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LETTER ARTICLE

Rapid Determination of Full and Empty Adeno-Associated Virus Capsid Ratio by Capillary Isoelectric Focusing

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Abstract: Adeno-associated virus (AAV) is one of the most promising gene transfer vector types featuring long-term gene expression and low toxicity. The lack of pathogenicity and the availability of many serotypes augmented the applicability of AAV virions in gene therapy applications. The recombinant AAV capsid includes the therapeutic protein-coding transgene as well as a promoter to initiate translation and a poly A sequence portion for stabilization. Current AAV manufacturing technologies, however, cannot guarantee the generation of only full capsids, i.e., including the entire required genome. Partially filled and empty capsids are also part of the product, decreasing in this way the efficacy and safety upon clinical translation. Therefore, rapid, accurate and QC friendly analysis of the full and empty capsid ratio is of high importance during AAV vector manufacturing and release testing. In this paper, an automated capillary isoelectric focusing technique is introduced, readily applicable in the biopharmaceutical industry for fast and efficient determination of the full and empty capsid ratio. The method also reveals information about the proportion of partially filled capsids. For higher resolution (<0.1 pI unit), mixtures of wide and narrow range ampholytes were utilized. The isoelectric point and peak area percentage reproducibility (RSD) of the mixed ampholyte assay were as low as 1.67% and 2.45 %, respectively, requiring only 65 nL of sample volume per injection.

Keywords: Adeno Associated Virus, capillary electrophoresis, Transmission electron microscopy, Isoelectric focusing, Full/empty capsid, Transgene.

1. INTRODUCTION

One of the most frequently utilized gene therapy vectors is Adeno-associated virus (AAV), featuring very low immunogenicity and different tropisms for multiple cell types [1, 2]. The virus comprises a protein shell, also referred to as the capsid with an icosahedral shape of approximately 20-25 nm in diameter, composed of 60 viral protein subunits [3, 4]. A single-stranded DNA molecule of approximately 4700-4800 bases is enclosed inside the capsid. This DNA in recombinant AAVs for gene therapy has three major elements, including the 145 nucleotide long inverted terminal repeats (ITR) at both ends, the promoter sequence and the therapeutic gene (transgene) [5]. During the manufacturing process of AAV vectors, many of the capsids either are not or are partially incorporated with the required DNA load (fragments or truncated). This can lead to a significantly low production yield of 10-20%, which results in a decrease in efficacy and an increase in potential safety risks. Hence, it is critical to be able to quantitatively determine the level of full, empty as well as partially filled AAV vectors.

Traditional methodologies used for the determination of the full vs empty AAV capsid ratios include spectrophotometry, analytical ultracentrifugation (AUC), electron microscopy (EM), ion-exchange chromatography (IEX), charge detection mass spectrometry (CDMS) as well as combined results obtained from both ELISA and qPCR data [6]. While some of these methods are well accepted and easy to use, they have limitations in accuracy and precision. Spectrophotometry based techniques use the UV absorbance ratio of the sample, measured at 260 nm and 280 nm to reveal the relationship between the protein to nucleic acid content [7]. While this approach is simple and fast, it suffers from impurity interferences and low accuracy. One of the broadly used analytical and preparative approaches capable of separating full, partially filled and empty capsids is analytical

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ultracentrifugation [8]. AUC has a large sample requirement and is difficult to utilize as a rapid quality control (QC) release assay system. Transmission and cryo-electron microscopy, as well as atomic force microscopy [9-12] are other frequently used methods capable of counting the number of full and empty capsids in a sample population, however, these methods have difficulties in determining the extent of DNA content in partially filled capsids. In addition, the evaluation of electron microscopy data is time-consuming, making it less desirable in the QC and release testing environment. EM methods also heavily rely on the analyst's interpretation of the data, which can be somewhat subjective. Ion-exchange chromatography (IEX) is a quantitative but low-resolution technique for full and empty capsid ratio determination [13, 14]. IEX also requires larger sample amounts and suffers from serotype dependence. Mass spectrometry (MS) based approaches, such as charge detection MS, can readily detect full, partially filled and empty capsids, yet commercially not available for routine use [15].

