. 193. A uniform Gaussian model was used with width at half height

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of either 1 or 0.8 Da. EX1006 (Shytuhina), p. 193. For spectra with width at half height of 1 Da, a maximum of 10 iterations were used. EX1006 (Shytuhina), p. 193. For spectra with width at half height of 0.8 Da, a maximum of 11 iterations were used. EX1006 (Shytuhina), pp. 193-94.

282. Post-translational modifications were identified by comparing observed masses to theoretical masses of expected N-glycoforms with and without acylation. EX1006 (Shytuhina), p. 194.

2. Results

283. Shytuhina discloses the steps they took to optimize their RP-HPLC-MS method. EX1006 (Shytuhina), p. 194.

(a) RP-HPLC

284. Shytuhina screened several HPLC columns with different combinations of mobile phases. EX1006 (Shytuhina), p. 194. They found that while capsid protein could be readily eluted off of the HPLC column with 0.1% TFA in acetonitrile, E1 tended to stick to the column and required a strong organic solvent such as 2-propanol to elute it. EX1006 (Shytuhina), p. 194.

285. In addition, TFA was necessary in the mobile phase to promote VLP interaction with the stationary phase. EX1006 (Shytuhina), p. 194. The lack of TFA in the mobile phases led to the elution of VLPs in the void volume of the column. EX1006 (Shytuhina), p. 194.

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286. Shytuhina notes that TFA, at the concentration used, did not cause aggregation of the Chikungunya proteins. EX1006 (Shytuhina), p. 194. Instead, Shytuhina discloses that TFA appeared to help disassemble the VLPs and facilitate the binding of proteins to the stationary phase. EX1006 (Shytuhina), p. 194.

287. Shytuhina found that a Waters XBridge BEH300 C4 column with 70% 2-propanol in the elution mobile phase had the best recovery for all three Chikungunya proteins, while column temperature of 60°C improved peak sharpness. EX1006 (Shytuhina), p. 194.

288. Shytuhina also screened different sample pre-treatment methods to optimize their separation. EX1006 (Shytuhina), p. 194. In particular, they initially observed carry-over of E1 and E2 between HPLC runs. EX1006 (Shytuhina), p. 194.

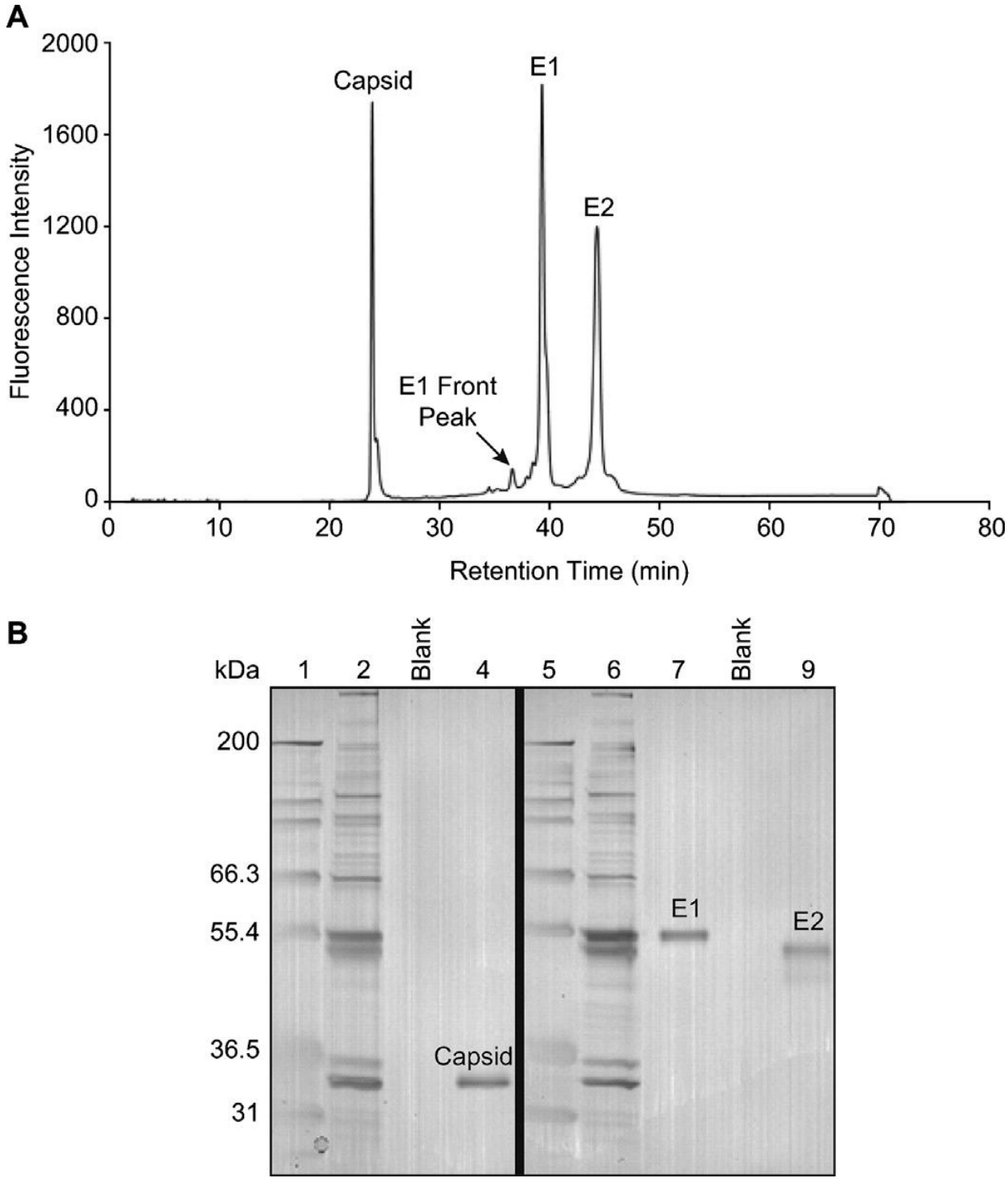
289. Shytuhina discovered that pre-incubation of sample with 5% Zwittergent 3-12 detergent increased the peak area of E1 by 130% and E2 by 60% and decreased the total carry-over to 4%. EX1006 (Shytuhina), p. 194. Shytuhina discloses that Zwittergent 3-12 detergent was believed to solubilize and stabilize the glycoproteins and prevent non-specific binding during chromatography. EX1006 (Shytuhina), p. 194. Shytuhina explains that incubation with 5% Zwittergent 3-12 detergent was therefore used as a sample pre-treatment method. EX1006 (Shytuhina), p. 194.

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290. To characterize the RP-HPLC chromatogram and identify which antigen protein was present in the individual peak, fractions were collected, concentrated and analyzed by SDS-PAGE and MALDI-ToF MS along with the corresponding unfractionated sample. EX1006 (Shytuhina), p. 194.

291. The proteins on the gel were identified as E1, E2, and capsid protein (from top to bottom in the gel in Fig. 1B) and the three peaks in the RP-HPLC chromatogram were confirmed as capsid, E1, and E2 from left to right (Fig. 1A), as shown below:

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EX1006 (Shytuhina), p. 194, Fig. 1.

(b) Intact LC-MS

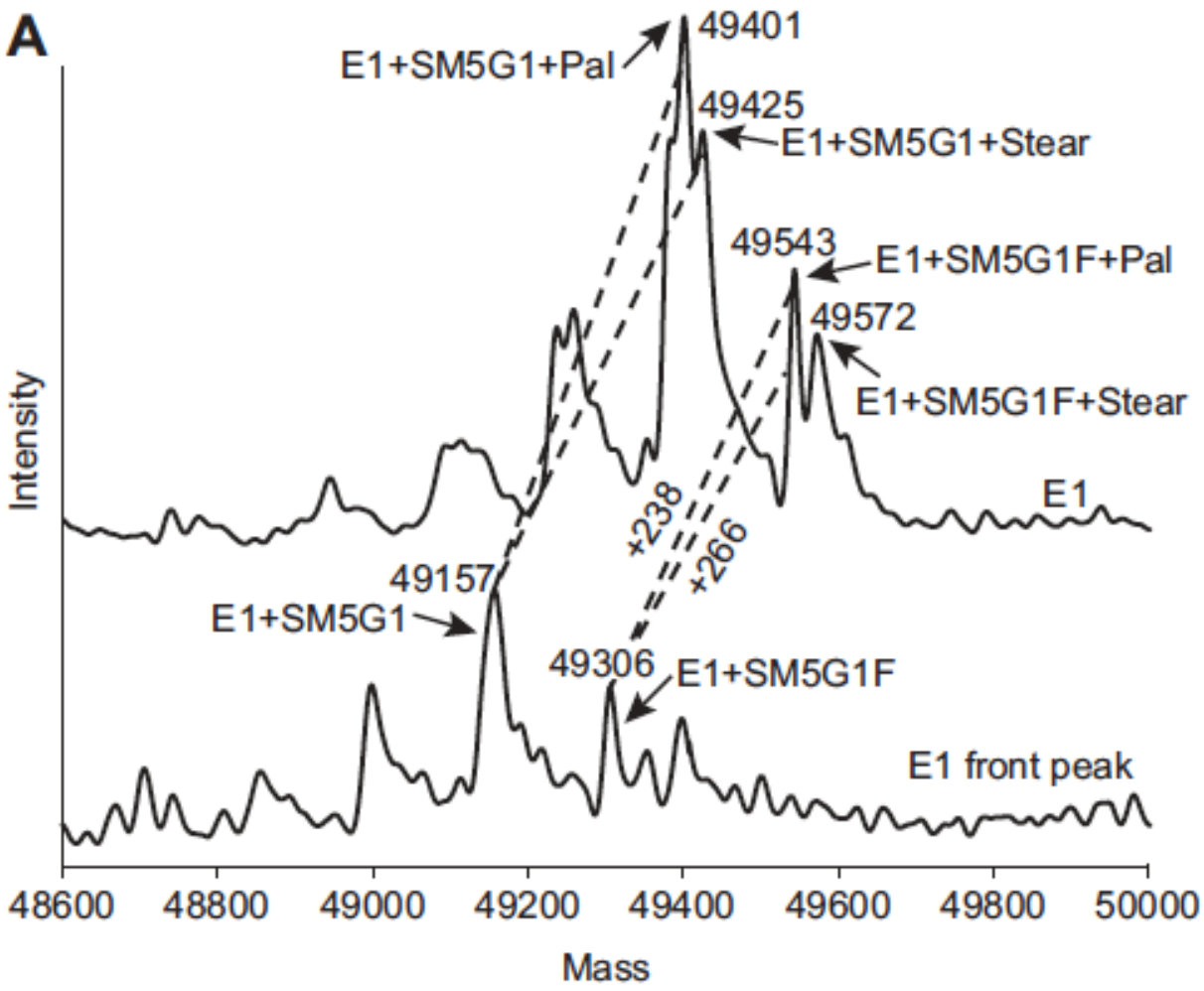
292. The ability to separate E1 and E2 by RP-HPLC chromatography allowed Shytuhina to characterize post-translational modifications of these viral glycoproteins by mass spectrometry. EX1006 (Shytuhina), p. 194.

293. Both E1 and E2 of Chikungunya were known to contain N-glycosylation, and both glycoproteins were also expected to contain acylation based on sequence similarity with another virus, Semliki Forest virus (SFV). EX1006 (Shytuhina), p. 194.

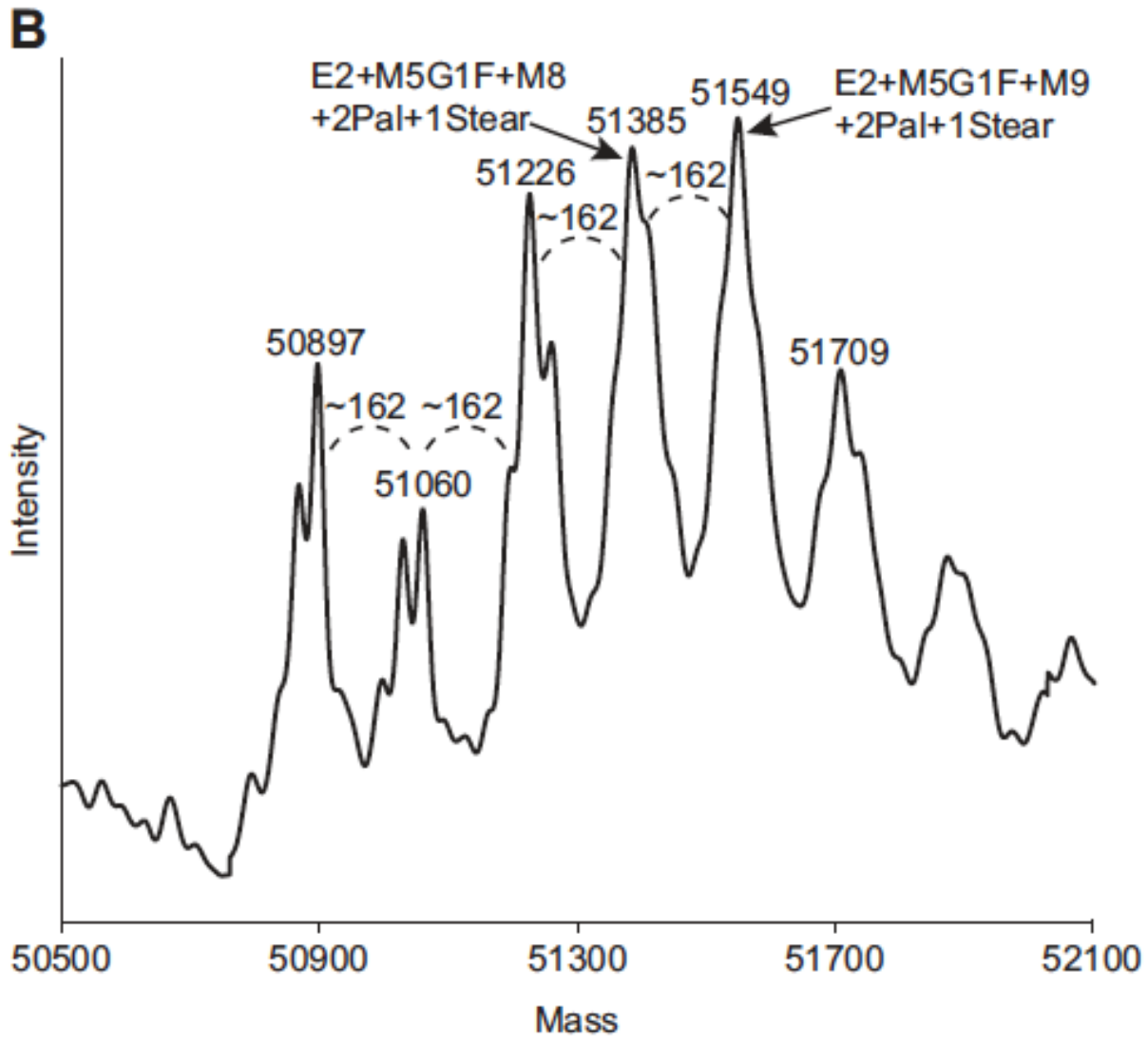
294. Shytuhina identified post-translational modifications present on Chinkungunya VLP proteins by LC-MS using accurate mass measurements of the intact protein antigens. EX1006 (Shytuhina), p. 194.

295. Fig. 2, reproduced below, shows the deconvoluted spectra for E1 (Fig. 2A) and E2 (Fig. 2B).

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EX1006 (Shytuhina), pp. 194-95, Fig. 2.

296. As shown in the figures above, multiple masses were observed with each representing different glycosylation and acylation modifications. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

297. Based on known glycoprotein acylation and expected N-linked glycans, Shytuhina identified glycoprotein modifications in several major peaks by matching

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the observed mass to the theoretical molecular weight. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

298. Shytuhina detected one N-glycosylation and one acylation – either palmitoylation (Pal) or stearoylation (Stear) – for the majority of E1 (Fig. 2A, top trace). EX1006 (Shytuhina), pp. 194-95, Fig. 2.

299. A small portion of glycosylated but deacylated E1 eluted slightly before the main population (E1 front peak in Figs. 1A and 2A bottom trace). EX1006 (Shytuhina), pp. 194-95, Fig. 2. Shytuhina explains that this result was not surprising, given that protein acylation is a reversible process, and enzymatic depalmitoylation of viral glycoprotein had been demonstrated. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

300. Fig. 2B shows that E2 contained two N-glycosylations and three acylations. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

3. Deamidation

301. Shytuhina states that they likely observed deamidation of one of the viral structural proteins they studied, the E2 protein of chikungunya virus. EX1006 (Shytuhina), p. 196. Specifically, in carrying out RP-HPLC, they observed an E2 degradant peak that eluted slightly earlier than the regular E2, suggesting that the degradant was more hydrophilic. EX1006 (Shytuhina), p. 194, Figure 1A, p. 196.

