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# Development and application of a reversed-phase high-performance liquid chromatographic method for quantitation and characterization of a Chikungunya virus-like particle vaccine

Anastasija Shytuhina [a], Pavlo Pristatsky [a], Jian He [a], Danilo R. Casimiro [a], Richard M. Schwartz [b], Van M. Hoang [a], Sha Ha [a,*]

[a] Vaccine Bioprocess Research & Development, Merck Research Laboratories, West Point, PA, 19486, United States
[b] Vaccine Production Program, Vaccine Research Center, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Gaithersburg, MD 20878, United States

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ABSTRACT

To effectively support the development of a Chikungunya (CHIKV) virus-like particle (VLP) vaccine, a sensitive and robust high-performance liquid chromatography (HPLC) method that can quantitate CHIKV VLPs and monitor product purity throughout the manufacturing process is needed. We developed a sensitive reversed-phase HPLC (RP-HPLC) method that separates capsid, E1, and E2 proteins in CHIKV VLP vaccine with good resolution. Each protein component was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) mass spectrometry (MS). The post-translational modifications on the viral glycoproteins E1 and E2 were further identified by intact protein mass measurements with liquid chromatography–mass spectrometry (LC–MS). The RP-HPLC method has a linear range of 0.51–12 μg protein, an accuracy of 96–106% and a precision of 12% RSD, suitable for vaccine product release testing. In addition, we demonstrated that the RP-HPLC method is useful for characterizing viral glycoprotein post-translational modifications, monitoring product purity during process development and assessing product stability during formulation development.

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## 1. Introduction

Chikungunya virus is a mosquito-borne alphavirus. It is an enveloped positive-stranded RNA virus with a diameter of 60–75 nm that causes acute illness including fever, rash and severe arthralgia. Although rarely fatal, CHIKV causes incapacitating and prolonged joint pain that presents serious economic and social impact. Since the first isolation of CHIKV in Tanzania in 1952, sporadic outbreaks occurred in Central and Southern Africa and South East Asia between 1960s and 1990s. Outbreaks resurged between 2004 and 2007 in Reunion Islands, India and Europe with hundreds of thousands of reported cases [1–3]. Due to the disease severity, the high infection rate during outbreaks, and the extensive geographic distribution, there is an urgent need for an effective CHIKV vaccine. In 2011, Nabel et al. reported the expression of CHIKV virus-like particles (VLPs) in human embryonic kidney cells [4]. Vaccination using these CHIKV VLPs protected Rhesus macaques from challenge with wild-type virus. Additionally, serum antibodies from the vaccinated macaques provided protection from a lethal dose of CHIKV in a mouse model. These results established the proof-of-concept that CHIKV VLPs were sufficient to elicit a protective humoral response against CHIKV infection. The success of these experiments warranted further characterization of the CHIKV VLPs.

Traditionally, vaccines composed of VLPs are characterized by SDS-PAGE for purity and quantified by colorimetric protein assays such as Bradford, bicinchoninic acid (BCA) or Lowry assay. Both approaches have disadvantages. SDS-PAGE is labor and time intensive. Colorimetric protein assays can be sensitive to detergents, reducing agents or certain salts. In addition, the colorimetric protein assays measure total protein concentration, and are therefore not specific for the antigenic components of the vaccine product. To effectively support process and formulation development, 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.

High-performance liquid chromatography (HPLC) has become an attractive analytical tool due to its high sensitivity and reproducibility. HPLC methods have been applied for the identification

* Corresponding author. Tel.: +1 215 652 9762.
*E-mail address:* sha_ha@merck.com (S. Ha).

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and quantitation of virus proteins and VLPs including influenza virus [5–8], lentivirus [9], Sendai virus [10], poliovirus [11,12], human papillomavirus VLP [13], adenovirus types 3 and 5 [14,15], and Hepatitis B VLP [16]. However, due to the hydrophobic nature of most viral glycoproteins and the presence of lipids with enveloped virus, it has been technically challenging to achieve good resolution and recovery for all the viral components [9,10,17].

The CHIKV VLP has three structural proteins and is 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 [18]. Our goal was to develop a RP-HPLC assay that would separate E1, E2 and capsid proteins of CHIKV VLPs. This method would serve to evaluate and quantitate the mass and purity of the vaccine product. Additionally, this method would be a tool to assess both protein degradation and post-translational modifications for formulation and process development.