The drawbacks of the above listed techniques called for more comprehensive, fast, easy to perform, robust approaches that are appropriate for QC/release testing to quantify the full and empty AAV capsid ratio with the possibility to reveal information on partially filled species, AAV charge heterogeneity and impurities. Capillary isoelectric focusing (cIEF) is a well-established bioanalytical technique in the biopharmaceutical industry, capable of separating closely related species, based on their isoelectric points, such as charge variants [16]. Due to their negatively charged DNA load, full AAV capsids generally have lower isoelectric points compared to their empty or partially filled counterparts [17], making cIEF a promising tool to fill the gaps described above. In this paper, we introduce an automated capillary isoelectric focusing method, featuring low sample volume requirements in conjunction with the use of narrow pH range ampholytes (≤0.1 pH unit resolution) for rapid (<45 min) QC friendly analysis of full, empty and partially filled AAV capsids.

2. MATERIALS AND METHODS

2.1. Chemicals

Phosphoric acid, sodium hydroxide, acetic acid, L-arginine, iminodiacetic acid and all other chemicals were from Sigma-Aldrich (St Louis, MO). AAV samples were from SignaGen Laboratories (Rockville, MD) and Vigene Biosciences (Rockville, MD) with the serotypes specified below. The components of the Advanced cIEF Starter Kit were used with the N-CHO coated capillary (Sciex, Framingham, MA), unless specified otherwise. All solutions were filtered through 5 µm syringe filters prior to use.

2.2. Sample Preparation

The master-mix solution was prepared by combining 200 µL of 3 M urea - cIEF gel solution, 12 µL of ampholytes (pH range specified in the relevant sections), 20 µL of cathodic stabilizer (500 mM L-Arginine), 2 µL of anodic stabilizer (200 mM iminodiacetic acid) and 2 µL of each pI marker used. The following AAV serotypes were used in the experiments from SignaGen Laboratories (Rockville, MD): AAV1-CMV-GFP (titer: 1.46 $x10^{13}$ GC/mL), AAV3-Cre-GFP (titer: 1.02 $x10^{13}$ GC/mL), AAV5-CMV-GFP (titer: 1.53 $x10^{13}$ GC/mL) and AAV9-CMV-GFP (titer: 3.12 $x10^{13}$ GC/mL). AAV8 of pAV-CMV-GFP Empty Capsid sample with a titer of 5.10 $x10^{12}$ GC/mL was used as enriched empty capsids, while packaged AAV8 of the pAV-CMV-GFP sample with a titer of 1.10 $x10^{13}$ GC/mL as enriched full capsids, both from Vigene Biosciences (Rockville, MD). 3 µL of AAV samples were mixed with 24 µL of master mix and transferred into nanoVials (Sciex) for cIEF analysis. Either the Pharmalyte, 3-10 wide pH range ampholyte (GE Healthcare) alone, or its mixtures with SERVALYT 6-8 narrow pH ampholyte (Serva, Heidelberg, Germany) or SH AESlytes 7-8 narrow pH ampholyte (Advanced Electrophoresis Solutions, Cambridge, Ontario) were used at various ratios to fine-tune resolution.

2.3. Capillary Isoelectric Focusing (cIEF)

All cIEF experiments were performed in normal polarity separation mode (anode at the injection side) on a PA 800 Plus Pharmaceutical Analysis system (SCIEX), equipped with a UV detector (280 nm filter). The separation capillary was filled with the relevant ampholyte solutions, as defined in the 'Results and Discussion' section. The anolyte was 200 mM phosphoric acid, while the catholyte was 300 mM sodium hydroxide. The chemical mobilizer was 350 mM acetic acid and the capillary cleaning solution was 4.3 M urea. The cathodic and anodic stabilizer solutions were 500 mM L-Arginine and 200 mM iminodiacetic acid (IDA), respectively. The N-CHO coated capillary was used for all cIEF analyses and the separation temperature was maintained at 20°C. The data were collected and analyzed by the 32 Karat Software version 10.3.

2.4. Transmission Electron Microscopy (TEM)

One drop (~5 µl) of AAV5 sample was placed onto a TEM copper grid covered by a thin (3-5 nm) continuous carbon film. The excess sample was soaked up with a paper tissue. Phosphotungstic acid (2%) was used for staining the sample for 30 seconds. A 200 kV FEI Talos F200X transmission electron microscope (equipped with a field-emission gun) was used for the measurements (Thermo Fisher, Waltham, MA). The TEM bright-field images were taken at random places and constant magnification (1 µm field of view) with a 4096 x 4096 pixel CMOS camera (FEI CETA). The evaluation was made by manual counting of at least 5000 virus particles.