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302. Regarding this degradant peak, Shytuhina states: "Proteins are prone to deamidation at high pH and deamidation introduces a net increase in surface charge. Thus, we speculate at pH 9.0 E2 undergoes deamidation and that this chemical modification affects CHIKV VLP antigenicity." EX1006 (Shytuhina), p. 196.

4. Monitoring by LC-MS to Support Process Development

303. Shytuhina explains that Chikungunya VLPs produced in different cell lines exhibit different post-translational modifications. EX1006 (Shytuhina), pp. 196-97. Shytuhina states that it is therefore important to monitor post-translational modifications to ensure product lot-to-lot consistency throughout the vaccine development cycle. EX1006 (Shytuhina), p. 197.

C. Yuan

304. Yuan was published in 1998, more than a year before the earliest possible priority date for the '313 patent (August 15, 2016), and is therefore AIA §102(a)(1) prior art. EX1037 (Yuan).

305. Yuan discloses an RP-HPLC method for analysis of virus-like particles (VLPs) of human papillomavirus (HPV) for process development. EX1037 (Yuan), Abstract.

306. In particular, Yuan's method is directed towards identifying the L1 capsid protein that comprises 90-95% of HPV capsids. EX1037 (Yuan), p. 21. Yuan

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explains that the HPV capsid is typically composed of 72 pentameric capsomers of L1 arranged on a skewed icosahedral lattice. EX1037 (Yuan), p. 21. A second structural protein, L2, reperesents the remaining 5-10% of the capsid. EX1037 (Yuan), p. 21.

307. Yuan states that their method is quantitative and can be used to facilitate HPV purification process development. EX1037 (Yuan), p. 21.

1. Methods

308. The first step in Yuan's analysis was denaturation of the HPV samples using a dissociation buffer of 8 M guanidine-HCl/50 mM Tris, pH 8.0. EX1037 (Yuan), p. 22. Then, 10% 2-mercaptoethanol was added to the samples, which were incubated at 55°C for 15 minutes. EX1037 (Yuan), p. 22.

309. The second step, HPLC, was then performed on a Hewlett-Packard 1100 Series liquid chromatography system equipped with a G1322A vacuum degasser, a G1311A quaternary pump, a G1315A diode array detection (DAD) system, a G1313A autosampler and a G1316A thermostatted column compartment. EX1037 (Yuan), p. 22.

310. Yuan states that its HPLC analyses were carried out on C_8 or C_4 reversed-phase columns (25 cm x 4.6 mm I.D., 5 µm particle size, 300 Å pore size) purchased from Vydac (Hesperia, CA, USA). EX1037 (Yuan), p. 22. Column temperature was set at 37°C. EX1037 (Yuan), p. 22.

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311. Yuan discloses that the solvents used were: solvent A was 0.1% TFA in water and solvent B was 0.075% TFA in acetonitrile. EX1037 (Yuan), p. 22. Gradient conditions used in the analysis are shown in the table below:

Table 1
Slow and fast gradient methods used for RP-HPLC

Slow gradient method		Fast gradient method	
Time (min)	% Solvent B	Time (min)	% Solvent B
0	5	0	40
10	5	5	40
15	40	15	55
30	60	17	100
32	100	20	100
37	100		

EX1037 (Yuan), p. 22, Table 1.

312. SDS-PAGE analysis was performed on the peak fractions. EX1037 (Yuan), p. 22.

2. Results

313. Yuan discusses the importance of the first step of dissociating the VLPs before placing them on the HPLC column. EX1037 (Yuan), p. 22. Yuan explains that the VLPs they used in the analysis were approximately 55 nm in diameter and contained a single capsid protein, L1, with a molecular mass of about 55,000. EX1037 (Yuan), p. 22.

314. According to Yuan, the VLPs are typically composed of 72 pentameric capsomers of L1 arranged on a skewed icosahedral lattice. EX1037 (Yuan), p. 22. The molecular mass of the intact recombinant particle is approximately 20 x 10^6. EX1037 (Yuan), p. 22.

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315. Yuan explains that to quantitate the L1 capsid protein, it was necessary to dissociate the VLPs prior to RP-HPLC analysis. EX1037 (Yuan), p. 22.

316. Yuan found that a buffer composed of 50 mM Tris, pH 8.0, containing 8 M guanidine-HCl and 10% 2-mercaptoethanol was effective in reducing disulfide bonds and destabilizing the VLPs to facilitate dissociation. EX1037 (Yuan), p. 22. The VLPs were incubated in this buffer at 55°C for 15 minutes before being loaded onto the column. EX1037 (Yuan), p. 22.

317. After incubation, the VLP sample containing monomeric L1 capsid protein was injected onto a Vydac C4 or C8 column. EX1037 (Yuan), p. 22.

318. The L1 capsid protein was eluted using an acetonitrile-water/0.1% TFA gradient, as discussed above. EX1037 (Yuan), pp. 22-23. A single, clearly resolved peak was obtained. EX1037 (Yuan), pp. 23-24, Fig. 1.

319. Yuan tested both C4 and C8 columns, noting that "either the C4 or C8 column can be used with equal efficiency of separation providing calibration is carried out with purified reference standard material." EX1037 (Yuan), p. 23.

320. Yuan noted that the retention time of L1 capsid protein on a C4 column was 0.2 minutes shorter than the retention time on a C8 column under fast gradient conditions. EX1037 (Yuan), p. 23.

321. Yuan confirmed the identity of the L1 capsid protein in the L1 containing peak fractions using SDS-PAGE. EX1037 (Yuan), p. 23.

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322. For quantitation, Yuan generated a calibration curve using HPV-18 VLPs purified by sucrose gradient centrifugation. EX1037 (Yuan), p. 23. Two C8 columns and one C4 column were used for analysis. EX1037 (Yuan), p. 23.

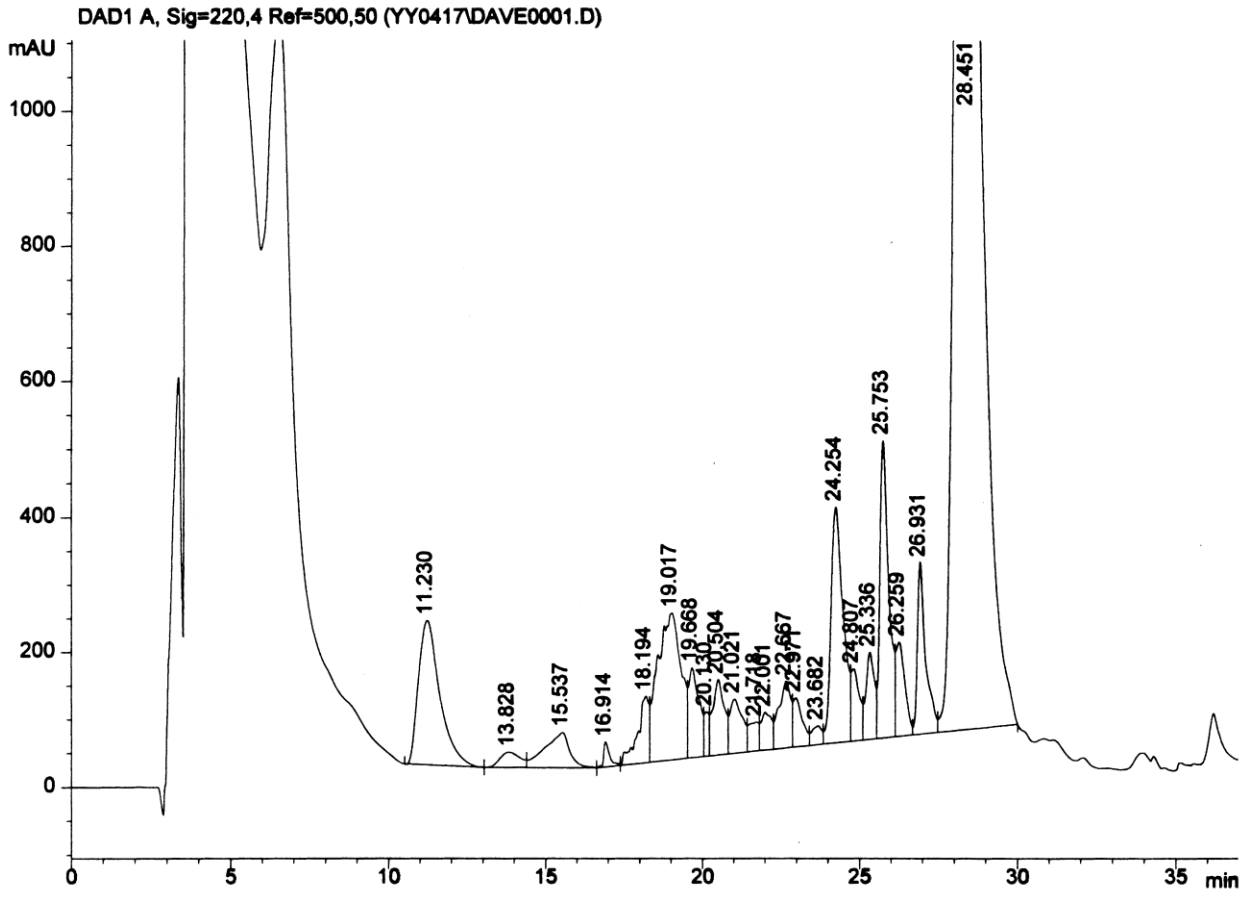
323. Yuan analyzed and quantitated HPV-18 in-process samples by both their RP-HPLC method and by Western blot. EX1037 (Yuan), p. 23.

324. They found that the L1 concentration for all samples was higher by RP-HPLC than by semi-quantitative Western blot. EX1037 (Yuan), p. 23.

325. Yuan also conducted spiking studies to evaluate whether their RP-HPLC method could be used for to quantitate partially purified samples for in-process development. EX1037 (Yuan), p. 23. They recoverd from 92% to 99% of the spiked protein, indicating that their method was suitable for quantitation of partially purified samples for process development. EX1037 (Yuan), p. 23.

326. Yuan applied their method to quantitate HPV produced in bioreactors (insect cells). EX1037 (Yuan), pp. 23, 25. They diluted the sample in dissociation buffer, then injected it onto a C_8 column using a slow gradient method. EX1037 (Yuan), pp. 23, 25. The chromatogram is shown below:

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EX1037 (Yuan), p. 26, Fig. 3.

327. The L1 capsid protein eluted in fractions from 23.8 to 25.0, as confirmed by SDS-PAGE. EX1037 (Yuan), p. 27.

D. Zabrouskov

328. Zabrouskov was published in 2005, more than a year before the earliest possible priority date for the ’313 patent (August 15, 2016), and is therefore AIA §102(a)(1) 102(a)(1) prior art. EX1038 (Zabrouskov).

329. Zabrouskov used top-down (intact) MS/MS to identify multiple deamidation sites, at asparagine and glutamine, on Ribonuclease A (RNase A). EX1038 (Zabrouskov), Abstract.

330. Zabrouskov analyzed the kinetics of deamidation at multiple different sites in RNase A, finding that deamidation at one site likely influenced deamidation at a nearby site. EX1038 (Zabrouskov), Abstract. This finding suggested that residual conformational effects persisted despite strongly denaturing conditions used. EX1038 (Zabrouskov), Abstract.

331. Zabrouskov also carried out site-specific quantitation of deamidation at the various sites in RNase A. EX1038 (Zabrouskov), Abstract.

332. As I discussed above, Zabrouskov states that top-down tandem mass spectrometry (MS/MS) was known to be uniquely useful for kinetic studies of multi-component systems. EX1038 (Zabrouskov), p. 987. Specifically, after MS separation of the target protein's molecular ions, further MS dissociation yields fragment ions whose mass shifts show which amino acids have been modified. EX1038 (Zabrouskov), p. 987.

333. Zabrouskov notes, nonetheless, that it was known in the art that deamidation poses a special challenge, in that the covalent $–NH_2 \rightarrow –OH$ modification produces only a 0.984 Da mass increase, closely matching the 1.002

Da spacing of the molecular ion isotope peaks. EX1038 (Zabrouskov), p. 987. As a result, it is difficult to distinguish deamidated forms from ^{13}C isotopic forms.

334. Despite this known difficulty, Zabrouskov successfully used top-down MS/MS to distinguish five stepwise deamidation sites in RNase A. EX1038 (Zabrouskov), p. 987.

335. According to Zabrouskov, rates of deamidation of asparagine and glutamine residues depend on protein primary sequence, three-dimensional structure, and solution parameters – increased pH and temperature and enhanced denaturation accelerate deamidation. EX1038 (Zabrouskov), p. 987.

336. Deamidation of Asn is generally favored over that of Gln, in part through operation of a cyclic imide reaction mechanism that also favors the Asn67-Gly68 sequence found in RNase A, while other neighboring residues also show an influence statistically. EX1038 (Zabrouskov), p. 987. However, some Asn and Gln residues are extremely resistant to *in vivo* deamidation. EX1038 (Zabrouskov), p. 987.

337. Zabrouskov states that in "bottom-up" MS proteomics, initial digestion of the protein gives peptides with mass spectra that often provide a fast, reliable identification of a protein, but are much less useful in characterizing post-translational modifications. EX1038 (Zabrouskov), p. 987.

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338. Zabrouskov further states that in top-down MS/MS, electrospray ionization (ESI) of a protein mixture introduces their gaseous molecular ions into a mass spectrometer. EX1038 (Zabrouskov), pp. 987-88.

339. For a specific protein, an accurate molecular mass differing from that of predicted values from sequence information indicates sequence errors, alternative splicing, protein or RNA editing, and/or post-translational modifications. EX1038 (Zabrouskov), p. 988.

340. These discrepancies can be identified and located by MS/MS separation and dissociation of the protein molecular ions, using methods such as collision-activated dissociation (CAD), infrared multiphoton dissociation (IRMPD), or electron capture dissociation (ECD). EX1038 (Zabrouskov), p. 988.

341. Zabrouskov found that for reduced RNase A, these techniques established five deamidation sites, as well their kinetic order of deamidation, indicating extensive conformational selectivity despite the strong denaturing conditions that they employed. EX1038 (Zabrouskov), p. 988.

1. Methods

342. Protein samples were reduced with 20 mM DTT^{red} and desalted on a reversed-phase protein trap (Michrom Bioresources Inc.), washed with 2 ml of a 0.1:99:0.5 (v/v/v) $MeCN/H_2O/CH_3COOH$ mixture, and eluted with 150 µl of a

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50:45:5 MeCN/H_2O/CH_3COOH mixture. EX1038 (Zabrouskov), p. 988. EX1038 (Zabrouskov), p. 988.

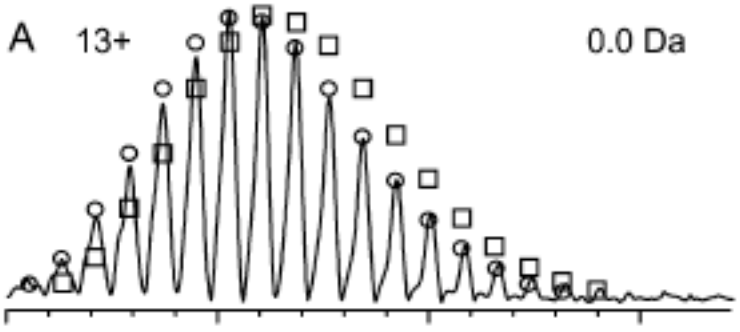
343. This eluent was loaded into a nanospray ESI emitter (2-4 ím inside diameter tip), with 1.0-1.5 kV versus the MS inlet producing a flow rate of 20-100 nL/min. The resulting ions were guided into the ion cell (10-9 Torr) of a modified 6 T Finnigan FT-MS device. EX1038 (Zabrouskov), p. 988.

344. Fragmentation was achieved by plasma ECD or IRMPD for ions entering the FT-MS cell, or by isolating specific ions in the cell using stored waveform inverse Fourier transform (SWIFT) followed by CAD. EX1038 (Zabrouskov), p. 988. Fragment assignments were made with THRASH. EX1038 (Zabrouskov), p. 988.