## 2. Materials and methods

### 2.1. Chemicals and reagents

HPLC grade acetonitrile (ACN), 2-propanol, trifluoroacetic acid (TFA), 0.1% TFA in acetonitrile, and 0.1% TFA in water were purchased from Fisher (Fair Lawn, NJ, USA). Zwittergent 3–12 detergent and C18 ZipTip were from Millipore (Billerica, MA, USA). Trypsin was from Promega (Madison, WI, USA). Formic acid, ammonium bicarbonate ($NH_4HCO_3$), α-cyano-4-hydrocinnamic acid (CHCA), iodoacetamide were from Sigma-Aldrich (St. Louis, MO, USA). Dithiothreitol (DTT) and GelCode Blue Stain Reagent were from Thermo Scientific (Pittsburgh, PA, USA). SilverQuest Staining Kit was from Invitrogen (Carlsbad, CA, USA). The expression and purification of CHIKV VLPs from the mammalian and the insect cell systems were described by Wagner et al. [19]. 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.

### 2.2. RP-HPLC

Samples containing CHIKV VLPs were analyzed on XBridge BEH300 C4 column (3.5 μm, 4.6 × 150 mm, 300 Å, Part # 186004504, from Waters) held at 60 °C with a linear AB gradient elution. Mobile phase A contained 0.1% TFA in water. Mobile phase B contained 30% ACN, 70% 2-propanol and 0.1% TFA. 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. Eluted proteins were detected by fluorescence at excitation at 280 nm and emission at 350 nm. Samples were incubated with 5% Zwittergent 3-12 detergent and injected at a volume of 100 μL. In the linearity study, the reference standard was diluted at 5.1–120 μg/mL in the presence of 5% Zwittergent 3-12 detergent. It was noted that in the early 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.

### 2.3. Protein identification: RP-HPLC peak collection, SDS-PAGE, in-gel digestion, and MALDI-ToF MS

CHIKV VLP fractions were collected from RP-HPLC run, dried in a Savant SpeedVac equipped with a cold acetone vapor trap. The fractions were then reconstituted in SDS-PAGE sample loading buffer and analyzed using a NOVEX gel apparatus in NOVEX 4–20% tris-glycine 1.5 mm gels. The gels were stained using either SilverQuest Staining Kit or GelCode Blue Stain Reagent.

For protein identification by MALDI-ToF MS, gels were first stained using GelCode Blue Stain. Protein bands were then excised along with a blank portion of the gel used as a negative control. Gel bands were placed into individual eppendorf tubes and crushed. They were destained by three cycles of dehydration with 25 mM ammonium bicarbonate ($NH_4HCO_3$)/50% ACN (v/v) and rehydration with 25 mM $NH_4HCO_3$. Destained gel pieces were dried in a Savant SpeedVac, reduced in 10 mM DTT/25 mM $NH_4HCO_3$ at 55 °C for 1 h, and alkylated with 55 mM iodoacetamide/25 mM $NH_4HCO_3$ at room temperature for 45 min in the dark. Gel pieces were washed with 25 mH $NH_4HCO_3$ and dried in a Savant SpeedVac. The pieces were incubated in a solution of high purity trypsin (5 ng/μL in 25 mM $NH_4HCO_3$) for 16 h at 37 °C. The resulting tryptic peptides were eluted from the gel pieces by vortexing with a solution of 5% formic acid/50% ACN (v/v). The extracted peptides were prepared for analyses by MALDI-ToF MS with C18 ZipTips per the manufacturer's instructions. Peptides were eluted from the ZipTips with 1 μL of matrix consisting of 8 mg/mL of CHCA in 0.05% TFA/50% ACN (v/v) and spotted onto a MALDI target. Samples were analyzed on a Bruker Autoflex III operated in reflector mode and spectra were acquired in positive ion mode in an *m*/*z* range of 700–4000 using a laser power of 76%. Proteins were identified by comparing observed masses to theoretical tryptic masses of the E1, E2, and capsid proteins.