3. RESULTS AND DISCUSSION

A pilot experiment was conducted first to understand the cIEF migration behavior of enriched full

and enriched empty AAV capsids, shown in Fig. **1**. In this experiment, the wide pI range ampholyte (pH 3-10) was used in the separation capillary with sodium hydroxide in the cathode and phosphoric acid in the anode reservoirs. The upper trace depicts the electropherogram of the enriched full capsid sample. As one can observe, besides the peak corresponding to the full capsid (peak 3), a few transitional products were also detected (peak group 2) along with a small peak representing, in this case, the empty capsid (peak 1), all focused between the pH 7.0 and 10.0 pI markers. The lower trace shows the separation of the sample containing the enriched empty capsid. Similar to the upper trace, peak 1 represents the empty, while peak group 2 represents the various partially filled transitional capsids and peak 3 the full capsid. These results suggested that the empty capsid focused at a higher pI value, while the full capsid at a lower pI value, the latter due to its negatively charged DNA load. The partially filled capsids focused between the empty and full capsid peaks, corresponding to the pI values defined by their actual DNA content.

After verifying the cIEF focusing order of the empty and full adeno associated virus capsids, five commercially available AAV samples of different serotypes (AAV1, AAV3, AAV5, AAV8 and AAV9) were analyzed using the wide pH range ampholyte (pH 3-10) as shown in Fig. **2**. Trace (a) depicts a blank run, while the upper traces of (b-f) show the corresponding AAV serotype associated electropherograms. The pI values of the separated peaks were determined by a calibration plot using a pI marker mixture and were found as follows: AAV1 = pI 7.8-8.0; AAV3 = pI 8.7-9.0; AAV5 = pI 7.0-7.2; AAV8 = pI 7.0-7.5; AAV9 = pI 7.3-7.5. Analysis of the AAV1 sample (trace b) revealed an overlapping peak distribution of the differently loaded capsids, i.e., empty, partially filled and full. The separation of the AAV3 (trace c) and AAV5 (trace d) samples featured two main peaks and a smaller one in between, considered to be corresponding to the empty, partially filled and full capsids. Again, the fact that the full capsids have lower pI values is due to the large amount of negative charges carried by the encapsulated DNA. AAV8 (trace e) and AAV9 (trace f) capsids focused on single broad peaks (the latter one with a shoulder), suggesting poor resolution of the variously filled capsid forms of these serotypes with the use of the wide pH range ampholyte.

As one can observe in Fig. **2**, with the use of the wide pH range ampholyte, the analysis of the AAV5 sample showed the well-resolved separation of the empty, partially filled and full capsid peaks (trace d). The full and empty percentage distribution of this sample from the processed cIEF data was determined as 43% and 57%, respectively. The calculation was made by applying the correction factor introduced by