2. Results

(a) Mass Spectrometry

345. The figure below shows the ESI mass spectrum of the 13+ molecular ions of reduced RNase A Zabrouskov obtained:

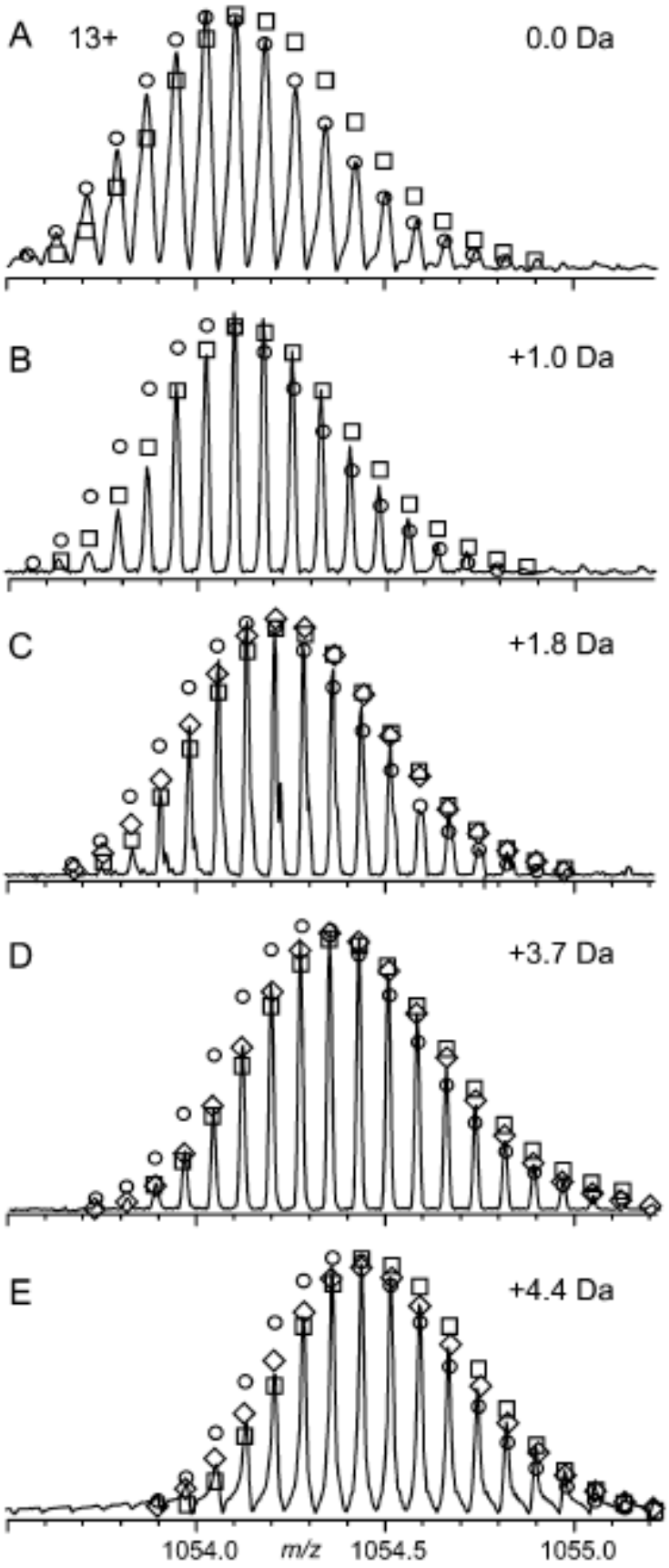


EX1038 (Zabrouskov), p. 988, Figure 1A.

346. The spectrum consists of a series of isotopic peaks (most abundant, 13 689.3-*8* Da; calcd 13 689.3-*8* Da) 1.0024 Da apart, with relative intensities that can be predicted (O) from the protein elemental composition. EX1038 (Zabrouskov), p. 988, Figure 1A.

347. The figures below show that deamidation produced a 0.9840 Da increase in mass ($–NH_2 \rightarrow –OH$) for each of the isotopic peaks, so that the mass spectrum of a mixture of deamidation products had peaks at virtually identical masses, but of overlapping peak intensities (I have reproduced Figure 1A again below to show the shift in the peaks):

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EX1038 (Zabrouskov), p. 988, Figure 1.

348. Purification by HPLC or isoelectric focusing ensured that each sample contained no more than two levels of deamidation, and the relative amounts of each that would give the observed isotopic peak intensities were calculated. EX1038 (Zabrouskov), p. 988.

349. Note that the alternative measurement of the deamidation peaks by high resolution is far more difficult than indicated by the nominal mass difference of 0.0183 Da. EX1038 (Zabrouskov), p. 989. Each of the "isotopic" peaks of Figure 1 from each degree of deamidation is also a composite of peaks of other isotopic compositions that are also isobaric (same nominal mass). EX1038 (Zabrouskov), p. 989.

350. Among these peaks within the 13 689.3-*8* Da "peak" of Figure 1A will be those containing $^{13}C_8{}^{15}N_0{}^{34}S_0$, $^{13}C_7{}^{15}N_1{}^{34}S_0$, and $^{13}C_6{}^{15}N_0{}^{34}S_1$ (with the other atoms as their most abundant isotopes)[3] with relative abundances of 100, 83, and 80%,

[3] The notations used here from Zabrouskov should be understood as follows: $^{13}C_8{}^{15}N_0{}^{34}S_0$ indicates 8 ^{13}C (carbon) atoms (8 C atoms with an atomic mass of 13), zero ^{15}N (nitrogen) atoms (zero N atoms with an atomic mass of 15), and zero ^{34}S (sulfur) atoms (zero S atoms with an atomic mass of 34). The total mass of

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respectively, with the last two lower in mass by 0.0064 and 0.0110 Da, respectively. EX1038 (Zabrouskov), p. 989.

351. Zabrouskov notes that high resolution could have been used on small fragment peaks that have few isobaric peaks, or on RNAse A synthesized from ^{13}C-, ^{15}N-, and ^{34}S-depleted precursors. EX1038 (Zabrouskov), p. 989.

(b) Deconvolution

352. RNase A was deamidated under denaturing alkaline conditions for 1, 3.5, and 4.2 h. EX1038 (Zabrouskov), p. 989. Further separation of the 1 h sample by cation exchange gave a chromatogram with three peaks in a relative area ratio of 5:43:52. EX1038 (Zabrouskov), p. 989. Zabrouskov states that these should represent products with different numbers of deamidated residues (*e.g.*, zero, one, and two), as each deamidation adds a negative charge to the protein. EX1038 (Zabrouskov), p. 989.

353. Zabrouskov also states that the ESI mass spectra of the last two fractions had the most abundant peak at 13 690.2-*8* and 13 691.6-*8* Da, respectively (Figure 1B,C; Figure 1A, 13 689.3-8 Da). EX1038 (Zabrouskov), pp. 988-89, Figure 1.

$^{13}C_8{}^{15}N_0{}^{34}S_0$ is therefore: 8x13=104. The total mass of $^{13}C_7{}^{15}N_1{}^{34}S_0$ is: 7x13 + 1x15 = 106. The total mass of $^{13}C_6{}^{15}N_0{}^{34}S_1$ is: 6x13 + 1x34 = 112.

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354. Zabrouskov found that the relative isotopic abundances of Figure 1B were in good agreement with the calculated abundance distribution (O) for reduced RNase A shifted by +1.0 Da (□). EX1038 (Zabrouskov), pp. 988-89, Figure 1B. Zabrouskov concludes that, therefore, most of fraction 2 was singly deamidated, consistent with the chromatographic separation. EX1038 (Zabrouskov), pp. 988-89, Figure 1B.

355. Zabrouskov also found that fraction 3 gave an isotopic abundance distribution (Figure 1C) intermediate between those calculated distributions corresponding to single (O) and double (□) deamidation, while the distribution calculated for a 1:4 ratio agreed closely with experiment [Figure 1C (◊)], indicating an average of 1.8 deamidations. EX1038 (Zabrouskov), pp. 988-89, Figure 1C.

356. Zabrouskov subjected the 3.5 and 4.2 h deamidation products to isoelectric focusing to select the ~25 and ~15% fractions, respectively, with mass spectra indicating that they represented more highly deamidated species. EX1038 (Zabrouskov), pp. 988-89, Figures 1D and 1E. These gave overlapping isotopic distributions corresponding to 3.7 (Figure 1D) and 4.4 (Figure 1E) deamidations, respectively. EX1038 (Zabrouskov), pp. 988-89, Figures 1D and 1E.

357. In Figure 1, the samples are designated by their deamidation degree: 0.0, 1.0, 1.8, 3.7, and 4.4 (Figure 1). EX1038 (Zabrouskov), pp. 988-89, Figure 1.

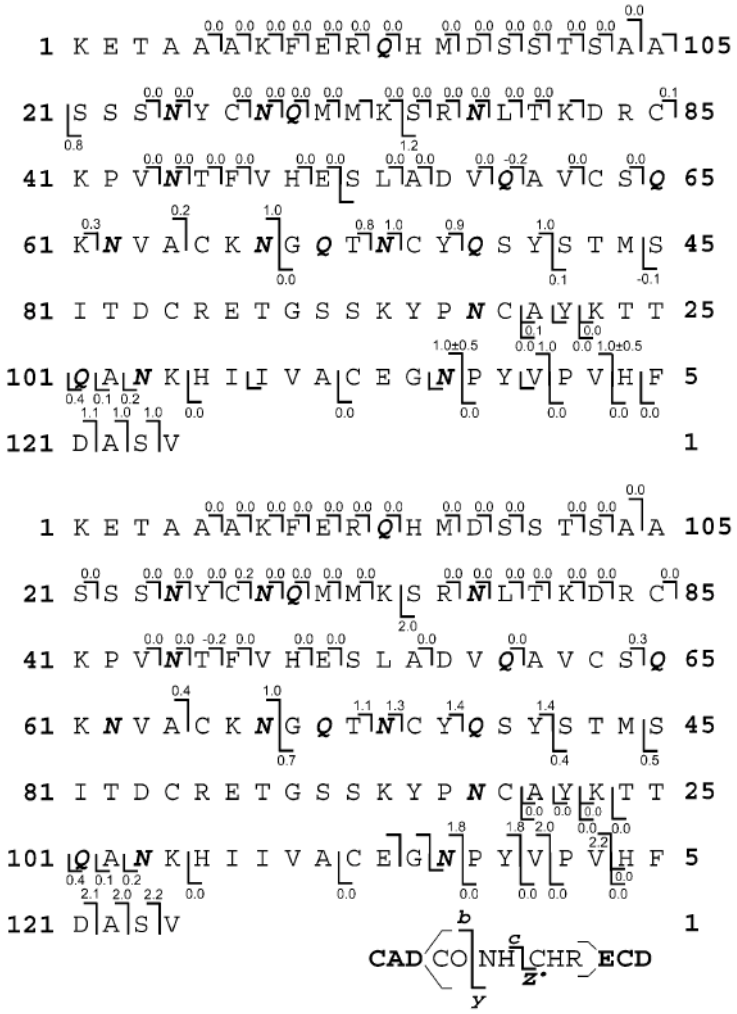
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358. Zabrouskov notes that the overlapping molecular ions that could represent more than one degree of deamidation were further dissociated by CAD and IRMPD to produce b and y fragment ions and by ECD to produce c, $z^{\bullet}$, and y fragment ions.[4] EX1038 (Zabrouskov), pp. 988-89, Figure 1.

359. Their overlapping fragment peak isotopic clusters were deconvoluted as in Figure 1, with the deamidation values shown in Figures 2 and 3. EX1038 (Zabrouskov), pp. 988-90, Figure 1, Figure 2, Figure 3. I have reproduced Figure 2 below, showing deamidation values at specific residues for samples of 1.0 (top figure) and 1.8 (bottom figure) deamidations:

[4] The notation used in Zabrouskov would have been understood by a POSA at the relevant time as standard notation for ions generated from breaking peptide bonds in mass spectrometry. Generally the ions that include the N-terminal portion of the peptide are referred to as "a, b, c" ions. The ions that include the C-terminal portion of the peptide are referred to as "x, y, z" ions.

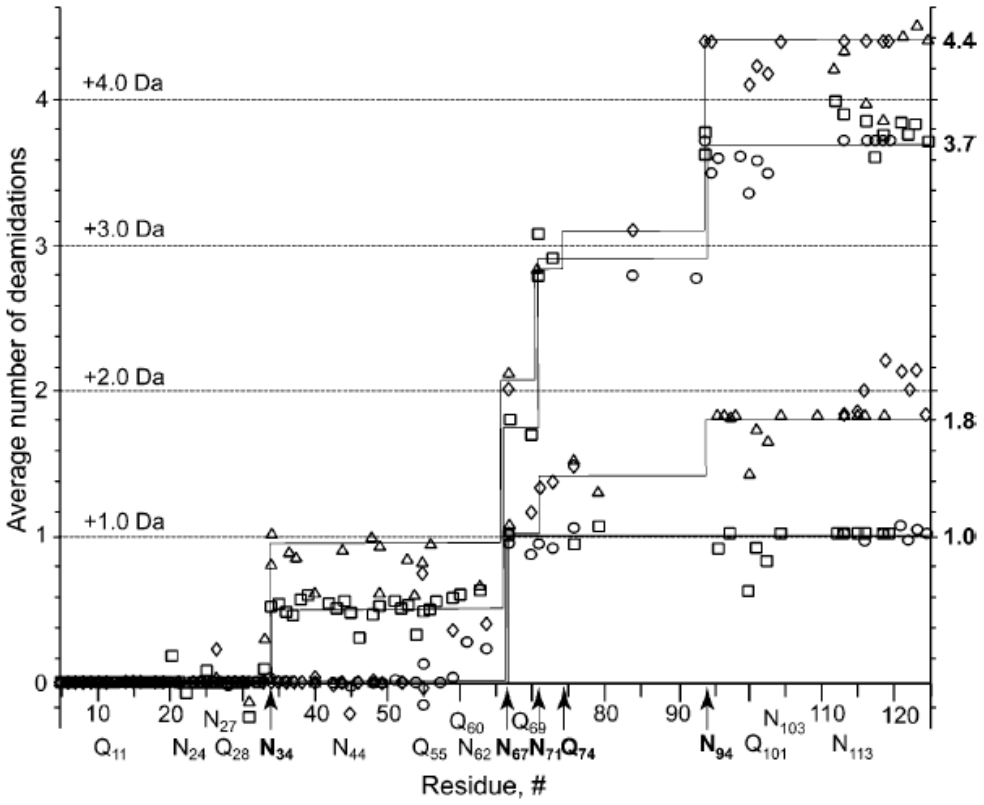
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EX1038 (Zabrouskov), p. 989, Figure 2.

360. Zabrouskov states that the precision of these values depends on peak intensities (not shown) and increases with a decrease in mass. EX1038 (Zabrouskov), p. 989.

361. I have reproduced below plots of these values (calculated to two decimal places) showing the increasing level of deamidation versus each of the Asn and Gln sites of RNase A for the four product samples:



EX1038 (Zabrouskov), p. 990, Figure 4. The legend to this figure explains: "Symbols for b or c and y or z• fragment ions, respectively: 1.0 (O and □), 1.8 (◊ and Δ), 3.7 (□ and O), and 4.4 deamidations (Δ and ◊). The deamidation value shown for a y or z• (C-terminal) ion represents the difference between its observed value and that of its parent ions." EX1038 (Zabrouskov), p. 990, Figure 4, Legend.

(c) Deamidation Percentages

362. Zabrouskov calculated percentages of deamidated products in the various reactions. EX1038 (Zabrouskov), p. 990. For example, Zabrouskov reported that, for the monodeamidated fraction, Asn67 was the first residue to deamidate and the deamidation of this reside was nearly complete in 1 hour (0:1:2

deamidations at a 5:43:52 ratio), with <10% monodeamidation at other sites. EX1038 (Zabrouskov), p. 990.

IX. CLAIM CONSTRUCTION

363. Challenged claims 1-27 of the ’313 patent recite methods of identifying and characterizing AAV particles, comprising denaturing the AAV particles, subjecting the denatured AAV particles to LC-MS intact protein analysis, and determining the masses of one or more of the AAV viral proteins (vp1, vp2, vp3), in the absence of a gel separation step. EX1001 (’313 patent), 83:1-85:7.

364. Certain claims further recite additional limitations, including detecting post-translational modifications, analyzing variant capsids, identifying different AAV serotypes, and using particular chromatographic methods such as RP-HPLC, and particular columns, such as C8 columns. EX1001 (’313 patent), 83:1-24, 28-40, 83:59-84:14, 84:64-67.

365. I have analyzed the terms recited in claims 1-27 according to their plain and ordinary meaning, unless otherwise noted.

X. GROUND 1: CLAIMS 1-27 ARE OBVIOUS OVER SATKUNANATHAN AND SHYTUHINA

366. In my opinion, claims 1-27 of the ’313 patent are obvious over Satkunanathan and Shytuhina.

367. A POSA would have been motivated to combine Satkunanathan and Shytuhina because both are directed towards efficient and precise methods for

analysis of viral particle composition for the purpose of process development. Both use liquid chromatography-mass spectrometry techniques to characterize purified viral preparations, identifying properties such as differences among different viral serotypes, or post-translational modifications.

368. Satkunanathan, however, enzymatically digested proteins before subjecting them to LC-MS. Satkunanathan sought to identify cellular proteins that co-purified with vectors based on different AAV serotypes. Satkunanathan cites the prior study by Dong, which used LC-MS to discover post-translational modifications in the AAV2 capsid and to identify various proteins that co-purified with AAV2.