### 2.4. Characterization of post-translational modifications: Intact protein mass analysis by LC–MS

The proteins in the VLP samples were separated and analyzed by LC–MS using a Waters Acquity LC system coupled to a Synapt G2 mass spectrometer (Waters, Milford, MA). The mobile phases were slightly modified as stated below. 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. Because TFA caused ion suppression, formic acid was used as the mobile phase modifier. In order to increase the signal intensity for intact protein accurate mass measurement, we used 10 consecutive injections of 25 μL of the sample. These injections were made with a short 2 min isocratic flow of 5% mobile B. 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. Mass spectra were obtained in positive mode by spraying the eluent into the mass spectrometer using an ESI source. The capillary, source cone, and extraction cone voltages were set at 3 kV, 20 V, and 4 V, respectively. Nitrogen was used as a desolvation gas at a flow rate of 800 L/h. The source and desolvation temperatures were set at 110 and 450 °C, respectively. The instrument was operated in Sensitivity mode and spectra were acquired in an *m*/*z* range of 1000–2500. 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.

## 3. Results and discussion

### 3.1. RP-HPLC development

CHIKV VLPs contain lipophilic and hydrophobic glycoproteins, making it challenging to develop an LC method that could elute the glycoproteins with minimal carry-over. We screened several HPLC columns with different combinations of mobile phases. We 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. In addition, TFA was necessary in the mobile phase to promote VLP interaction with the stationary phase. The lack of TFA in the mobile phases led to the elution of VLPs in the void volume of the column. It is interesting to note that TFA, at the concentration used, did not cause aggregation of the CHIKV proteins. Instead, TFA appeared to help disassemble the VLPs and facilitate the binding of proteins to the stationary phase. A Waters XBridge BEH300 C4 column with 70% 2-propanol in the elution mobile phase had the best recovery for all three CHIKV proteins, while column temperature of 60 °C improved peak sharpness.

Even with these optimized HPLC condition, we still observed carry-over of E1 and E2 between HPLC runs. In order to minimize carry-over we screened different sample pre-treatment methods. We 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%. Zwittergent 3-12 detergent is believed to solubilize and stabilize the glycoproteins and prevent non-specific binding during chromatography. Thus, incubation with 5% Zwittergent 3-12 detergent was used as a routine sample pre-treatment method.

In order 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. 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).

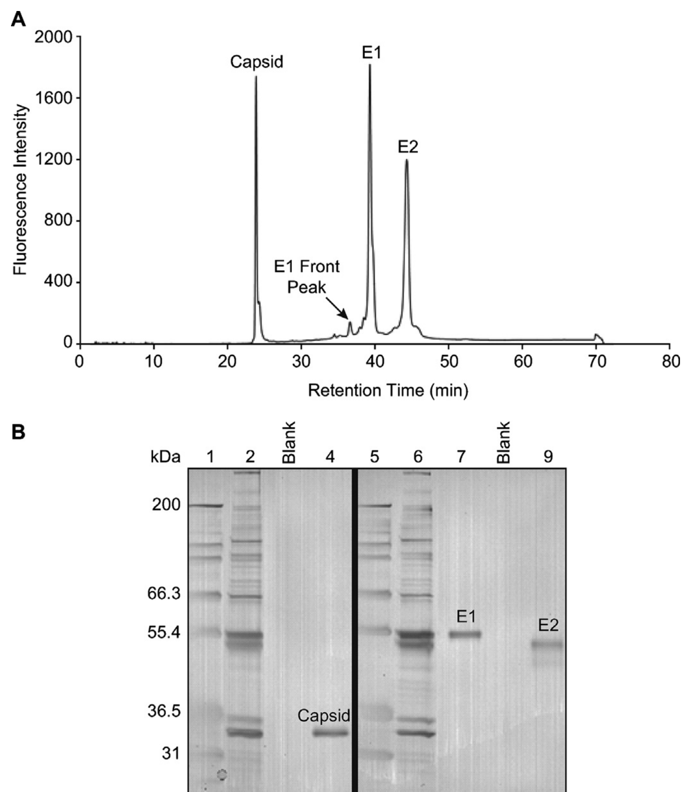


**Fig. 1.** (A) RP-HPLC chromatogram with fluorescent detection of a representative CHIKV VLP at 12 μg load. The sample was prepared with 5% Zwittergent 3–12 detergent and analyzed on XBridge BEH300 C4 cloumn at 60 °C. Structural proteins—capsid, E1, and E2 were baseline separated. (B) SDS-PAGE gel for peak identification. Lanes 1 and 5 show the molecular weight markers. Lanes 2 and 6 show the whole unfractionated sample. Lanes 4, 7, and 9 show the collected RP-HPLC fractions along with their identification as the structural CHIKV VLP proteins capsid, E1, and E2. E1 front peak was collected and analyzed. However, it was not visible on the gel due to its low abundance.