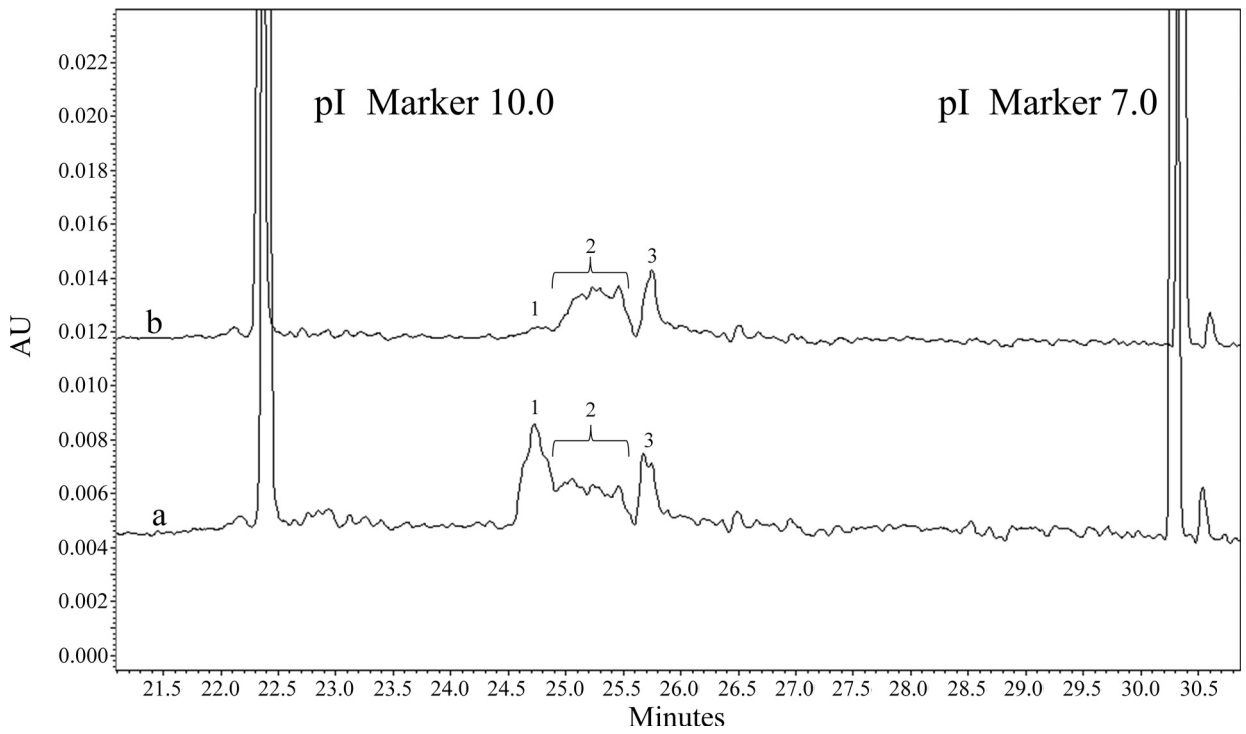

Fig. (1). Capillary isoelectric focusing of enriched empty (trace a) and enriched full (trace b) AAV capsids for migration/focusing order determination. Peaks: 1-empty, 2-transitional and 3-full capsids. Conditions: 20 cm effective length N-CHO capillary (50 µm ID and 30 cm total length), filled with 3-10 wide pH range ampholyte containing background electrolyte. Catholyte: 300 mM sodium hydroxide, Anolyte: 200 mM phosphoric acid. Applied electric potential: 25 kV for the 15 min for the focusing step. Chemical mobilization step: 30 kV with 350 mM acetic acid for 30 min from the outlet reservoir. The separation temperature of the capillary cartridge was set at 20°C. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