369. Using LC-MS/MS, Satkunathan extended the work of Dong to study differences in co-purifying proteins among three different AAV serotypes (AAV2, AAV5, and AAV8). Satkunanathan identified 44 proteins that co-purified with AAV vectors.

370. Satkunanathan then focused on a single protein among the 44, YB1, determining that down-regulation of this protein resulted in increased physical vector genome titers of AAV2 and AAV8 vectors. Satkunanathan identified the different capsid protein for each serotype, and disclosed that each different serotype had different collections of cellular proteins associated with it.

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371. Satkunanathan teaches that the mechanism through which YB1 affects AAV production should be investigated further, to enhance the production, quality, and safety of AAV vectors.

372. Satkunanathan also identified capsid proteins for each of the three serotypes studied, AAV2, AAV5, and AAV8.

373. In addition, Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM.

374. A POSA at the time would have been motivated to continue the investigation and characterization of co-purifying cellular factors such as YB1 that could enhance production of AAV vectors in a serotype-dependent manner.

375. A POSA at the time would have further been motivated to improve methods for characterizing AAV proteins for process development, as Satkunanathan teaches, including identifying and characterizing different serotypes, identifying and characterizing PTMs, and identifying and characterizing individual AAV capsid proteins (vp1, vp2, and vp3). As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

376. A POSA would have understood that more precise, accurate characterization to improve process development would involve the capability to distinguish AAV serotypes, identify PTMs, and monitor degradation products.

377. As I discussed above, a POSA would have understood that LC-MS analysis involving enzymatic digestion before MS analysis (*e.g.*, "bottom-up analysis"), would not have enabled reliable and accurate separation and characterization of the AAV viral capsid proteins from one another, and from the capsid proteins of different serotypes.

378. In particular, a POSA would have understood that the intact LC-MS method of Shytuhina would have allowed efficient, reliable, accurate analysis of individual AAV capsid proteins, including distinguishing vp1, vp2, and vp3 from one another and from those of other serotypes, and assessing PTMs and other variations and truncations.

A. Claim 1

1. "A method of detecting post-translational modifications of one or more viral proteins (VPs) in a preparation of adeno-associated virus (AAV) particles, the method comprising"

379. Satkunanathan in combination with Shytuhina meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV

vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

380. Using LC-MS/MS, Satkunanathan identified capsid proteins from each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. In addition, Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification. EX1005 (Satkunanathan), p. 931. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. Moreover, as I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

381. Shytuhina uses intact LC-MS to identify various post-translational modifications, glycosylation, and acylation, of viral structural proteins. EX1006 (Shytuhina), p. 194. Shytuhina observed several different glycosylation and acylation modifications on each of the two viral structural proteins analyzed by intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Shytuhina therefore used intact LC-MS to detect post-translational modifications of viral proteins in a preparation of viral particles.

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382. A POSA would have understood that applying Shytuhina's intact LC-MS method to Satkunanathan's study of variations among different AAV serotypes would have provided a method of detecting post-translational modifications of the AAV capsid proteins in a preparation of AAV particles.

383. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 1.

2. "a) denaturing the AAV particles;"

384. Satkunanathan in combination with Shytuhina meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

385. Shytuhina teaches carrying out two denaturing steps – treatment with Zwittergent and RP-HPLC – before subjecting the denatured viral particles to LC-MS intact protein analysis. EX1006 (Shytuhina), pp. 193-94.

386. For the first step, Shytuhina teaches incubating the samples with 5% Zwittergent 3-12 detergent. EX1006 (Shytuhina), pp. 193-94. Shytuhina states that pre-treatment with Zwittergent improved recovery, and that "Zwittergent 3-12

detergent is believed to solubilize and stabilize the glycoproteins [viral structural proteins] and prevent non-specific binding during chromatography." EX1006 (Shytuhina), p. 194. A POSA at the time would have understood that "solubilizing" glycoproteins involves denaturing them.

387. For the second step, Shytuhina carried out an RP-HPLC separation, before subjecting the denatured samples to LC-MS, which then involved another RP-HPLC column coupled to the mass spectrometer. EX1006 (Shytuhina), p. 193. Shytuhina states that for the initial RP-HPLC separation (before LC-MS), they used TFA in the mobile phase on the column, and that "TFA appeared to help disassemble the VLPs and facilitate the binding of proteins to the stationary phase." EX1006 (Shytuhina), p. 194.

388. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 1.

3. "b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;"

389. Satkunanathan in combination with Shytuhina meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and

the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

390. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. As discussed above regarding limitation 1(a), Shytuhina teaches an intact LC-MS method involving pre-treatment of samples with Zwittergent 3-12 detergent followed by denaturation on a RP-HPLC column with TFA as part of the mobile phase. EX1006 (Shytuhina), pp. 193-94.

391. After the initial RP-HPLC, the denatured viral particles are then separated and analyzed by intact LC-MS using a Waters Acquity LC system coupled to a Synapt G2 mass spectrometer. The denatured viral particles are therefore subjected to LC-MS intact protein analysis.

392. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 1.

4. "c) determining the masses of the one or more VPs; and"

393. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and

the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

394. Using LC-MS/MS, Satkunanathan determined the masses of capsid proteins for each of three different AAV serotypes, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

395. Shytuhina determined the masses of two viral structural proteins containing various different PTMs using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

396. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 1.

5. "d) determining any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs that have not undergone post-translational modifications to detect a deviation in the compared masses,"

397. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

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398. Shytuhina states that "[b]ased on known glycoprotein acylation and expected N-linked glycans, we identified glycoprotein modifications in several major peaks by matching the observed mass to the theoretical molecular weight." EX1006 (Shytuhina), p. 194.

399. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 1.

6. "wherein the VPs comprise VP1, VP2 and VP3 capsid proteins, and wherein the method is performed in the absence of a gel separation step."

400. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

401. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

402. Shytuhina identified and distinguished two structural viral proteins, E1 and E2, using intact LC-MS in the absence of a gel separation step. EX1006

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(Shytuhina), pp. 193-95, Fig. 2. As discussed above, Shytuhina used RP-HPLC to separate the viral proteins, a capsid protein and two other structural proteins, before subjecting the structural proteins to intact LC-MS. EX1006 (Shytuhina), pp. 193-95, Fig. 2. No gel separation step was used at any point as part of this process.

403. Satkunanathan identified capsid proteins using LC-MS/MS for each of three different AAV serotypes. Moreover, the masses of the AAV capsid proteins (87, 73, and 62 kDa) are similar to the masses of the Chikungunya structural proteins (approximately 55 and 33 kDa) studied by Shytuhina. As a result, a POSA would have understood that it would have been straightforward to apply Shytuhina's intact LC-MS method to identify AAV capsid proteins.

404. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 1.

B. Claim 2: "The method of claim 1, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination."

405. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 2. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV

serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

406. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have understood that methionine oxidation was a post-translational modification of AAV capsid proteins. EX1022 (Dong), p. 2, Figure S1.

407. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

408. Shytuhina uses intact LC-MS to identify various post-translational modifications, including glycosylation, of viral structural proteins. EX1006 (Shytuhina), p. 194.

409. Therefore, the combination of Satkunanathan and Shytuhina meets the additional limitation of dependent claim 2.

C. Claim 3: "The method of claim 2, wherein the post-translational modification is N-terminal acetylation."

410. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 3. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to

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improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

411. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have understood that methionine oxidation was a post-translational modification of AAV capsid proteins. EX1022 (Dong), p. 2, Figure S1.

412. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

413. Shytuhina uses intact LC-MS to identify various post-translational modifications, including glycosylation, of viral structural proteins. EX1006 (Shytuhina), p. 194.

414. A POSA would have expected that, given Shytuhina's identification of post-translational modifications including various different glycosylations and acylations, Shytuhina's intact LC-MS method would identify the known post-translational modification of N-terminal acetylation of AAV capsid proteins.

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415. Therefore, the combination of Satkunanathan and Shytuhina meets the additional limitation of dependent claim 3.

D. Claim 4: "The method of claim 1, wherein the AAV particles are denatured using detergent, heat, high salt or buffer with low or high pHs."

416. Satkunanathan in combination with Shytuhina meets the additional limitation of dependent claim 4. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

417. As discussed above for claim element 1(a), Shytuhina teaches incubating the VLP samples with 5% Zwittergent 3-12 detergent as one of two denaturing steps preceding LC-MS. EX1006 (Shytuhina), pp. 193-94. Shytuhina states that pre-treatment with Zwittergent improved recovery, and that "Zwittergent 3-12 detergent is believed to solubilize and stabilize the glycoproteins [viral structural proteins] and prevent non-specific binding during chromatography." EX1006 (Shytuhina), p. 194.

418. A POSA at the time would have understood that "solubilizing" glycoproteins with the detergent Zwittergent 3-12 involves denaturing them.

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419. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 4.

E. Claim 5: "The method of claim 1, wherein the liquid chromatography is reverse phase chromatography."

420. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 5. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

421. Shytuhina's method involves separation of the viral proteins by RP-HPLC coupled to mass spectrometry. EX1006 (Shytuhina), pp. 193-195. Shytuhina used an initial RP-HPLC separation, and then a second RP-HPLC separation coupled to the mass spectrometer, with slightly modified mobile phases. EX1006 (Shytuhina), p. 193.

422. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 5.

F. Claim 6: "The method of claim 5, wherein the reverse phase chromatography is C8 reverse phase chromatography."

423. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 6. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

424. Shytuhina teaches carrying out two denaturing steps – treatment with Zwittergent and RP-HPLC – before subjecting the denatured viral particles to LC-MS intact protein analysis. EX1006 (Shytuhina), pp. 193-94.

425. For the first step, Shytuhina teaches incubating the samples with 5% Zwittergent 3-12 detergent. EX1006 (Shytuhina), pp. 193-94. Shytuhina states that pre-treatment with Zwittergent improved recovery, and that "Zwittergent 3-12 detergent is believed to solubilize and stabilize the glycoproteins [viral structural proteins] and prevent non-specific binding during chromatography." EX1006 (Shytuhina), p. 194. A POSA at the time would have understood that "solubilizing" glycoproteins involves denaturing them.

426. For the second step, Shytuhina carried out an RP-HPLC separation, before subjecting the denatured samples to LC-MS intact protein analysis, which

then involved another RP-HPLC column coupled to the mass spectrometer. EX1006 (Shytuhina), p. 193. Shytuhina states that for the initial RP-HPLC separation (before LC-MS), they used TFA in the mobile phase on the column, and that "TFA appeared to help disassemble the VLPs and facilitate the binding of proteins to the stationary phase." EX1006 (Shytuhina), p. 194.

427. Shytuhina discloses that the specific RP-HPLC column they used prior to LC-MS was a C4 column. EX1006 (Shytuhina), p. 193. Although Shytuhina does not expressly disclose the column used for the RP-HPLC coupled to the mass spectrometer (the LC-MS intact protein analysis), a POSA would have understood it was likely that they used the same C4 column that had successfully separated E1 and E2 in the prior RP-HPLC step.

428. In any event, a POSA at the time would have understood that it was generally necessary to test a few different columns to optimize the desired separation. EX1037 (Yuan), pp. 22-23.

429. As I discussed above, Yuan discloses a RP-HPLC method to detect a capsid protein of HPV, the L1 protein. EX1037 (Yuan), Abstract. Yuan tested both C4 and C8 columns, finding that either column could be used with equal efficiency to purify the L1 capsid protein. EX1037 (Yuan), p. 23.

430. Satkunanathan used a reversed phase C18 column for LC-MS/MS. EX1005 (Satkunanathan), p. 931. Mahoney used a C8 column for RP-HPLC of large

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peptides. EX1029 (Mahoney), pp. 11199-200, Table I, Table II (testing various different organic solvents including acetonitrile and 2-propanol on a C8 column, and comparing their ability to separate large denatured peptides with and without TFA).

431. A POSA would have understood that these columns, C4, C8, and C18, were commonly used columns for separation of viral structural proteins, including AAV capsid proteins. A POSA would therefore have likely tested all of them, in applying Shytuhina's intact LC-MS method to the separation and identification of AAV capsid proteins.

432. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 6.

G. Claim 7: "The method of claim 1, further comprising determining the sequence of one or more VPs that has undergone post-translational modifications."

433. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 7. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

434. A POSA would further have understood that Satkunanathan's LC-MS/MS method determined the sequences of the fragments in the mixtures of protein fragments analyzed. For example, using LC-MS/MS, Satkunanathan identified AAV capsid proteins from three different serotypes by matching the sequence of the proteins as determined by MS/MS against a database, specifically protein FASTA databases. EX1005 (Satkunanathan), p. 931, Supplementary Table S1.

435. Given that Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification, and that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2, Figure S1. And, as I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

436. As demonstrated in references such as Zabrouskov, for example, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

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437. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract.

438. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

439. The combination of Satkunanathan and Shytuhina therefore discloses the additional limitation of dependent claim 7.

H. Claim 8: "The method of claim 7, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination."

440. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 8. As I discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

441. Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification. EX1005 (Satkunanathan), p. 931. Given that Dong had

determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2, Figure S1.

442. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

443. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

444. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Shytuhina separated and characterized glycosylated forms of E1 and E2 and acylated forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

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445. Moreover, Shytuhina separated glycosylated but deacylated E1 from glycosylated and acylated E1, and characterized both forms. EX1006 (Shytuhina), pp. 194-95, Fig. 2A.

446. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize modified forms of at least some capsid proteins, such as those studied in Satkunanathan, from one another.

447. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2, including glycosylated forms of both proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

448. As further demonstrated in references such as Zabrouskov, for example, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

449. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov

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identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

450. A POSA would therefore have understood how to use Shytuhina's intact LC-MS method to identify post-translational modifications on viral structural proteins such as the AAV capsid proteins, and also how to combine this method with MS/MS, such as that used in Satkunanathan and Zabrouskov.

451. A POSA would have understood Zabrouskov's teaching that deamidation was a particularly challenging post-translational modification to characterize, given "the special challenge" that "its covalent $–NH_2 \rightarrow –OH$ modification causes an only 0.984 Da mass increase, closely matching the 1.002 Da spacing of the molecular ion isotope peaks." EX1038 (Zabrouskov), p. 987.

452. A POSA would therefore have understood that N-terminal truncation and acetylation in AAV capsid proteins would have been more easily characterized by intact LC-MS/MS than the deamidation modifications characterized by Zabrouskov.

453. Therefore, Satkunanathan, in combination with Shytuhina, meets the additional limitation of dependent claim 8.

I. Claim 9: "The method of claim 8, wherein the post-translational modification is N-terminal acetylation."

454. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 9. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

455. Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification. EX1005 (Satkunanathan), p. 931. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2, Figure S1.

456. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

457. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS, for example, Shytuhina separated and

characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Shytuhina separated and characterized glycosylated forms of E1 and E2 and acylated forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

458. Moreover, Shytuhina separated glycosylated but deacylated E1 from glycosylated and acylated E1, and characterized both forms. EX1006 (Shytuhina), pp. 194-95, Fig. 2A.

459. A POSA would further have understood, particularly in light of Shytuhina's separation and characterization of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize modified forms of at least some capsid proteins, such as those studied in Satkunanathan.

460. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2, including glycosylated forms of both proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

461. As further demonstrated in references such as Zabrouskov, for example, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the

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sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

462. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

463. A POSA would therefore have understood how to use Shytuhina's intact LC-MS method to identify post-translational modifications on viral structural proteins such as the AAV capsid proteins, and also how to combine this method with MS, such as that used in Satkunanathan and Zabrouskov.

464. A POSA would have understood Zabrouskov's teaching that deamidation was a particularly challenging post-translational modification to characterize, given "the special challenge" that "its covalent $–NH_2 \rightarrow –OH$ modification causes an only 0.984 Da mass increase, closely matching the 1.002 Da spacing of the molecular ion isotope peaks." EX1038 (Zabrouskov), p. 987.

465. A POSA would therefore have understood that the known post-translational modifications of AAV capsid proteins, N-terminal truncation and

acetylation, would have been more easily characterized by intact LC-MS/MS than the deamidation modifications characterized by Zabrouskov.

466. Therefore, Satkunanathan, in combination with Shytuhina, meets the additional limitation of dependent claim 9

J. Claim 10: "The method of claim 7, wherein the sequences of VP1, VP2 and VP3 are determined."

467. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 10. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

468. A POSA would further have understood that Satkunanathan's LC-MS/MS method determined the sequences of the fragments in the mixtures of protein fragments analyzed. For example, using LC-MS/MS, Satkunanathan identified AAV capsid proteins from three different serotypes by matching the sequence of the proteins as determined by MS against a database, specifically protein FASTA databases. EX1005 (Satkunanathan), p. 931, Supplementary Table S1.

469. Given that Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification, and that Dong had determined that AAV

capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2, Figure S1.

470. As demonstrated in references such as Zabrouskov, for example, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

471. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract.

472. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

473. The combination of Satkunanathan and Shytuhina therefore discloses the additional limitation of dependent claim 10.

K. Claim 11:

1. "A method of determining the heterogeneity of viral particles in a preparation of adeno-associated virus (AAV) particles comprising VP1, VP2 and VP3 capsid proteins, the method comprising"

474. Satkunanathan in combination with Shytuhina meets this limitation of claim 11. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

475. I note that "heterogeneity" is defined in the '313 patent as follows:

> "Heterogeneity" when used in reference to an AAV capsid refers to an AAV capsid characterized by one or more capsid polypeptides observed to deviate from a reference mass of a VP1, VP2, and/or VP3 polypeptide, or fragment thereof. A reference mass may include, without limitation, a theoretical, predicted, or expected mass of a VP1, VP2, and/or VP3 polypeptide, e.g., of a known AAV serotype. For example, an AAV capsid may be said to display heterogeneity if it demonstrates one or more of the following properties (without limitation): a mixed serotype, a variant capsid, a capsid amino acid substitution, a truncated capsid, or a modified capsid.

EX1001 ('313 patent), 19:54-65.

476. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

477. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have understood that methionine oxidation was a PTM of AAV capsid proteins.

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EX1022 (Dong), p. 2. A POSA would therefore have understood that Satkunanathan likely identified certain AAV capsid proteins carrying this post-translational modification.

478. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

479. A POSA at the time would further have understood that PTMs vary from protein molecule to protein molecule. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Shytuhina discloses that, using intact LC-MS, they identified multiple different glycosylation and acylation modifications for each of the viral structural proteins they studied. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

480. A POSA would therefore have understood that Shytuhina's intact LC-MS method, which identified heterogeneous populations of viral structural proteins, could have been applied to identify heterogeneous populations of the capsid proteins Satkunanathan studied, some of which likely carried PTMs, including N-terminal acetylation and truncation.

481. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 11.

2. "a) denaturing the AAV particles;"

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482. Satkunanathan in combination with Shytuhina meets this limitation of claim 11. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

483. Shytuhina teaches carrying out two denaturing steps – treatment with Zwittergent and RP-HPLC – before subjecting the denatured viral particles to LC-MS intact protein analysis. EX1006 (Shytuhina), pp. 193-94.

484. For the first step, Shytuhina teaches incubating the samples with 5% Zwittergent 3-12 detergent. EX1006 (Shytuhina), pp. 193-94. Shytuhina states that pre-treatment with Zwittergent improved recovery, and that "Zwittergent 3-12 detergent is believed to solubilize and stabilize the glycoproteins [viral structural proteins] and prevent non-specific binding during chromatography." EX1006 (Shytuhina), p. 194. A POSA at the time would have understood that "solubilizing" glycoproteins involves denaturing them.

485. For the second step, Shytuhina carried out an RP-HPLC separation, before subjecting the denatured samples to LC-MS, which then involved another RP-HPLC column coupled to the mass spectrometer. EX1006 (Shytuhina), p. 193.

Shytuhina states that for the initial RP-HPLC separation (before LC-MS), they used TFA in the mobile phase on the column, and that "TFA appeared to help disassemble the VLPs and facilitate the binding of proteins to the stationary phase." EX1006 (Shytuhina), p. 194.

486. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 11.

3. "b) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis, thereby separating the peaks of the VP1, VP2 and VP3 capsid proteins;"

487. Satkunanathan in combination with Shytuhina meets this limitation of claim 11. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

488. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. As discussed above regarding limitation 11(a), Shytuhina teaches an intact LC-MS method involving pre-treatment of samples with Zwittergent 3-12 detergent followed by denaturation on a RP-HPLC column with TFA as part of the mobile phase. EX1006 (Shytuhina), pp. 193-94.

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489. After the initial RP-HPLC, the denatured viral particles are then separated and analyzed by intact LC-MS using a Waters Acquity LC system coupled to a Synapt G2 mass spectrometer. The denatured viral particles are therefore subjected to LC-MS intact protein analysis.

490. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Shytuhina also separated and characterized a small amount of glycosylated but deacylated E1 protein by intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 1, Fig. 2A.

491. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

492. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have understood that methionine oxidation was a PTM of AAV capsid proteins. EX1022 (Dong), p. 2. A POSA would therefore have understood that Satkunanathan likely identified certain AAV capsid proteins carrying this post-translational modification.

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493. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

494. A POSA would have understood that the AAV capsid protein masses differ from one another by substantially more than the masses of the post-translationally modified viral structural proteins in Shytuhina differ from one another or from the unmodified proteins. A POSA would therefore have also understood that the intact LC-MS method of Shytuhina, applied to the capsid proteins identified by LC-MS/MS in Satkunanathan, would have identified the three different capsid proteins.

495. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 11.

4. "c) deconvoluting the peaks of the VP1, VP2 and VP3 capsid proteins; and"

496. Satkunanathan in combination with Shytuhina meets this limitation of claim 11. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

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497. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Shytuhina also separated and characterized a small amount of glycosylated but deacylated E1 protein by intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 1, Fig. 2A.

498. Shytuhina used software to deconvolute the MS results. EX1006 (Shytuhina), pp. 193-94. Shytuhina states:

> Data acquisition and analysis (deconvolution) were performed with Waters MassLynx 4.1 software. Protein spectra were deconvoluted to obtain the observed intact protein masses. MaxEnt deconvolution parameters were set with output mass range of 40,000-60,000 and resolution of 0.1 Da/channel. Minimum intensity ratios were 33% for both the left and right parameters. A uniform Gaussian model was used with width at half height of either 1 or 0.8 Da. For spectra with width at half height of 1 Da, a maximum of 10 iterations were used. For spectra with width at half height of 0.8 Da, a maximum of 11 iterations were used. Post-translational modifications were identified by comparing observed masses to theoretical masses of expected N-glycoforms with and without acylation.

EX1006 (Shytuhina), pp. 193-94.

499. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 11.

5. "d) determining the masses of one or more of the VP1, VP2 and VP3 capsid proteins and additional capsid proteins within one or more of the deconvoluted peaks,"

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500. Satkunanathan in combination with Shytuhina meets this limitation of claim 11. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

501. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have understood that methionine oxidation was a post-translational modification of AAV capsid proteins. EX1022 (Dong), p. 2, Figure S1.

502. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

503. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS,

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for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A.

504. A POSA would have understood that the AAV capsid protein masses differ from one another by substantially more than the masses of the post-translationally modified viral structural proteins in Shytuhina differ from one another or from the unmodified proteins. A POSA would therefore have also understood that the intact LC-MS method of Shytuhina, applied to the capsid proteins identified by LC-MS/MS in Satkunanathan, would have identified the three different capsid proteins.

505. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize varying forms of at least some capsid proteins, such as those studied in Satkunanathan, from one another.

506. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 11.

6. "wherein the method is performed in the absence of a gel separation step."

507. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 11. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on

Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

508. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

509. Shytuhina identified and distinguished two structural viral proteins, E1 and E2, using intact LC-MS in the absence of a gel separation step. EX1006 (Shytuhina), pp. 193-95, Fig. 2. As discussed above, Shytuhina used RP-HPLC to denature and separate the viral proteins, a capsid protein and two other structural proteins, before subjecting the denatured viral structural proteins to intact LC-MS. EX1006 (Shytuhina), pp. 193-95, Fig. 2. No gel separation step was used as part of this process.

510. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 11.

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L. Claim 12: "The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are variant[5] capsids."

511. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 12. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

512. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have understood that methionine oxidation was a PTM of AAV capsid proteins. EX1022 (Dong), p. 2, Figure S1. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some

[5] For my analysis of all claims of the '313 patent that involve the terms "variant" or "variants," I have used those terms consistently with Genzyme's use in its infringement contentions in the related litigation involving the '313 patent.

capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2, Figure S1.

513. As I discussed above, and as a POSA would further have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

514. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2 from one another and from unmodified forms of the proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

515. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein, in addition to identifying E1 unmodified protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

516. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had

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sufficiently high resolution to separate and characterize at least some capsid protein variants (*e.g.*, N-terminal truncated and acetylated capsid proteins) of the AAV serotypes studied in Satkunanathan, from one another.

517. Satkunanathan, in combination with Shytuhina, therefore meets this additional limitation of dependent claim 12.

M. Claim 13: "The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are capsid amino acid substitutions."

518. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 13. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

519. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

520. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral

structural proteins E1 and E2 from one another and from unmodified forms of the proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

521. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein, in addition to identifying E1 unmodified protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

522. As I discussed above, a POSA would have understood that a variety of different AAV serotypes, and versions of those serotypes with amino acid modifications to the capsid, including single amino acid substitutions and larger insertions, deletions, and truncations, had been discovered and engineered.

523. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate capsid proteins with amino acid substitutions from capsid proteins without such substitutions and to characterize those amino acid substitutions.

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524. Satkunanathan, in combination with Shytuhina, therefore meets this additional limitation of dependent claim 13.

N. Claim 14: "The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are truncated capsids."

525. Satkunanathan in combination with Shytuhina meets this limitation of dependent claim 14. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

526. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

527. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2 from one another and from unmodified forms of the proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

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528. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein, in addition to identifying E1 unmodified protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

529. As also discussed above, a POSA would have understood that a variety of different AAV serotypes, and versions of those serotypes with amino acid modifications to the capsid, including single amino acid substitutions and larger insertions, deletions, and truncations, had been discovered and engineered.

530. As I also discussed above, a POSA at the time would have further understood that AAV capsid proteins are post-translationally modified by N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920. Becerra states that after truncation of the N-terminal methionine, the following alanine becomes acetylated *in vivo*. EX1011 (Becerra), p. 7920.

531. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize varying forms of at least

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some capsid proteins, such as those studied in Satkunanathan, from one another, including AAVs with truncated capsid proteins.

532. A POSA would have been motivated to use Shytuhina's intact LC-MS method to monitor AAV composition for process development, including identifying truncated capsids.

533. Satkunanathan, in combination with Shytuhina, therefore meets this additional limitation of dependent claim 14.

O. Claim 15: "The method of claim 11, wherein the additional capsid proteins within one or more of the deconvoluted peaks are modified capsids."

534. Satkunanathan in combination with Shytuhina meets this limitation of dependent claim 15. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

535. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. Satkunanathan uses LC-MS/MS with methionine oxidation set as a dynamic modification. EX1005 (Satkunanathan), p. 931. A POSA at the time would have

understood that methionine oxidation was a PTM of AAV capsid proteins. EX1022 (Dong), p. 2, Figure S1. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2, Figure S1.

536. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

537. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein, in addition to identifying E1 unmodified protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

538. As I also discussed above, a POSA at the time would have further understood that AAV capsid proteins are post-translationally modified by N-terminal acetylation *in vivo*. EX1011 (Becerra), p. 7920. Becerra states that after

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truncation of the N-terminal methionine, the following alanine becomes acetylated *in vivo*. EX1011 (Becerra), p. 7920.

539. A POSA would have expected that, given Shytuhina's identification of post-translational modifications, Shytuhina's intact LC-MS method would identify the known post-translational N-terminal truncation and acetylation of AAV capsid proteins.

540. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to and characterize modified forms of at least some capsid proteins (e.g., N-terminally truncated and acetylated capsid proteins), such as those studied in Satkunanathan, from one another.

541. A POSA would have been motivated to use Shytuhina's intact LC-MS method to monitor AAV composition for process development, including identifying modified capsids.

542. Satkunanathan, in combination with Shytuhina, therefore meets this additional limitation of dependent claim 15.

P. Claim 16: "The method of claim 15, wherein the modifications of the modified capsids are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination."

543. Satkunanathan in combination with Shytuhina meets this limitation of dependent claim 16. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

544. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

545. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

546. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein, in addition to identifying E1 unmodified protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and

identifying within the deconvoluted peaks modified forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

547. As I also discussed above, a POSA at the time would have further understood that AAV capsid proteins are post-translationally modified by N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920. Becerra states that after truncation of the N-terminal methionine, the following alanine becomes acetylated *in vivo*. EX1011 (Becerra), p. 7920.

548. A POSA would have expected that, given Shytuhina's identification of post-translational modifications, Shytuhina's intact LC-MS method would identify the known post-translational N-terminal truncation and acetylation of AAV capsid proteins.

549. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate modified forms of at least some capsid proteins (e.g., N-terminally truncated and acetylated capsid proteins), such as those studied in Satkunanathan, from one another.

550. A POSA would have been motivated to use Shytuhina's intact LC-MS method to monitor AAV composition for process development, including

identifying modified capsids with modification such as glycosylation and N-terminal acetylation.

551. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of dependent claim 16.

Q. Claim 17: "The method of claim 16, wherein the modification is N-terminal acetylation."

552. Satkunanathan in combination with Shytuhina meets this limitation of dependent claim 17. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

553. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

554. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

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555. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein, in addition to identifying E1 unmodified protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

556. As I also discussed above, a POSA at the time would have further understood that AAV capsid proteins are post-translationally modified by N-terminal acetylation *in vivo*. EX1011 (Becerra), p. 7920. Becerra states that after truncation of the N-terminal methionine, the following alanine becomes acetylated *in vivo*. EX1011 (Becerra), p. 7920.

557. A POSA would have expected that, given Shytuhina's identification of post-translational modifications, Shytuhina's intact LC-MS method would identify the known post-translational N-terminal truncation and acetylation of AAV capsid proteins.

558. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize modified forms of at least

some capsid proteins (e.g., N-terminally truncated and acetylated capsid proteins), such as those studied in Satkunanathan, from one another.

559. A POSA would have been motivated to use Shytuhina's intact LC-MS method to monitor AAV composition for process development, including identifying modified capsids with modification such as glycosylation and N-terminal acetylation.

560. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of dependent claim 17.

R. Claim 18: "The method of claim 11, wherein the liquid chromatography is reverse phase chromatography."

561. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 18. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

562. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis. EX1006 (Shytuhina), p. 193. For the RP-HPLC

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coupled to the mass spectrometer, they slightly modified the mobile phases, relative to those used in their earlier RP-HPLC separation, by substituting formic acid for TFA, because TFA led to ion suppression. EX1006 (Shytuhina), p. 193.

563. For this second RP-HPLC coupled to the mass spectrometer, Mobile phase A was 0.1% formic acid (v/v) in water, and Mobile phase B was 0.1% formic acid (v/v) in 30% ACN/70% isopropanol.

564. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 18.

S. Claim 19: "The method of claim 18, wherein the reverse phase chromatography is C8 reverse phase chromatography."

565. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 19. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

566. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis.

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567. Shytuhina discloses that the specific RP-HPLC column they used for the first RP-HPLC step was a C4 column. EX1006 (Shytuhina), p. 193. Although Shytuhina does not expressly disclose the column used for the second RP-HPLC, coupled to the mass spectrometer, a POSA would have understood it was likely that they used the same C4 column that had successfully separated E1 and E2 in the prior RP-HPLC step.

568. In any event, a POSA at the time would have understood that it was generally necessary to test a few different columns to optimize the desired separation. EX1037 (Yuan), pp. 22-23.

569. As I discussed above, Yuan discloses a RP-HPLC method to detect a capsid protein of HPV, the L1 protein. EX1037 (Yuan), Abstract. Yuan tested both C4 and C8 columns, finding that either column could be used with equal efficiency to purify the L1 capsid protein. EX1037 (Yuan), p. 23.

570. Satkunanathan used a reversed phase C18 column for LC-MS/MS. EX1005 (Satkunanathan), p. 931. Mahoney used a C8 column for RP-HPLC of large peptides. EX1029 (Mahoney), pp. 11199-200, Table I, Table II (testing various different organic solvents including acetonitrile and 2-propanol on a C8 column, and comparing their ability to separate large denatured peptides with and without TFA).

571. A POSA would have understood that these columns, C4, C8, and C18, were commonly used columns for separation of viral structural proteins, including

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AAV capsid proteins. A POSA would therefore have likely tested all of them, in applying Shytuhina's intact LC-MS method to the separation and identification of AAV capsid proteins.

572. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 19.

T. Claim 20

1. "A method of preparing a pharmaceutical composition of adeno-associated virus (AAV) particles, the method comprising:"

573. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

574. Satkunanathan is expressly directed towards enhanced production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract.

575. Satkunanathan states: "The significance of our findings should be considered in the context of future development of production methods for AAV-based gene therapy products." EX1005 (Satkunanathan), p. 930.

576. A POSA at the time would therefore have understood Satkunanathan to be directed towards methods of preparing a pharmaceutical composition of AAV particles.

577. Shytuhina is similarly expressly directed towards an analytical method for monitoring viral particles for process development of pharmaceuticals, including vaccines. EX1006 (Shytuhina), Abstract.

578. Shytuhina states:

> We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. . . . This method can be applied as a release test for dose of CHIKV VLP vaccine product. Because this method provides good separation, we are able to characterize the post-translational modifications of the two viral glycoproteins. ***We demonstrated that this RP-HPLC method could support process development by monitoring product purity, and support formulation development by monitoring the product protein degradation***.