### 3.2. Intact protein mass analysis of E1 and E2

The ability to separate E1 and E2 by RP-HPLC chromatography allows us to characterize post-translational modifications of these viral glycoproteins by mass spectrometry. Both E1 and E2 of CHIKV contain N-glycosylation and both glycoproteins are expected to contain acylation based on sequence similarity with Semliki Forest virus (SFV) [18,20]. N-glycosylation directs the trafficking of glycoproteins from endoplasmic reticulum (ER) to Golgi and then to the cell membrane. Elimination of glycosylation negatively impacts virus infectivity and replication [21]. Acylation is a post-translational modification of cysteine with fatty acids via a thioester bond. It is proposed to assist E2 C-terminus transit from insertion in the endoplasmic reticulum bilayer to anchoring on the cytoplasmic face. As acylation is required to form a critical epitope for nucleocapsid binding [22], the lack of this post-translational modification results in defective virus assembly and budding [23].

Post-translational modifications present on CHIKV VLP proteins were identified by LC–MS using accurate mass measurements of the intact protein antigens. Fig. 2 shows the deconvoluted spectra for E1 and E2. Multiple masses were observed with each representing different glycosylation and acylation modifications. Based 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. Due to the mass heterogeneity of both E1 and E2, the deconvoluted masses of the minor peaks were less accurate. Therefore those peaks were not assigned though we expected other types of N-glycosylation or acylation to exist as well. We detected one N-glycosylation and one acylation—either palmitoylation (Pal) or stearoylation (Stear), for the majority of E1 (Fig. 2A, top trace). A small portion of glycosylated but deacylated E1 eluted slightly before the main population (E1 front peak in Figs. 1A and 2A bottom trace). This is not surprising as protein acylation is a reversible process and enzymatic depalmitoylation of viral glycoprotein has been demonstrated [24]. Fig. 2B shows that E2 contained two N-glycosylations and three acylations. The observation that E2 contained one hybrid glycan and one oligomannose is consistent with the glycan composition of the well-studied Sindbis virus (SINV) expressed in mammalian cells [25]. We did not detect a preference of either palmitoylation or stearoylation for CHIKV E1. This is in contrast to the report of exclusive selection of stearate for SFV E1 [20,26]. It is unknown whether the selection of different fatty acids is due to the difference in sequence or the expression host.

### 3.3. Evaluation of E1 and E2 recoveries from RP-HPLC

Recovery of viral membrane protein from HPLC has been a technical challenge for many researchers. For example, 5–50% recovery