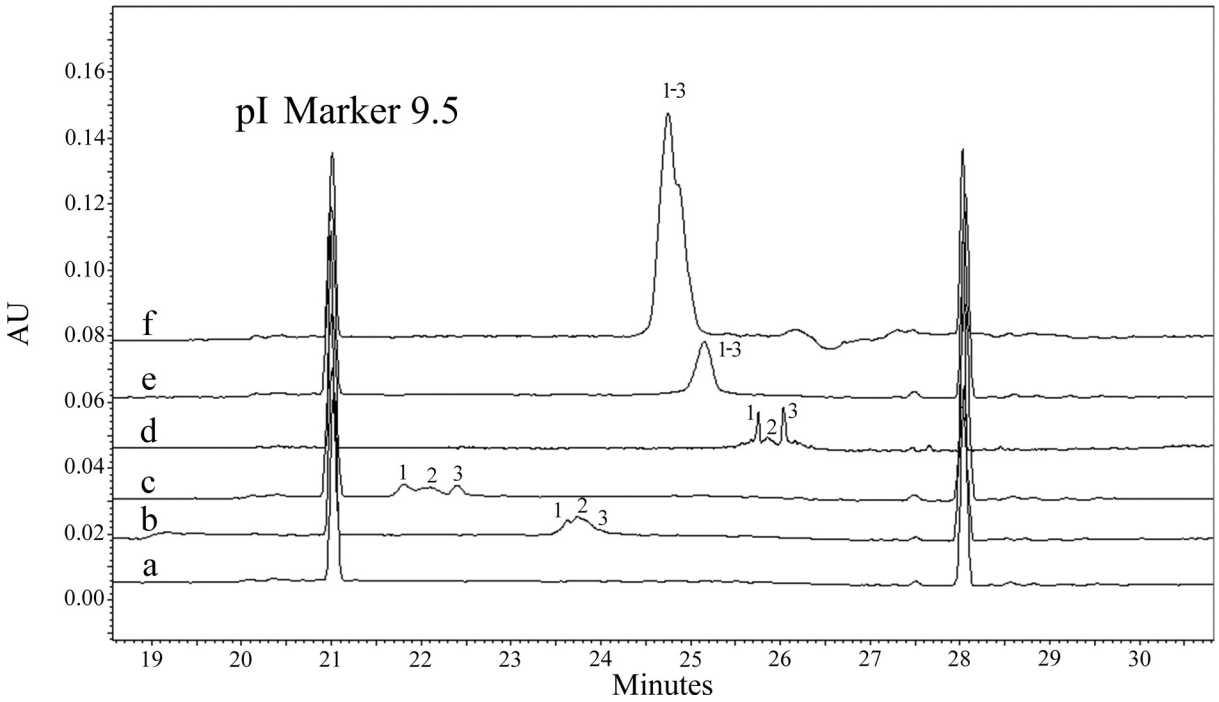

Fig. (2). Capillary isoelectric focusing analysis of five AAV capsid samples with different serotypes using the wide pH range ampholyte of pH 3-10. Traces: a) Blank, b) AAV1, c) AAV3, d) AAV5, e) AAV8 and f) AAV9. Peaks: 1-empty, 2-partially filled and 3-full capsids. Conditions were the same as in Fig. **1**. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

Goricar *et al*., [18] to alleviate any possible overestimation of the amounts of the full capsids that might have been caused by the 280 nm UV detection. This particular serotype was also subject to analysis by transmission electron microscopy (TEM), an orthogonal AAV capsid characterization method. Figure **3** shows a representative section of the TEM results, where the green circles and squares depict examples of full and empty capsid images, respectively. The arrows show the transitional capsids. The full to empty distribution using this method was assessed as 35% to 65%, by manually counting the capsid types, *i.e*., reasonably similar to that obtained with the cIEF method.

To quantify the capsid load distribution in some of the serotypes that resulted in either broad co-focused (AAV1) or non-separated single peaks (AAV8 and 9) with the use of the wide range ampholyte of pH 3-10, the resolution of the analysis was fine-tuned by mixing the wide range type with narrow range ones. Trace (a) in Figure **4** depicts the results obtained with the use of 1:1 ratio of the wide range (pH 3-10) and narrow range (pH 6-8) ampholytes for the analysis of the AAV1 sample. As one can observe, in contrast to the results obtained with the use of the wide range ampholyte only (Fig. **2**, trace b), this particular sample was resolved into five separated peaks, apparently corresponding to the empty (peak 1), partially filled (peak 2) and full (peak 3) capsids. Peaks 4 and 5 are probably sample related impurities. The middle trace (b) in Fig. **4** shows the separation of the AAV8 capsid sample with the 2:1 ratio of the wide and narrow range ampholytes resulting in a significant resolution increase in comparison to trace e in Fig. **2**. The multiple peaks were obtained assumable corresponding to the empty