EX1006 (Shytuhina), p. 197 (emphasis added).

579. Because Shytuhina discloses a method to support both process and formulation development, a POSA would therefore have understood Shytuhina to disclose a method for preparing a pharmaceutical composition.

580. As discussed above, a POSA at the time would have applied Shytuhina's method to process and formulation development of AAV vectors for gene therapy.

581. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 20.

2. "monitoring AAV particles for consistency and/or identity;"

582. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

583. Satkunanathan is expressly directed towards enhanced production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract.

584. Satkunanathan states: "The significance of our findings should be considered in the context of future development of production methods for AAV-based gene therapy products." EX1005 (Satkunanathan), p. 930.

585. A POSA at the time would therefore have understood Satkunanathan to be directed towards methods of preparing a pharmaceutical composition of AAV particles.

586. Shytuhina is similarly expressly directed towards an analytical method for monitoring viral particles for process development of pharmaceuticals, including vaccines. EX1006 (Shytuhina), Abstract.

587. Shytuhina states:

> We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. . . . This method can be applied as a release test for dose of CHIKV VLP vaccine product. Because this method provides good separation, we are able to characterize the post-translational modifications of the two viral glycoproteins. ***We demonstrated that this RP-HPLC method could support process development by monitoring product purity, and support formulation development by monitoring the product protein degradation***.

EX1006 (Shytuhina), p. 197 (emphasis added).

588. Because Shytuhina discloses a method to support both process and formulation development, a POSA would therefore have understood Shytuhina to disclose a method for preparing a pharmaceutical composition.

589. A POSA would have further understood that applying Shytuhina's method, as discussed above, for monitoring product degradation, monitors the viral particles for consistency and identity. EX1006 (Shytuhina), p. 197.

590. As discussed above, a POSA at the time would have applied Shytuhina's method to process and formulation development of AAV vectors for gene therapy.

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591. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 20.

3. "wherein the AAV particles comprise viral proteins (VPs) comprising VP1, VP2 and VP3 capsid proteins of an AAV particle capsid,"

592. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 1. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

593. Shytuhina identified and distinguished two structural viral proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 193-95, Fig. 2. As discussed above, Shytuhina used RP-HPLC to separate the viral proteins, a capsid protein and two other structural proteins, before subjecting the structural proteins to intact LC-MS. EX1006 (Shytuhina), pp. 193-95, Fig. 2.

594. Satkunanathan identified capsid proteins using LC-MS/MS for each of three different AAV serotypes, AAV2, AAV5, and AAV8. Moreover, the masses of the AAV capsid proteins (87, 73, and 62 kDa) are similar to the masses of the Chikungunya structural proteins (approximately 55 and 33 kDa) studied by

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Shytuhina. As a result, a POSA would have understood that it would have been straightforward to apply Shytuhina's intact LC-MS method to identify AAV capsid proteins.

595. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 20.

4. "wherein the AAV particle is monitored for consistency and/or identity by:"

596. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes.

597. Satkunanathan is expressly directed towards enhanced production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract.

598. Satkunanathan states: "The significance of our findings should be considered in the context of future development of production methods for AAV-based gene therapy products." EX1005 (Satkunanathan), p. 930.

599. A POSA at the time would therefore have understood Satkunanathan to be directed towards methods of preparing a pharmaceutical composition of AAV particles.

600. Shytuhina is similarly expressly directed towards an analytical method for monitoring viral particles for process development of pharmaceuticals, including vaccines. EX1006 (Shytuhina), Abstract.

601. Shytuhina states:

> We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. . . . This method can be applied as a release test for dose of CHIKV VLP vaccine product. Because this method provides good separation, we are able to characterize the post-translational modifications of the two viral glycoproteins. ***We demonstrated that this RP-HPLC method could support process development by monitoring product purity, and support formulation development by monitoring the product protein degradation***.

EX1006 (Shytuhina), p. 197 (emphasis added).

602. Because Shytuhina discloses a method to support both process and formulation development, a POSA would therefore have understood Shytuhina to disclose a method for preparing a pharmaceutical composition.

603. A POSA would have further understood that applying Shytuhina's method, as discussed above, for monitoring product degradation, monitors the viral particles for consistency and identity. EX1006 (Shytuhina), p. 197.

604. Moreover, Shytuhina states expressly that it is important to monitor PTMs "to ensure product lot-to-lot consistency." EX1006 (Shytuhina), p. 197.

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605. As discussed above, a POSA at the time would have applied Shytuhina's method to process and formulation development of AAV vectors for gene therapy.

606. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 20.

5. "a) extracting an aliquot of an AAV particle preparation;"

607. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

608. Satkunanathan discloses that the samples of viral particles subjected to LC-MS/MS analysis were first "extracted." EX1005 (Satkunanathan), p. 931. A POSA would have understood that the samples had been obtained by removing an aliquot or aliquots from Satkunanathan's purified preparation. EX1005 (Satkunanathan), pp. 930-31.

609. Shytuhina discloses that they prepared VLP samples from mammalian and insect cells:

> Briefly, HEK293 cells were transiently transfected with a plasmid DNA encoding the CHIKV structural genes. The cell culture supernatant was clarified, concentrated and purified with Q Sepharose XL anion exchange column. In the insect cell system, the high-pH adapted *Spodoptera frugiperda* insect cells (*Sf*Basic) were infected by baculovirus encoding the CHIKV structural genes. The culture supernatant was clarified, concentrated and purified with Sephacryl S-400 HR size exclusion column and Q-Sepharose XL anion exchange column.

EX1006 (Shytuhina), p. 193.

610. Shytuhina further discloses that after these purifications, "[s]amples containing CHIKV VLPs" were analyzed on the initial RP-HPLC column, before being subected to LC-MS. EX1006 (Shytuhina), p. 193.

611. A POSA would have understood that obtaining these "samples" for analysis would have involved removing or extracting an aliquot from the VLP preparations.

612. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 20.

6. "b) denaturing the AAV particles;"

613. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and

the fact that the AAV capsid proteins were known to be subject to post-translational modification.

614. Shytuhina teaches carrying out two denaturing steps – treatment with Zwittergent and RP-HPLC – before subjecting the denatured viral particles to LC-MS intact protein analysis. EX1006 (Shytuhina), pp. 193-94.

615. For the first step, Shytuhina teaches incubating the samples with 5% Zwittergent 3-12 detergent. EX1006 (Shytuhina), pp. 193-94. Shytuhina states that pre-treatment with Zwittergent improved recovery, and that "Zwittergent 3-12 detergent is believed to solubilize and stabilize the glycoproteins [viral structural proteins] and prevent non-specific binding during chromatography." EX1006 (Shytuhina), p. 194. A POSA at the time would have understood that "solubilizing" glycoproteins involves denaturing them.

616. For the second step, Shytuhina carried out an RP-HPLC separation, before subjecting the denatured samples to LC-MS, which then involved another RP-HPLC column coupled to the mass spectrometer. EX1006 (Shytuhina), p. 193. Shytuhina states that for the initial RP-HPLC separation (before LC-MS), they used TFA in the mobile phase on the column, and that "TFA appeared to help disassemble the VLPs and facilitate the binding of proteins to the stationary phase." EX1006 (Shytuhina), p. 194.

617. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 20.

7. "c) subjecting the denatured AAV particles to liquid chromatography/mass spectrometry (LC/MS) intact protein analysis;"

618. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

619. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. As discussed above regarding limitation 20(b), Shytuhina's intact LC-MS method involves pre-treatment of samples with Zwittergent 3-12 detergent followed by denaturation on a RP-HPLC column with TFA as part of the mobile phase. EX1006 (Shytuhina), pp. 193-94.

620. After the initial RP-HPLC, the denatured viral particles are then separated and analyzed by intact LC-MS using a Waters Acquity LC system coupled to a Synapt G2 mass spectrometer. The denatured viral particles are therefore subjected to LC-MS intact protein analysis.

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621. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 20.

8. "d) determining the masses of one or more VPs of the AAV particles; and"

622. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes.

623. Using LC-MS/MS, Satkunanathan determined the masses of capsid proteins for each of three different AAV serotypes, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

624. Using intact LC-MS, Shytuhina determined the masses of, and separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

625. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 20.

9. "e) comparing the determined masses of the one or more VPs to theoretical masses of corresponding VPs, wherein the theoretical masses of corresponding VPs are those VPs of known AAV serotypes and/or those that have not undergone undesired post-translational modifications; and"

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626. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

627. Using LC-MS/MS, Satkunanathan determined the masses of capsid proteins for each of three different AAV serotypes, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

628. Using intact LC-MS, Shytuhina determined the masses of, and separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

629. Shytuhina states that "[b]ased on known glycoprotein acylation and expected N-linked glycans, we identified glycoprotein modifications in several major peaks by matching the observed mass to the theoretical molecular weight." EX1006 (Shytuhina), p. 194.

630. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 20.

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10. "f) determining if there is any deviation of the determined masses of the one or more VPs from the theoretical masses of the corresponding VPs;"

631. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

632. Using LC-MS/MS, Satkunanathan determined the masses of capsid proteins for each of three different AAV serotypes, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1.

633. Using intact LC-MS, Shytuhina determined the masses of, and separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

634. Shytuhina states that "[b]ased on known glycoprotein acylation and expected N-linked glycans, we identified glycoprotein modifications in several major peaks by matching the observed mass to the theoretical molecular weight." EX1006 (Shytuhina), p. 194.

635. Satkunanathan, in combination with Shytuhina, thus meets this limitation of claim 20.

11. "wherein the determination of any deviation of the determined masses of the one or more VPs from the theoretical masses of corresponding VPs thereby monitors the AAV particles for consistency and/or identity;"

636. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

637. Satkunanathan is expressly directed towards enhanced production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract.

638. Satkunanathan states: "The significance of our findings should be considered in the context of future development of production methods for AAV-based gene therapy products." EX1005 (Satkunanathan), p. 930.

639. A POSA at the time would therefore have understood Satkunanathan to be directed towards methods of preparing a pharmaceutical composition of AAV particles.

640. Shytuhina is similarly expressly directed towards an analytical method for monitoring viral particles for process development of pharmaceuticals, including vaccines. EX1006 (Shytuhina), Abstract.

641. Shytuhina states:

> We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. . . . This method can be applied as a release test for dose of CHIKV VLP vaccine product. Because this method provides good separation, we are able to characterize the post-translational modifications of the two viral glycoproteins. ***We demonstrated that this RP-HPLC method could support process development by monitoring product purity, and support formulation development by monitoring the product protein degradation***.

EX1006 (Shytuhina), p. 197 (emphasis added).

642. Because Shytuhina discloses a method to support both process and formulation development, a POSA would have understood Shytuhina to disclose a method for preparing a pharmaceutical composition.

643. A POSA would have further understood that applying Shytuhina's method, as discussed above, for monitoring product degradation, monitors the viral particles for consistency and identity. EX1006 (Shytuhina), p. 197.

644. Moreover, Shytuhina states expressly that it is important to monitor PTMs "to ensure product lot-to-lot consistency." EX1006 (Shytuhina), p. 197.

645. Shytuhina expressly discloses, as discussed above, that they compared the masses they determined through intact LC-MS with known theoretical masses

for the E1 and E2 viral proteins, and, through that comparison, identified PTMs. EX1006 (Shytuhina), p. 194.

646. As discussed above, a POSA at the time would have applied Shytuhina's method to process and formulation development of AAV vectors for gene therapy.

647. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 20.

12. "wherein the monitoring for consistency and/or identity is performed in the absence of a gel separation step; and"

648. Satkunanathan, in combination with Shytuhina, meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

649. Satkunanathan is expressly directed towards enhanced production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract.

650. Satkunanathan states: "The significance of our findings should be considered in the context of future development of production methods for AAV-based gene therapy products." EX1005 (Satkunanathan), p. 930.

651. A POSA at the time would therefore have understood Satkunanathan to be directed towards methods of preparing a pharmaceutical composition of AAV particles.

652. Shytuhina is similarly expressly directed towards an analytical method for monitoring viral particles for process development of pharmaceuticals, including vaccines. EX1006 (Shytuhina), Abstract.

653. Shytuhina states:

> We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. . . . This method can be applied as a release test for dose of CHIKV VLP vaccine product. Because this method provides good separation, we are able to characterize the post-translational modifications of the two viral glycoproteins. ***We demonstrated that this RP-HPLC method could support process development by monitoring product purity, and support formulation development by monitoring the product protein degradation***.

EX1006 (Shytuhina), p. 197 (emphasis added).

654. Because Shytuhina discloses a method to support both process and formulation development, a POSA would have understood Shytuhina to disclose a method for preparing a pharmaceutical composition.

655. A POSA would have further understood that applying Shytuhina's method, as discussed above, for monitoring product degradation, monitors the viral particles for consistency and identity. EX1006 (Shytuhina), p. 197.

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656. Moreover, Shytuhina states expressly that it is important to monitor PTMs "to ensure product lot-to-lot consistency." EX1006 (Shytuhina), p. 197.

657. Shytuhina expressly discloses, as discussed above, that they compared the masses they determined through intact LC-MS with known theoretical masses for the E1 and E2 viral proteins, and, through that comparison, identified PTMs. EX1006 (Shytuhina), p. 194.

658. As discussed above, a POSA at the time would have applied Shytuhina's method to process and formulation development of AAV vectors for gene therapy.

659. Shytuhina's method for using LC-MS intact protein analysis to monitor viral structural proteins for consistency and/or identity was performed in the absence of a gel separation step – no gel separation step was used at any point in the process.

660. Satkunanathan, in combination with Shytuhina, therefore meets this limitation of claim 20.

13. "wherein if less than an undesirable amount of deviation is determined during the monitoring for consistency and/or identity, the AAV particles are combined with one or more pharmaceutically acceptable excipients to form the pharmaceutical composition."

661. Satkunanathan in combination with Shytuhina meets this limitation of claim 20. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on

Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

662. Satkunanathan is expressly directed towards enhanced production of AAV vectors for gene therapy. EX1005 (Satkunanathan), Abstract.

663. Satkunanathan states: "The significance of our findings should be considered in the context of future development of production methods for AAV-based gene therapy products." EX1005 (Satkunanathan), p. 930.

664. A POSA at the time would therefore have understood Satkunanathan to be directed towards methods of preparing a pharmaceutical composition of AAV particles.

665. Shytuhina is similarly expressly directed towards an analytical method for monitoring viral particles for process development of pharmaceuticals, including vaccines. EX1006 (Shytuhina), Abstract.

666. Shytuhina states:

> We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. . . . This method can be applied as a release test for dose of CHIKV VLP vaccine product. Because this method provides good separation, we are able to characterize the post-translational modifications of the two viral glycoproteins. ***We demonstrated that this RP-HPLC method could support process development by***

monitoring product purity, and support formulation development by monitoring the product protein degradation.

EX1006 (Shytuhina), p. 197 (emphasis added).

667. As discussed above, a POSA at the time would have applied Shytuhina's method to process and formulation development of AAV vectors for gene therapy.

668. Moreover, Shytuhina states expressly that it is important to monitor PTMs "to ensure product lot-to-lot consistency." EX1006 (Shytuhina), p. 197.

669. A POSA would have understood that each lot of viral particles monitored in this fashion would be evaluated to determine whether the purity was sufficient to use the viral particle preparation as part of the pharmaceutical preparation.

670. A POSA would further have understood that this process of monitoring PTMs to ensure product lot-to-lot consistency would involve evaluating the amount of deviation from the theoretical masses of the viral proteins to determine whether the amount of deviation was less than a particular threshold deemed undesirable.

671. A POSA would further have understood that if the amount of deviation from the theoretical viral protein masses was below this threshold, then the preparation of viral particles would continue to move through the process to be combined with pharmaceutically acceptable excipients to form the pharmaceutical composition.

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672. Therefore, the combination of Satkunanathan and Shytuhina meets this limitation of claim 20.

U. Claim 21: "The method of claim 20, wherein the monitoring of the AAV particles for consistency and/or identity includes determining the serotype of the AAV particles based on the comparison of the determined masses of the VPs to the theoretical masses of the corresponding VPs."

673. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 21. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

674. Using LC-MS/MS, Satkunanathan identified capsid proteins from each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. Satkunanathan, moreover, taught the importance of differences among the serotypes for development of therapies based on AAV vectors. EX1005 (Satkunanathan), p. 930 ("We reason that by analyzing host cellular proteins coproduced and copurified with AAV vectors, we may identify differences in protein composition among the three AAV serotypes AAV2, AAV5,

and AAV8; better understand the role of cellular proteins in AAV assembly; and ultimately improve AAV vector production").

675. Shytuhina discloses LC-MS intact protein analysis of viral structural proteins. EX1006 (Shytuhina), pp. 193-94. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

676. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A.

677. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to characterize AAV capsid proteins from at least some different serotypes, such as those studied in Satkunanathan (AAV2, AAV5, and AAV8).

678. A POSA would further have understood that numerous AAV serotypes had been discovered, with varying degrees of homology to known serotypes and to one another. *See, e.g.*, EX1017 (Chen), Abstract, pp. 14783-87, Fig. 2, Fig. 3, Fig. 4. A POSA would have been motivated to use Shytuhina's intact LC-MS method to

monitor AAV composition for process development, including characterizing different AAV serotypes.

679. Satkunanathan, in combination with Shytuhina, therefore meets this additional limitation of dependent claim 21.

V. Claim 22: "The method of claim 20, wherein a determination of any actual deviation in masses reflects heterogeneity in the AAV particle preparation."

680. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 22. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

681. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

682. A POSA at the time would have further understood that PTMs vary from protein molecule to protein molecule. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Shytuhina discloses that, using intact LC-MS, they characterized different

glycosylation and acylation modifications for each of the viral structural proteins they studied. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

683. A POSA would therefore have understood that Shytuhina's intact LC-MS method, which identified heterogeneous populations of viral structural proteins, could be applied to identify heterogeneous populations of the capsid proteins Satkunanathan studied, some of which likely carried PTMs. A POSA would further have understood that different PTMs in the capsid proteins of each different AAV serotype would have led to heterogeneity within that population of particles.

684. Therefore, the combination of Satkunanathan and Shytuhina meets this additional limitation of claim dependent 22.

W. Claim 23: "The method of claim 22, wherein the heterogeneity in the AAV particle preparation is due to mixed AAV capsid serotypes, variant AAV capsid proteins, AAV capsid protein amino acid substitutions, truncated AAV capsid proteins or modified AAV capsid proteins."

685. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 23. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

686. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

687. A POSA at the time would have further understood that PTMs vary from protein molecule to protein molecule. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Shytuhina discloses that, using intact LC-MS, they characterized different glycosylation and acylation modifications for each of the viral structural proteins they studied. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

688. A POSA would therefore have understood that Shytuhina's intact LC-MS method, which identified heterogeneous populations of viral structural proteins, could be applied to identify heterogeneous populations of the capsid proteins Satkunanathan studied, some of which likely carried PTMs. A POSA would further have understood that different PTMs in the capsid proteins of each different AAV serotype would have led to heterogeneity within that population of particles.

689. Therefore, the combination of Satkunanathan and Shytuhina meets this additional limitation of dependent claim 23.

X. Claim 24: "The method of claim 21, wherein the undesired post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination."

690. Satkunanathan in combination with Shytuhina meets this additional limitation of dependent claim 24. As discussed above, a POSA would have been

motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

691. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

692. A POSA at the time would have further understood that PTMs vary from protein molecule to protein molecule. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Shytuhina discloses that, using intact LC-MS, they characterized different glycosylation and acylation modifications for each of the viral structural proteins they studied. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

693. A POSA would therefore have understood that Shytuhina's intact LC-MS method, which identified heterogeneous populations of viral structural proteins, could be applied to identify heterogeneous populations of the capsid proteins Satkunanathan studied, some of which likely carried PTMs. A POSA would further have understood that different PTMs in the capsid proteins of each different AAV serotype would have led to heterogeneity within that population of particles.

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694. Satkunanathan, in combination with Shytuhina, therefore meets this additional limitation of dependent claim 24.

Y. Claim 25: "The method of claim 21, wherein the AAV particles are denatured using detergent, heat, high salt or buffer with low or high pHs."

695. Satkunanathan in combination with Shytuhina meets the additional limitation of dependent claim 25. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

696. Shytuhina teaches incubating the VLP samples with 5% Zwittergent 3-12 detergent as one of two denaturing steps preceding LC-MS. EX1006 (Shytuhina), pp. 193-94. Shytuhina states that pre-treatment with Zwittergent improved recovery, and that "Zwittergent 3-12 detergent is believed to solubilize and stabilize the glycoproteins [viral structural proteins] and prevent non-specific binding during chromatography." EX1006 (Shytuhina), p. 194.

697. A POSA at the time would have understood that "solubilizing" glycoproteins with the detergent Zwittergent 3-12 involves denaturing them.

698. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 25.

Z. Claim 26: "The method of claim 21, wherein the liquid chromatography is reverse phase chromatography."

699. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 26. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

700. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis. EX1006 (Shytuhina), p. 193. For the RP-HPLC coupled to the mass spectrometer, they slightly modified the mobile phases, relative to those used in their earlier RP-HPLC separation, by substituting formic acid for TFA, because TFA led to ion suppression. EX1006 (Shytuhina), p. 193.

701. For this second RP-HPLC coupled to the mass spectrometer, Mobile phase A was 0.1% formic acid (v/v) in water, and Mobile phase B was 0.1% formic acid (v/v) in 30% ACN/70% isopropanol.

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702. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 26.

AA. Claim 27: "The method of claim 26, wherein the reverse phase chromatography is C8 reverse phase chromatography."

703. The combination of Satkunanathan and Shytuhina discloses the additional limitation of dependent claim 27. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

704. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis.

705. Shytuhina discloses that the specific RP-HPLC column they used for the first RP-HPLC step was a C4 column. EX1006 (Shytuhina), p. 193. Although Shytuhina does not expressly disclose the column used for the second RP-HPLC, coupled to the mass spectrometer, a POSA would have understood it was likely that they used the same C4 column that had successfully separated E1 and E2 in the prior RP-HPLC step.

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706. In any event, a POSA at the time would have understood that it was generally necessary to test a few different columns to optimize the desired separation. EX1037 (Yuan), pp. 22-23.

707. As I discussed above, Yuan discloses a RP-HPLC method to detect a capsid protein of HPV, the L1 protein. EX1037 (Yuan), Abstract. Yuan tested both C4 and C8 columns, finding that either column could be used with equal efficiency to purify the L1 capsid protein. EX1037 (Yuan), p. 23.

708. Satkunanathan used a reversed phase C18 column for LC-MS/MS. EX1005 (Satkunanathan), p. 931. Mahoney used a C8 column for RP-HPLC of large peptides. EX1029 (Mahoney), pp. 11199-200, Table I, Table II (testing various different organic solvents including acetonitrile and 2-propanol on a C8 column, and comparing their ability to separate large denatured peptides with and without TFA).

709. A POSA would have understood that these columns, C4, C8, and C18, were commonly used columns for separation of viral structural proteins, including AAV capsid proteins. A POSA would therefore have likely tested all of them, in applying Shytuhina's intact LC-MS method to the separation and identification of AAV capsid proteins.

710. Satkunanathan, in combination with Shytuhina, therefore meets the additional limitation of dependent claim 27.

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BB. A POSA Would Have Had a Reasonable Expectation of Success in Making the Claimed Combination

711. A POSA would have had a reasonable expectation of success in combining Satkunanathan with Shytuhina. The techniques required to make the claimed combination, namely, RP-HPLC, intact LC-MS, intact LC-MS/MS, and application of software to deconvolute and interpret MS data, were well known to people of ordinary skill in the art at the time and would have required nothing more than routine experimentation.

712. Shytuhina successfully separated glycoproteins by liquid chromatography, specifically RP-HPLC, and analyzed them by intact LC-MS, successfully identifying several different post-translational modifications.

713. I note that intact LC-MS analysis of AAV capsid proteins would have been even more straightforward for a POSA than working with the glycoproteins Shytuhina analyzed. Glycoproteins are more variable and therefore more difficult to analyze than AAV capsid proteins by intact LC-MS.

714. A POSA furthermore, using nothing more than routine experimentation, would have been able to select the best column, C4 or C8, for example, to optimize the separation by RP-HPLC, and then the analysis by MS.

715. Moreover, a POSA would have a reasonable chance of success in separating AAV capsid proteins and characterizing them, including any PTMs, by intact LC-MS.

716. Satkunanathan successfully identified and distinguished three different AAV serotypes, and Shytuhina successfully separated and characterized viral structural glycoproteins with multiple different PTMs.

717. Moreover, researchers in the field for years before the relevant date, as I discussed above, had been successfully separating reduced monoclonal antibodies and identifying PTMs using intact LC-MS. Applying intact LC-MS to AAV capsid proteins at the relevant date would have been considerably more routine and straightforward than applying the technique to monoclonal antibodies.

718. Researchers in the field foryears before the relevant date, as I also discussed above, had also been using intact LC-MS/MS to characterize PTMs such as deamidation, obtaining sequencing information as part of the analysis.

CC. Secondary Considerations Do Not Change the Conclusion of Obviousness

719. For evidence of "secondary considerations" to be informative of obviousness, I understand that there must be a "nexus" or link between the alleged secondary consideration and the subject matter recited in the Asserted Claims. I am not aware of any secondary considerations of non-obviousness with the required nexus to the claims of the '313 patent. For example, I am not aware of any commercial success attributable to an analytical method of monitoring an AAV

preparation using the well known technique of intact LC-MS.[6] Similarly, I am not aware of any licenses directed specifically to the '313 patent or the subject matter recited in challenged claims 1-27.

720. Finally, I am not aware of any unexpected results having a nexus to the claimed subject matter. The '313 patent does not disclose unexpected properties of the claimed formulation. As I have discussed, intact LC-MS had been used successfully for years before the relevant date as an analytical technique to monitor proteins, including monoclonal antibodies, and viral structural proteins, for process development. The use of LC-MS/MS to identify the capsid proteins of different AAV serotypes had been disclosed in Satkunanathan, and the use of intact LC-MS to monitor viral structural proteins, including characterizing their PTMs, for process development had been disclosed in Shytuhina.

721. To the extent Patent Owner attempts to raise secondary considerations that have only a marginal nexus, if any, to claims 1-27 of the '313 patent, such

[6] If Patent Owner attempts to rely on the commercial success of Sarepta's gene therapy treatment for Duchenne muscular dystrophy – Elevidys® – there is no nexus to the challenged claims of the '313 patent. There is no nexus between the commercial success of Elevidys® and the analytical method recited in the challenged claims.

evidence of secondary considerations should not outweigh the compelling evidence of obviousness, discussed above. Thus, secondary considerations do not alter my opinion that claims 1-27 of the '313 patent are obvious over the combination of Satkunanathan and Shytuhina.

XI. GROUND 2: CLAIMS 6, 19, AND 27 ARE OBVIOUS OVER THE COMBINATION OF SATKUNANATHAN, SHYTUHINA, AND YUAN

722. In my opinion, dependent claims 6, 19, and 27 are also obvious over the combination of Satkunanathan, Shytuhina, and Yuan. In addition to setting out my opinions below, I refer back to my discussion of Ground 1 in its entirety.

A. Claim 6: "The method of claim 5, wherein the reverse phase chromatography is C8 reverse phase chromatography."

723. In my opinion, the combination of Satkunanathan, Shytuhina, and Yuan discloses the additional limitation of dependent claim 6.

724. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

725. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the

mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis.

726. Shytuhina discloses that the specific RP-HPLC column they used for the first RP-HPLC step was a C4 column. EX1006 (Shytuhina), p. 193. Although Shytuhina does not expressly disclose the column used for the second RP-HPLC, coupled to the mass spectrometer, a POSA would have understood it was likely that they used the same C4 column that had successfully separated E1 and E2 in the prior RP-HPLC step.

727. In any event, a POSA at the time would have understood that it was generally necessary to test a few different columns to optimize the desired separation. EX1037 (Yuan), pp. 22-23.

728. As I discussed above, Yuan discloses a RP-HPLC method to detect a capsid protein of HPV, the L1 protein. EX1037 (Yuan), Abstract. Yuan tested both C4 and C8 columns, finding that either column could be used with equal efficiency to purify the L1 capsid protein. EX1037 (Yuan), p. 23.

729. Satkunanathan used a reversed phase C18 column for LC-MS/MS. EX1005 (Satkunanathan), p. 931.

730. A POSA would have understood that these columns, C4, C8, and C18, were commonly used columns for separation of viral structural proteins, including AAV capsid proteins. A POSA would therefore have likely tested all of them, in

applying Shytuhina's intact LC-MS method to the separation and identification of AAV capsid proteins, in combination with Yuan's method of testing columns such as C4 and C8, to optimize the LC-MS separation and protein identification.

731. Satkunanathan, in combination with Shytuhina and Yuan, therefore meets the additional limitation of dependent claim 6.

B. Claim 19: "The method of claim 18, wherein the reverse phase chromatography is C8 reverse phase chromatography."

732. In my opinion, the combination of Satkunanathan, Shytuhina, and Yuan discloses the additional limitation of dependent claim 19.

733. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

734. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis.

735. Shytuhina discloses that the specific RP-HPLC column they used for the first RP-HPLC step was a C4 column. EX1006 (Shytuhina), p. 193. Although

Shytuhina does not expressly disclose the column used for the second RP-HPLC, coupled to the mass spectrometer, a POSA would have understood it was likely that they used the same C4 column that had successfully separated E1 and E2 in the prior RP-HPLC step.

736. In any event, a POSA at the time would have understood that it was generally necessary to test a few different columns to optimize the desired separation. EX1037 (Yuan), pp. 22-23.

737. As I discussed above, Yuan discloses a RP-HPLC method to detect a capsid protein of HPV, the L1 protein. EX1037 (Yuan), Abstract. Yuan tested both C4 and C8 columns, finding that either column could be used with equal efficiency to purify the L1 capsid protein. EX1037 (Yuan), p. 23.

738. Satkunanathan used a reversed phase C18 column for LC-MS/MS. EX1005 (Satkunanathan), p. 931.

739. A POSA would have understood that these columns, C4, C8, and C18, were commonly used columns for separation of viral structural proteins, including AAV capsid proteins. A POSA would therefore have likely tested all of them, in applying Shytuhina's intact LC-MS method to the separation and identification of AAV capsid proteins, in combination with Yuan's method of testing columns such as C4 and C8, to optimize the LC-MS separation and protein identification.

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740. Satkunanathan, in combination with Shytuhina and Yuan, therefore meets the additional limitation of dependent claim 19.

C. Claim 27: "The method of claim 26, wherein the reverse phase chromatography is C8 reverse phase chromatography."

741. In my opinion, the combination of Satkunanathan, Shytuhina, and Yuan discloses the additional limitation of dependent claim 27.

742. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins were known to be subject to post-translational modification.

743. Shytuhina carried out an initial RP-HPLC step, which separated and denatured the proteins, and then carried out a second RP-HPLC step, coupled to the mass spectrometer – this second step subjected the denatured viral structural proteins to LC-MS intact protein analysis.

744. Shytuhina discloses that the specific RP-HPLC column they used for the first RP-HPLC step was a C4 column. EX1006 (Shytuhina), p. 193. Although Shytuhina does not expressly disclose the column used for the second RP-HPLC, coupled to the mass spectrometer, a POSA would have understood it was likely that

they used the same C4 column that had successfully separated E1 and E2 in the prior RP-HPLC step.

745. In any event, a POSA at the time would have understood that it was generally necessary to test a few different columns to optimize the desired separation. EX1037 (Yuan), pp. 22-23.

746. As I discussed above, Yuan discloses a RP-HPLC method to detect a capsid protein of HPV, the L1 protein. EX1037 (Yuan), Abstract. Yuan tested both C4 and C8 columns, finding that either column could be used with equal efficiency to purify the L1 capsid protein. EX1037 (Yuan), p. 23.

747. Satkunanathan used a reversed phase C18 column for LC-MS/MS. EX1005 (Satkunanathan), p. 931.

748. A POSA would have understood that these columns, C4, C8, and C18, were commonly used columns for separation of viral structural proteins, including AAV capsid proteins. A POSA would therefore have likely tested all of them, in applying Shytuhina's intact LC-MS method to the separation and identification of AAV capsid proteins, in combination with Yuan's method of testing columns such as C4 and C8, to optimize the LC-MS separation and protein identification.

749. Satkunanathan, in combination with Shytuhina and Yuan, therefore meets the additional limitation of dependent claim 27.

D. A POSA Would Have Had a Reasonable Expectation of Success in Making the Claimed Combination

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750. A POSA would have had a reasonable expectation of success in combining Satkunanathan and Shytuhina with Yuan. The techniques required to make the claimed combination, namely, RP-HPLC, intact LC-MS, and application of software to deconvolute and interpret MS data, were well known to people of skill in the art at the time and would have required nothing more than routine experimentation.

751. In particular, optimizing RP-HPLC for a particular separation would have required only routine experimentation. As Yuan demonstrates, researchers at the time routinely tested columns such as C4 and C8 columns, including for separation of viral structural proteins, to determine which column separated most effectively and efficiently.

752. Yuan successfully used both C4 and C8 columns to detect the L1 capsid protein of HPV. Yuan expressly found that either the C4 or C8 column could be used with equal efficiency of separation.

753. Shytuhina successfully separated glycoproteins by liquid chromatography, specifically RP-HPLC, and analyzed them by intact LC-MS, successfully identifying several different post-translational modifications.