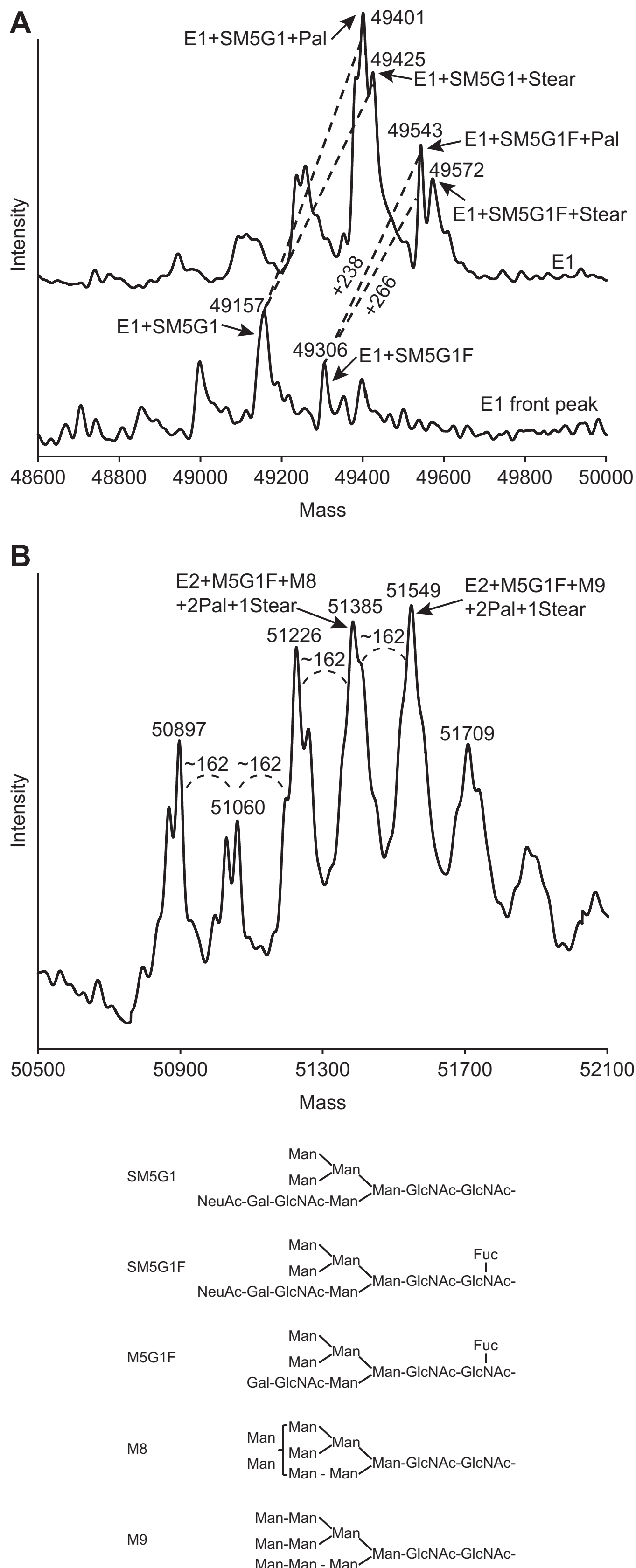


**Fig. 2.** Intact protein mass analyses of E1 and E2 by LC–MS. The deconvoluted protein masses of E1 and E1 front peak are shown in (A), and the deconvoluted protein mass of E2 is shown in (B). E1 front peak is labeled in Fig. 1A chromatogram. The post-translational modifications are identified for the major E1 and E2 peaks.

**Table 1**
Assay precision as evaluated by the total peak area.

| Injection # | Day 1 | Day 2 | Day 3 |
|---|---|---|---|
| 1 | $1.33 \times 10^9$ | $1.32 \times 10^9$ | $1.10 \times 10^9$ |
| 2 | $1.35 \times 10^9$ | $1.34 \times 10^9$ | $1.08 \times 10^9$ |
| 3 | $1.34 \times 10^9$ | $1.34 \times 10^9$ | $1.08 \times 10^9$ |
| Average | $1.34 \times 10^9$ | $1.33 \times 10^9$ | $1.09 \times 10^9$ |
| Repeatability | 0.84% | 1.1% | 0.89% |
| Intermediate precision | | 12% | |

was shown with RP-HPLC for most membrane associated Sendai virus proteins [10], and less than 32% recovery for influenza viral proteins [27]. The use of a high concentration of formic acid in the mobile phase was reported to solubilize membrane proteins and improve recovery [11,12]. However, acid induced cleavage along the peptide backbone was a concern. Recently, the uses of non-porous silica-based column and monolithic column have been reported to minimize non-specific adsorption and increase recovery for influenza virus protein analysis [5,7].

We evaluated the recoveries of E1 and E2 from RP-HPLC in comparison to capsid protein using UV absorbance. Capsid protein was selected as the reference since it eluted readily from the chromatography with minimal carry-over issue. Although the fluorescent signal detected in the RP-HPLC assay provides high sensitivity, the fluorescence is sensitive to the local environment of the protein. Because of this, the signal is not proportional to the number of tryptophan residues in each protein. To calculate the relative recovery, we measured the UV absorbance of each peak at 280 nm and calculated the stoichiometry of E1, E2 and capsid protein using the theoretical extinction coefficient of each protein [28]. With Zwittergent 3-12 detergent pre-treatment, we achieved 94% recovery for E1 and 95% recovery for E2. This brought the detected molar ratio of E1:E2:capsid to 1:1:1, consistent with the theoretical composition of the VLPs.

### *3.4. RP-HPLC assay performance assessment*

The International Conference on Harmonisation (ICH) outlines expectations on pharmaceutical product development and registration. ICH Q2(R1) requires that an analytical assay that is intended for quantitative measurement of the active moiety of a drug substance or drug product needs to demonstrate linearity, accuracy and precision [29].

To evaluate linearity, a series of CHIKV VLP reference standards of 5.1–120 μg/mL were injected at 100 μL volume onto the column. The total peak area of capsid, E1 and E2 showed a linear response to the protein load with an $R^2$ of 1.000.