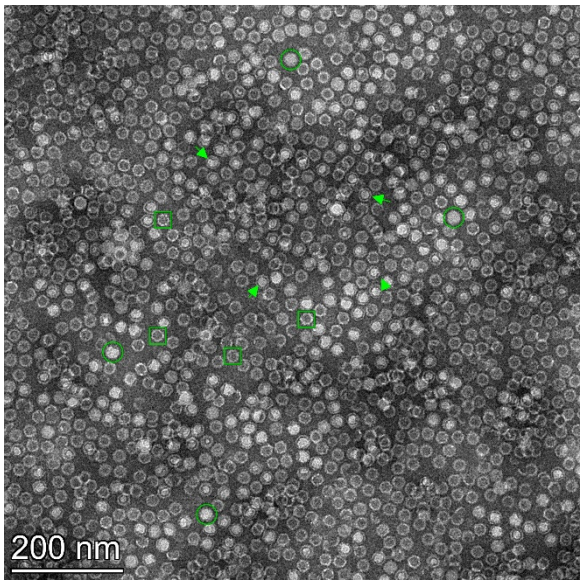

Fig. (3). Analysis of the full and empty capsid distribution of the AAV5 sample by transmission electron microscopy (TEM). Green Circles: examples of full capsid images; Green Squares: examples of empty capsid images; Green Arrows: examples of transitional capsids. Conditions: TEM: 200 kV FEI Talos F200X transmission electron microscope with bright-field images taken at random places and constant magnification (1 µm field of view) with a 4096 x 4096 pixel CMOS camera. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(peak 1), partially filled (peaks 2 and 3) and full (peak 4) capsids. Another example of the usefulness of such pH range optimization is delineated in the upper trace of Fig. **4** (c), where a good separation of the AAV9 sample is shown with the use of 2:1 ratio mixture of the wide and narrow range ampholytes. Please note when using the wide range ampholyte alone for this particular sample, only a single peak with a shoulder was observed (Fig. **2**, trace f). However, applying the 2:1 mixture of pH 3-10 and 6-8 ampholytes resulted in excellent separation of all major components, probably corresponding to the empty (peak 1), partially filled (peaks 2 and 3) and full (peak 4) capsids. Peak 5 here was also considered as sample related impurity. This particular sample apparently contained mostly partially filled capsids, with various DNA loads represented by the individual peaks of 2 and 3). The assay reproducibility was also evaluated for this sample based on 6 consecutive runs and the pI and peak area percent RSD values were 1.67 and 2.45%, respectively.

Interestingly, in some instances, further cIEF optimization did not bring about better separations. As an example, additional optimization by mixing the pH 3-10 ampholyte with an ultra-narrow range type of pH 7-8 in 2:1 ratio (Fig. **5**, trace b) did not result in better separation of the AAV5 serotype as with the use of the 2:1 ratio of pH 3-10 and pH 6-8 ampholyte mixture (trace a). Albeit, trace (b) in this example shows 9 peaks, some of them might be just focusing on artifacts or spikes (*e.g.*, peaks 2, 3 and 4). Trace (a), on the other hand, while featuring only five peaks, can be better evaluated from cIEF point of view with the empty (peak 1), full (peak 5) and partially filled (peaks 2-4) capsids.

CONCLUSION

In this paper, an automated cIEF based rapid and high-resolution method was introduced to determine the full/empty capsid ratios of different serotype AAV vectors. The full capsids focused at the lower, while the empty ones at the higher pI regime, respectively, based on their DNA load. In addition, the generated data also provided information about the presence of partially filled capsids, focused in between. Mixing wide and narrow range ampholytes resulted in better separation of otherwise co-focusing full, transitional and empty capsids with very small pI value differences. The reproducibility of the assay, even with the mixed range ampholytes was as low as 1.67% RSD for isoelectric point and 2.45% for peak area percentage determination. It is also important to note that the cIEF method required less than 5 µL of the total sample size for multiple runs, considering the typical injection volume of 65 nL.

LIST OF ABBREVIATIONS

AAV	=	Adeno Associated Virus
AUC	=	Analytical ultracentrifugation
cIEF	=	capillary isoelectric focusing
GC	=	genome copy