754. I note that intact LC-MS analysis of AAV capsid proteins would have been even more straightforward for a POSA than working with the glycoproteins

Shytuhina analyzed. Glycoproteins are more variable and therefore more difficult to analyze than AAV capsid proteins by intact LC-MS.

755. A POSA therefore, using nothing more than routine experimentation, would have been able to select the best column, C4 or C8, for example, to optimize the separation by RP-HPLC, and then the analysis by MS.

756. A POSA would have had a reasonable chance of success that a C8 column would have successfully separated the AAV capsid proteins. I note that the HPV L1 capsid protein is approximately the same size as the AAV capsid proteins (55 kDa), further demonstrating that a POSA would have had a reasonable chance of success in separating the AAV capsid proteins on a C8 column.

757. Moreover, a POSA would have a reasonable chance of success in separating AAV capsid proteins and identifying them, including any PTMs, by intact LC-MS.

758. Satkunanathan successfully identified and distinguished three different AAV serotypes, and Shytuhina successfully separated and characterized viral structural glycoproteins with multiple different PTMs.

759. Moreover, researchers in the field for years before the relevant date, as I discussed above, had been successfully separating and characterizing reduced monoclonal antibodies and identifying PTMs using intact LC-MS. Applying intact LC-MS to AAV capsid proteins at the relevant date would have been considerably

more routine and straightforward than applying the technique to monoclonal antibodies.

E. Secondary Considerations Do Not Change the Conclusion of Obviousness

760. For evidence of "secondary considerations" to be informative of obviousness, I understand that there must be a "nexus" or link between the alleged secondary consideration and the subject matter recited in the Asserted Claims. I am not aware of any secondary considerations of non-obviousness with the required nexus to the claims of the '313 patent. For example, I am not aware of any commercial success attributable to an analytical method meeting the limitations of dependent claims 6, 19, and 27.[7] Similarly, I am not aware of any licenses directed specifically to the '313 patent or the subject matter recited in dependent claims 6, 19, and 27.

761. Finally, I am not aware of any unexpected results having a nexus to the claimed subject matter. The '313 patent does not disclose unexpected properties of

[7] If Patent Owner attempts to rely on the commercial success of Sarepta's gene therapy treatment for Duchenne muscular dystrophy – Elevidys® – there is no nexus to the challenged claims of the '313 patent. There is no nexus between the commercial success of Elevidys® and the analytical method recited in challenged claims 6, 19, and 27.

the claimed method. As I have discussed, researchers in the field at the relevant time routinely tested different columns, such as C4 and C8 columns, to optimize the separation of proteins, including viral capsid proteins similar in molecular weight to the AAV capsid proteins.

762. In addition, intact LC-MS had been used successfully for years before the relevant date as an analytical technique to monitor proteins, including monoclonal antibodies, and viral structural proteins, for process development. The use of LC-MS/MS to identify the capsid proteins of different AAV serotypes had been disclosed in Satkunanathan, and the use of intact LC-MS to monitor viral structural proteins, including identifying their PTMs, for process development had been disclosed in Shytuhina.

763. To the extent Patent Owner attempts to raise secondary considerations that have only a marginal nexus, if any, to claims 6, 19, and 27 of the ’313 patent, such evidence of secondary considerations should not outweigh the compelling evidence of obviousness, discussed above. Thus, secondary considerations do not alter my opinion that claims 6, 19, and 27 of the ’313 patent would have been obvious over the combination of Satkunanathan, Shytuhina, and Yuan.

XII. GROUND 3: CLAIMS 7, 8, 9, AND 10 ARE OBVIOUS OVER THE COMBINATION OF SATKUNANATHAN, SHYTUHINA, AND ZABROUSKOV

764. In my opinion, dependent claims 7, 8, 9, and 10 are also obvious over the combination of Satkunanathan, Shytuhina, and Zabrouskov. In addition to setting out my opinions below, I refer back to my discussion of Ground 1 in its entirety.

A. Claim 7: "The method of claim 1, further comprising determining the sequence of one or more VPs that has undergone post-translational modifications."

765. The combination of Satkunanathan and Shytuhina with Zabrouskov discloses the additional limitation of dependent claim 7. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

766. A POSA would further have understood that Satkunanathan's LC-MS/MS method determined the sequences of the fragments in the mixtures of protein fragments analyzed. For example, using LC-MS/MS, Satkunanathan identified AAV capsid proteins from three different serotypes by matching the sequence of the

proteins as determined by MS/MS against a database, specifically protein FASTA databases. EX1005 (Satkunanathan), p. 931, Supplementary Table S1.

767. Given that Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification, and that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2. And, as I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

768. Relying on Zabrouskov, a POSA would have understood to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

769. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract.

770. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

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771. The combination of Satkunanathan and Shytuhina with Zabrouskov therefore discloses the additional limitation of dependent claim 7.

B. Claim 8: "The method of claim 7, wherein the post-translational modifications are selected from the group consisting of acetylation, deacetylation, deamidation, glycosylation, truncation and ubiquitination."

772. The combination of Satkunanathan and Shytuhina with Zabrouskov discloses the additional limitation of dependent claim 8. As I discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

773. Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification. EX1005 (Satkunanathan), p. 931. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2.

774. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known

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since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

775. Using intact LC-MS, Shytuhina separated and characterized different post-translationally modified forms of viral structural proteins E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

776. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Shytuhina separated and characterized glycosylated forms of E1 and E2 and acylated forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

777. Moreover, Shytuhina separated glycosylated but deacylated E1 from glycosylated and acylated E1, and characterized both forms. EX1006 (Shytuhina), pp. 194-95, Fig. 2A.

778. A POSA would further have understood, particularly in light of Shytuhina's identification and separation of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize modified forms of at least some capsid proteins, such as those studied in Satkunanathan, from one another.

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779. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2, including glycosylated forms of both proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

780. Relying on Zabrouskov, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

781. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

782. A POSA would therefore have understood how to use Shytuhina's intact LC-MS method to identify post-translational modifications on viral structural proteins such as the AAV capsid proteins, and also how to combine this method with MS/MS, such as that used in Satkunanathan and Zabrouskov.

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783. A POSA would have understood Zabrouskov's teaching that deamidation was a particularly challenging post-translational modification to characterize, given "the special challenge" that "its covalent $–NH_2 \rightarrow –OH$ modification causes an only 0.984 Da mass increase, closely matching the 1.002 Da spacing of the molecular ion isotope peaks." EX1038 (Zabrouskov), p. 987.

784. A POSA would therefore have understood that the known post-translational modifications of AAV capsid proteins, N-terminal truncation and acetylation, would have been more easily characterized by intact LC-MS/MS than the deamidation modifications characterized by Zabrouskov.

785. The combination of Satkunanathan and Shytuhina with Zabrouskov therefore discloses the additional limitation of dependent claim 8.

C. Claim 9: "The method of claim 8, wherein the post-translational modification is N-terminal acetylation."

786. The combination of Satkunanathan and Shytuhina with Zabrouskov discloses the additional limitation of dependent claim 9. As discussed above, a POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

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787. Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification. EX1005 (Satkunanathan), p. 931. Given that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2.

788. Satkunanathan identified capsid proteins for each of the three serotypes tested, AAV2, AAV5, and AAV8. EX1005 (Satkunanathan), Supplementary Table S1. As I discussed above, and as a POSA would have understood, it had been known since the 1980s that AAV capsid proteins undergo N-terminal truncation and acetylation *in vivo*. EX1011 (Becerra), p. 7920.

789. Shytuhina characterized post-translational modifications for each of the two viral structural proteins, E1 and E2, using intact LC-MS. EX1006 (Shytuhina), pp. 194-95, Fig. 2. Using intact LC-MS, for example, Shytuhina separated and characterized two different PTMs for the E1 structural protein. EX1006 (Shytuhina), pp. 194-95, Fig. 2A. Shytuhina separated and characterized glycosylated forms of E1 and E2 and acylated forms of E1 and E2. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

790. Moreover, Shytuhina separated glycosylated but deacylated E1 from glycosylated and acylated E1, and characterized both forms. EX1006 (Shytuhina), pp. 194-95, Fig. 2A.

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791. A POSA would further have understood, particularly in light of Shytuhina's separation and characterization of glycosylated but deacylated E1 from glycosylated and acylated E1, that Shytuhina's intact LC-MS method had sufficiently high resolution to separate and characterize modified forms of at least some capsid proteins, such as those studied in Satkunanathan.

792. Specifically, a POSA would have understood that Shytuhina's intact LC-MS method involved, as discussed above, deconvoluting the peaks obtained using software and identifying within the deconvoluted peaks modified forms of E1 and E2, including glycosylated forms of both proteins. EX1006 (Shytuhina), pp. 194-95, Fig. 2.

793. Relying on Zabrouskov, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2, vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

794. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract. As part of the MS/MS analysis, Zabrouskov

determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

795. A POSA would therefore have understood how to use Shytuhina's intact LC-MS method to identify post-translational modifications on viral structural proteins such as the AAV capsid proteins, and also how to combine this method with MS/MS, such as that used in Satkunanathan and Zabrouskov.

796. A POSA would have understood Zabrouskov's teaching that deamidation was a particularly challenging post-translational modification to characterize, given "the special challenge" that "its covalent $–NH_2 \rightarrow –OH$ modification causes an only 0.984 Da mass increase, closely matching the 1.002 Da spacing of the molecular ion isotope peaks." EX1038 (Zabrouskov), p. 987.

797. A POSA would therefore have understood that the known post-translational modifications of AAV capsid proteins, N-terminal truncation and acetylation, would have been more easily characterized by intact LC-MS/MS than the deamidation modifications characterized by Zabrouskov.

798. The combination of Satkunanathan and Shytuhina with Zabrouskov therefore discloses the additional limitation of dependent claim 9.

D. Claim 10: "The method of claim 7, wherein the sequences of VP1, VP2 and VP3 are determined."

799. The combination of Satkunanathan and Shytuhina with Zabrouskov discloses the additional limitation of dependent claim 10. As discussed above, a

POSA would have been motivated to apply Shytuhina's efficient and precise intact LC-MS method to improve on Satkunanathan's use of LC-MS/MS to optimize process development for AAV vectors, particularly in light of differences among the different AAV serotypes and the fact that the AAV capsid proteins (vp1, vp2, vp3) were known to be subject to post-translational modification.

800. A POSA would further have understood that Satkunanathan's LC-MS/MS method determined the sequences of the fragments in the mixtures of protein fragments analyzed. For example, using LC-MS/MS, Satkunanathan identified AAV capsid proteins from three different serotypes by matching the sequence of the proteins as determined by MS against a database, specifically protein FASTA databases. EX1005 (Satkunanathan), p. 931, Supplementary Table S1.

801. Given that Satkunanathan's settings included the PTM methionine oxidation as a dynamic modification, and that Dong had determined that AAV capsid proteins undergo this PTM, Satkunanathan's analysis likely included the sequence of some capsid proteins containing this PTM. EX1005 (Satkunanathan), p. 931; EX1022 (Dong), Abstract, p. 2.

802. Relying on Zabrouskov, a POSA at the time would have understood how to combine Shytuhina's intact LC-MS method with Satkunanathan's LC-MS/MS method, to determine the sequences of the AAV capsid proteins, vp1, vp2,

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vp3, and further characterize their post-translational modifications. EX1038 (Zabrouskov), Abstract.

803. Zabrouskov used MS/MS of intact proteins ("top-down MS/MS") to analyze deamidation of RNase A. EX1038 (Zabrouskov), Abstract. Zabrouskov identified multiple deamidation sites, at asparagine and glutamine, on RNase A. EX1038 (Zabrouskov), Abstract.

804. As part of the MS/MS analysis, Zabrouskov determined the sequences of the fragments generated from the intact RNase A proteins during MS/MS analysis. EX1038 (Zabrouskov), Figures 2-4, pp. 989-90.

805. The combination of Satkunanathan and Shytuhina with Zabrouskov therefore discloses the additional limitation of dependent claim 10.

E. A POSA Would Have Had a Reasonable Expectation of Success in Making the Claimed Combination

806. A POSA would have had a reasonable expectation of success in combining Satkunanathan with Shytuhina and Zabrouskov. The techniques required to make the claimed combination, namely, RP-HPLC, intact LC-MS, intact LC-MS/MS, and application of software to deconvolute and interpret MS data, were well known to people of ordinary skill in the art at the time and would have required nothing more than routine experimentation.

807. Shytuhina successfully separated glycoproteins by liquid chromatography, specifically RP-HPLC, and analyzed them by intact LC-MS, successfully identifying several different post-translational modifications.

808. I note that intact LC-MS analysis of AAV capsid proteins would have been even more straightforward for a POSA than working with the glycoproteins Shytuhina analyzed. Glycoproteins are more variable and therefore more difficult to analyze than AAV capsid proteins by intact LC-MS.

809. A POSA furthermore, using nothing more than routine experimentation, would have been able to select the best column, C4 or C8, for example, to optimize the separation by RP-HPLC, and then the analysis by MS.

810. Moreover, a POSA would have a reasonable chance of success in separating AAV capsid proteins and characterizing them, including any PTMs, by intact LC-MS.

811. Satkunanathan successfully identified and distinguished three different AAV serotypes, and Shytuhina successfully separated and characterized viral structural glycoproteins with multiple different PTMs.

812. Moreover, researchers in the field for years before the relevant date, as I discussed above, had been successfully separating reduced monoclonal antibodies and identifying PTMs using intact LC-MS. Applying intact LC-MS to AAV capsid

proteins at the relevant date would have been considerably more routine and straightforward than applying the technique to monoclonal antibodies.

813. A POSA would also have had a reasonable expectation of success in further combining Satkunanathan with Shytuhina and Zabrouskov to carry out intact LC-MS/MS to provide further characterization of post-translational modifications of AAV capsid proteins by obtaining the sequences of the modified capsid proteins through tandem MS. As demonstrated by Zabrouskov, researchers in the field for years before the earliest possible priority date for the '313 patent had been using intact LC-MS/MS to characterize post-translational modifications of proteins through obtaining the masses and sequences of the modified proteins.

F. Secondary Considerations Do Not Change the Conclusion of Obviousness

814. For evidence of "secondary considerations" to be informative of obviousness, I understand that there must be a "nexus" or link between the alleged secondary consideration and the subject matter recited in the Asserted Claims. I am not aware of any secondary considerations of non-obviousness with the required nexus to the claims of the '313 patent. For example, I am not aware of any commercial success attributable to an analytical method of monitoring an AAV

preparation using the well known technique of intact LC-MS or intact LC-MS/MS.[8] Similarly, I am not aware of any licenses directed specifically to the '313 patent or the subject matter recited in challenged claims 1-27.

815. Finally, I am not aware of any unexpected results having a nexus to the claimed subject matter. The '313 patent does not disclose unexpected properties of the claimed formulation. As I have discussed, intact LC-MS had been used successfully for years before the relevant date as an analytical technique to monitor proteins, including monoclonal antibodies, and viral structural proteins, for process development. The use of LC-MS/MS to identify the capsid proteins of different AAV serotypes had been disclosed in Satkunanathan, the use of intact LC-MS to monitor viral structural proteins, including characterizing their PTMs, for process development had been disclosed in Shytuhina, and the use of intact LC-MS/MS to provide additional characterization of post-translationally modified proteins, by

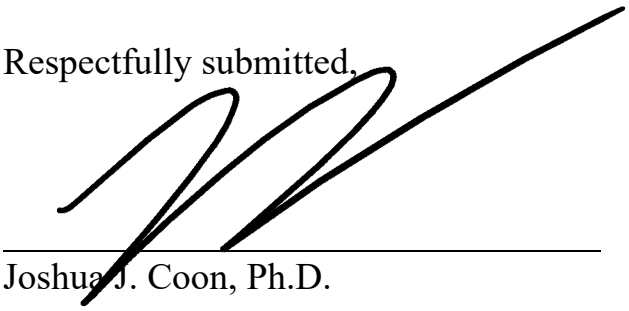
[8] If Patent Owner attempts to rely on the commercial success of Sarepta's gene therapy treatment for Duchenne muscular dystrophy – Elevidys® – there is no nexus to the challenged claims of the '313 patent. There is no nexus between the commercial success of Elevidys® and the analytical method recited in the challenged claims.

providing their sequences in addition to analyzing their masses, had been disclosed in Zabrouskov.

816. To the extent Patent Owner attempts to raise secondary considerations that have only a marginal nexus, if any, to claims 1-27 of the ’313 patent, such evidence of secondary considerations should not outweigh the compelling evidence of obviousness, discussed above. Thus, secondary considerations do not alter my opinion that claims 1-27 of the ’313 patent are obvious over the combination of Satkunanathan with Shytuhina and Zabrouskov.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code.

Respectfully submitted,



Joshua J. Coon, Ph.D.

Date: November 26, 2025