Precision was determined by injecting a sample containing 107 μg/mL CHIKV VLP in triplicate each day in three different days. The repeatability, calculated as the relative standard deviation (RSD) of the total peak area within each day, was 1.1%. The intermediate precision, calculated as the RSD of the averaged peak area within three days, was 12% (Table 1). The retention time did not drift during the analysis.

Accuracy was evaluated by measuring the same sample prepared at low, middle and high concentrations within the standard curve range. The accuracy was defined as the percentage of measured mass compared to the theoretical mass. The accuracy was 96–106% across the linear range of the assay (Table 2).

In summary, this RP-HPLC method is linear, precise and accurate between 0.51 and 12 μg. The linear range of this method is appropriate for CHIKV VLP drug substance or drug product testing. For example, in the recent phase I clinical study of CHIKV VLPs sponsored by National Institute of allergy and Infectious Diseases (NIAID), the drug product concentration is 40 μg/mL VLP. If we

**Table 2**
Assay accuracy evaluated at three concentration levels.

| | Theoretical mass (μg) | Measured mass (μg) | Accuracy |
|---|---|---|---|
| Low | 0.64 | 0.61 | 96% |
| Middle | 3.2 | 3.1 | 98% |
| High | 10.7 | 11.4 | 106% |

inject 100 μL volume of the Zwittergent3-12 detergent treated drug product for RP-HPLC analysis, the total protein mass is 2.0 μg which is within the linear range of this quantitative method.

### 3.5. Impurity monitoring to support process development

Monitoring impurity clearance throughout the manufacturing process is vital for successful vaccine product development. Traditionally, SDS-PAGE has been used to monitor purity for subunit vaccines. We demonstrate here that RP-HPLC can be applied to analyze purification intermediate samples to support process development. Fig. 3 shows an overlay of two process intermediate samples. The chromatograms clearly show that capsid, E1 and E2 are enriched in the downstream sample (solid line). This method can also be used to assess lot-to-lot consistency for qualitative and quantitative analysis of impurity. Because RP-HPLC can be automated and has a high throughput, it is an ideal alternative to the traditional SDS-PAGE method.

### 3.6. Monitoring E2 degradation to support formulation development

The function and stability of alphaviruses are greatly impacted by pH. At pH 6.0, alphaviruses undergo an irreversible conformational change that exposes the E1 fusion loop and triggers membrane fusion [18]. Consistent with this we observed that CHIKV VLPs aggregated when exposed to pH 6.0 (data not shown). In order to develop a stable vaccine, formulation studies were carried out under various conditions. Storing CHIKV VLPs at 25 °C and pH 9 for one week led to a ~20% decrease in reactivity/binding to a neutralizing Ab. Interestingly, the pH 9.0 stressed sample presented an altered elution profile on RP-HPLC (Fig. 4). While capsid and E1 eluted similarly as the control, an E2 degradant peak was detected that eluted slightly earlier than the regular E2, suggesting that the degradant is more hydrophilic. 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. RP-HPLC can then be used as a rapid method to monitor the chemical stability of CHIKV VLP.

### 3.7. Monitoring glycoprotein post-translational modifications as a result of a change in cell substrates

Cell substrate influences viral glycoprotein post-translational modification. For example, the N-glycosylation pathway in insect

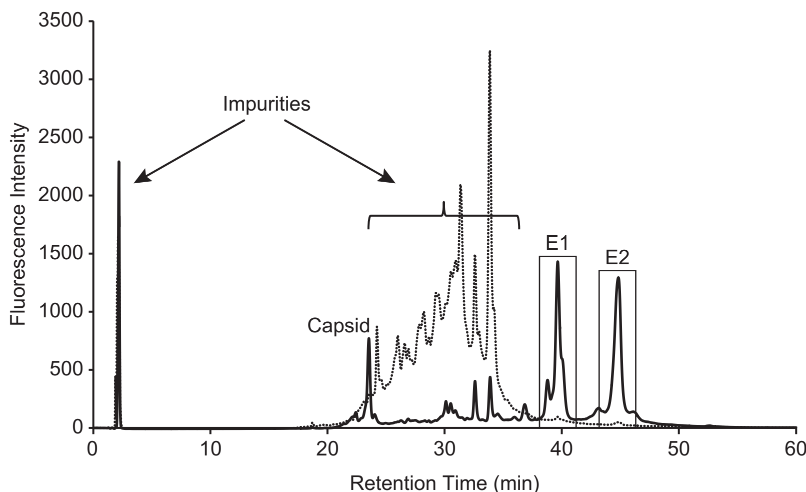


**Fig. 3.** Overlay of two RP-HPLC traces representing an upstream process intermediate sample (dotted line) and a downstream process intermediate sample (solid line). It is noted that the E1 and E2 peaks from the upstream sample, although small, are detectable above the baseline. It is evident that the RP-HPLC can be used to track impurity clearance throughout the process and monitor product purity.