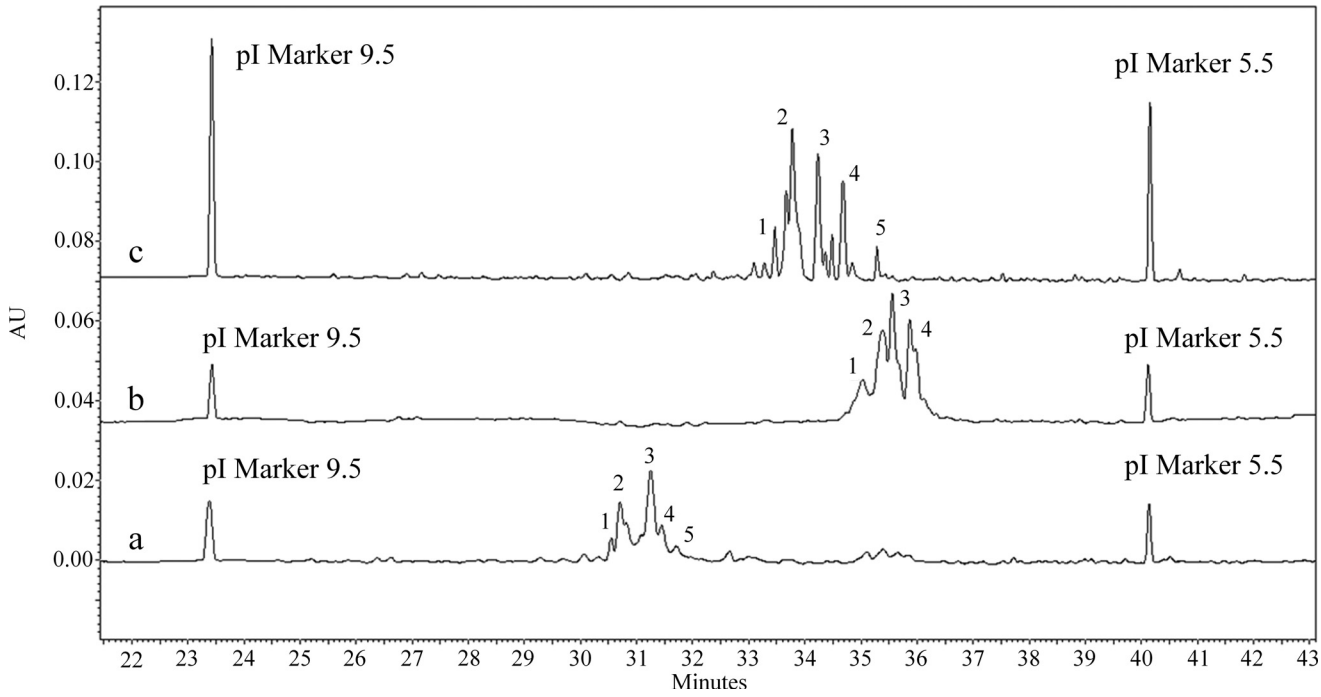

Fig. (4). Optimization of the different pH range ampholyte mixtures (pH 3-10 and pH 6-8) for high-resolution cIEF analysis of the AAV1, 8 and 9 serotypes. Traces: a) AAV1 with 1:1, b) AAV8 with 2:1 and 3) AAV9 with 2:1 mixture of wide and narrow range ampholytes. Peaks: 1-empty, 2,3-partially filled, 4-full capsids, 5-sample related impurity. All other conditions were the same as in Figure 1. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

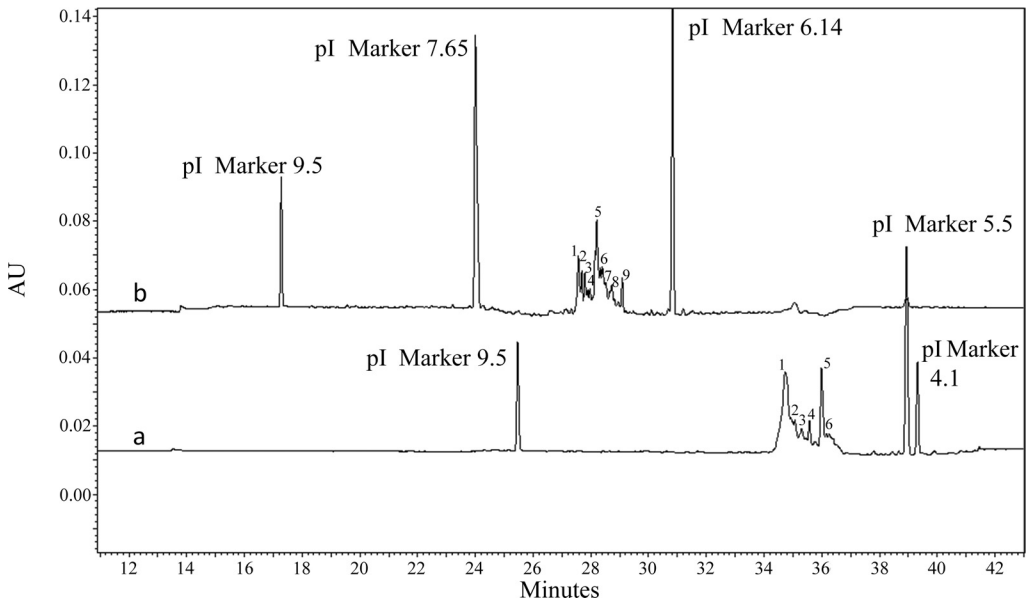

Fig. (5). Effect of the addition of an ultra-narrow range ampholyte on the resolution of the cIEF analysis of the AAV5 serotype using 2:1 ratios of the pH 3-10 plus pH 6-8 (trace a) and the pH 3-10 plus pH 7-8 (trace b) mixtures. Peaks in trace a) 1: empty, 2-4: partially filled, 5: full; trace b) 1: empty, 2-4: empty related focusing artefacts, 5-8: partially filled and 9: full capsids. Conditions were the same as in Figure 1, with the exception of the ampholyte mixtures used. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

IEX = ion exchange chromatography

TEM = Transmission electron microscopy

ETHICAL APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

HUMAN AND ANIMAL RIGHTS PARTICIPATE

No animals/humans were used for studies that are the basis of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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