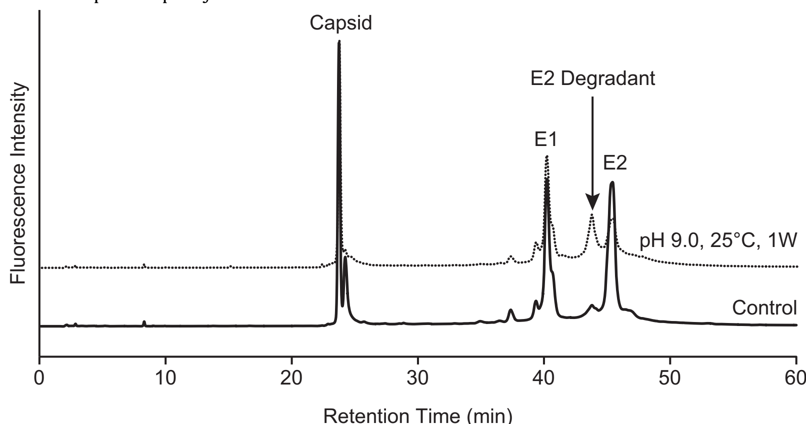


**Fig. 4.** RP-HPLC trace of a CHIKV VLP sample after storage at pH 9.0, 25 °C for one week. The E2 degradant peak elutes earlier than E2, suggesting E2 undergoes deamidation degradation.

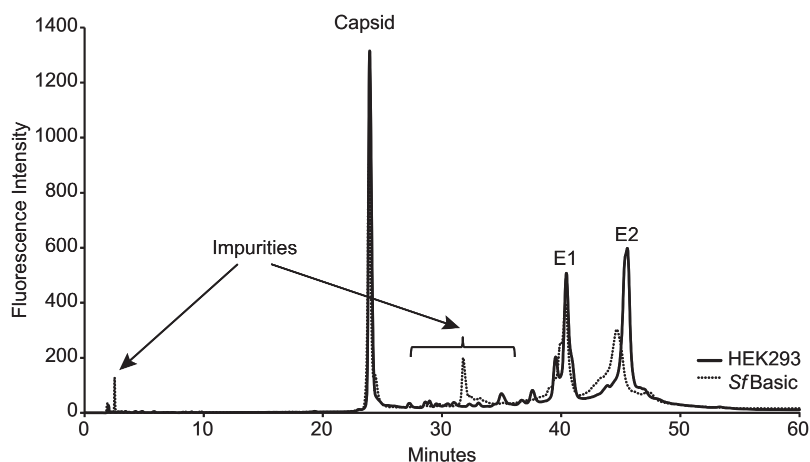


**Fig. 5.** RP-HPLC traces of two purified CHIKV VLP samples derived from two different cell substrates. The change in E2 retention time is likely due to different post-translational modifications. Additionally, different impurity profiles are seen in these two samples.

cells diverges from mammalian starting from the intermediate of $GlcNAcMan_3GlcNAc_2Fuc$ in the Golgi. Instead of elongating the intermediate to produce complex glycans as in mammalian cells, insect cells trim the non-reducing GlcNAc to produce the major N-glycan $Man_3GlcNAc_2Fuc$ (paucimannose) [30,31]. To express CHIKV VLP in insect cells, Wagner et al. developed a novel insect cell line—*Sf*Basic by adapting Sf21 in elevated culture pH [19,32,33]. E2 from VLPs expressed in this cell substrate had different elution profile compared to HEK293 expressed VLP (Fig. 5). Further studies are being conducted to characterize the post-translational modification of the glycoproteins derived from *Sf*Basic cell line. While the impact of post-translational modifications on immunogenicity or stability of CHIKV VLPs is unknown, it is important to monitor these modifications to ensure product lot-to-lot consistency throughout the vaccine development cycle.

## 4. Conclusion

We developed a RP-HPLC method that separates capsid, E1 and E2, and allowed the characterization and quantitation of CHIKV VLP components. This assay is accurate, and has a linear range of 0.51–12 μg protein and a precision of 12% RSD. 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.

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