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Validation of a high performance liquid chromatography method for quantitation of foot-and-mouth disease virus antigen in vaccines and vaccine manufacturing

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ABSTRACT

Foot-and-mouth disease (FMD) is an infectious viral disease that affects the main meat and dairy production animals, including cattle, sheep, goats and swine. It is readily transmissible and countries where the disease is present suffer harsh international trade restrictions on livestock products and serious economic losses. Vaccines are important tools to contain outbreaks and maintain the status of free with or without vaccination, as defined by the World Organization for Animal Health (OIE). The efficacy of vaccines is reliant on the content and integrity of inactivated virus particles. The long-established method to quantify the viral content of vaccines along the manufacturing process and in the final product is the 140S sucrose density gradient analysis. This method has been a valuable tool for many decades. However, it requires gradient preparation for each sample, a lengthy ultracentrifugation and a manual UV reading of the gradient, rendering it highly operator dependent and almost impossible to automate. We present a method to quantify FMDV particles in vaccines and intermediate process samples that is based on separation of components by size exclusion high performance liquid chromatography (SE-HPLC) and measurement of virus by absorption at 254 nm. The method has been extensively validated; it is accurate, precise and linear. It is applicable to all FMDV strains and sample materials and has a good concordance with the 140S test. The proposed method uses off the shelf HPLC equipment and columns. It is easily automated for high throughput operation, affording a useful process analytical technology and a novel tool for control of final product by manufacturers and regulatory agencies.

1. Introduction

Foot-and-mouth disease (FMD) is an acute viral infection that affects cloven-hoofed *Artiodactyla*, including cattle, sheep, goats and swine as well as several wild species. Due to its highly contagious nature and concomitant economic losses, it is arguably the most serious disease of the livestock industry.

Stringent control programs and potent vaccines have assisted in eradicating the disease in most developed countries but the disease is still endemic in several regions of the world. Periodically, OIE publishes disease distribution and outbreak maps and the FMD status as reported by the OIE has a severe impact in economies with high reliance on meat trade. The occurrence of an outbreak usually entails the withdrawal by the OIE of the "free from FMD without vaccination" or "free from FMD with vaccination" status from the affected nation or region. Normally, national states responses involve a total or partial ban of imports of meat or products of animal origin from the afflicted country or zone [1]. Despite these strict international trade restrictions, major outbreaks have occurred relatively recently in Europe (2000, 2001) [1], in Japan (2000, 2010) [2], Korea (2000, 2002, 2010, 2011, 2014) [3–5] and the disease is widespread in mainland China, Southeastern Asia and Africa [1].

Most vaccines commercially available at this time are based on whole virus particles prepared in BSL-3 biosafety facilities and chemically inactivated.[1] Over two billion vaccine doses are manufactured and applied worldwide annually [1].

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[1] A few exceptions being vaccines using synthetic peptides as antigens approved and distributed only in the mainland China market (China Animal Husbandry Industry Co., Ltd., Lanzhou; Tecon Biology Co., Ltd., Ili Kazakh; China Agricultural Vet Bio Science and Technology Co., Ltd., Lanzhou; Shang Hai Shen Lian Biomedical Co. Ltd., Shanghai).

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The efficacy of inactivated virus vaccines is highly dependent on virus integrity [6,7]. FMDV is a non-enveloped virus with icosahedral symmetry and about 30 nm diameter. It is very labile in vitro, as whole particles dissociate into monomers at temperatures above 56 °C, pH below 7 [8,9] and pH above 11 [10]. The 140S quantitative sucrose density gradient analysis as developed by Barteling et al. in 1974 [11], is the recommended method [12,13] to measure the viral antigen concentration, both in intermediate manufacturing materials and in final vaccines. For more than forty years, this methodology provided a tool for virus concentration measurement. It entails the ultracentrifugation of the sample in a sucrose concentration gradient from about 20% to 45% and the scanning of gradient absorbance at 254 nm using a flow-through UV monitor. The sucrose gradients are prepared either by layering sucrose solutions of decreasing concentration with a pipette or using automatic gradient formers that easily produce linear gradients with good reproducibility. An international effort to standardize the method has been carried out more than three decades ago [14] but there is currently no uniform protocol adhered to by manufacturers or regulatory agencies [15]. The technical burden of the method, the requirement of specialized equipment and staff training has certainly contributed to this state of affairs.

The lack of a standardized method to measure the active ingredient in vaccines is a main contributor to the continued requirement of expensive and cumbersome tests for vaccine registration and batch release. Such tests involve the use of a substantial number of large animals from the target species, special biosafety facilities, lengthy trials lasting from 30 to 90 days and a great deal of animal suffering.

Liquid chromatography has been extensively used for analytical and preparative purposes in the field of viral and VLP-based vaccines for human use or gene therapy viral vectors [16–19]. As an analytical technique it allows fast measurement of the analyte of interest as well as contaminants that may be present in the sample. Size exclusion chromatography, also called gel permeation chromatography (GPC) is a separation principle based only on particle size and is independent of particle charge, buffer pH and ionic strength, making it particularly suitable as an analytical technology applicable across a wide range of process stages. Previously, we described a low pressure methodology for FMDV quantification based on Fast Protein Liquid Chromatography (FPLC) [20]. In the present work we describe a high throughput method based on High Performance Liquid Chromatography (HPLC) which was developed, deployed and validated for use in FMDV vaccine manufacturing and quality control.

2. Materials and methods

2.1. Virus strains

South American FMDV vaccine strains O1 Campos, A24 Cruzeiro, A2001 Argentina and C3 Indaial, originally derived from field isolates were obtained by Biogénesis Bagó S.A. from SENASA (Argentine National Animal Health Service). The received viruses were propagated in BHK21 Clone 13 cell to form the master virus seed bank (MVSB). Once the virus identity and purity in MVSB were confirmed to current Good Manufacturing Practices (cGMP) requirements, working virus seed banks (WVSB) were prepared for infection of the industrial scale BHK21 Clone 13 cell suspension cultures used in vaccine production. Virus suspensions were inactivated with ethyleneimine as described elsewhere [21].

2.2. Samples of manufacturing process materials

For SE-HPLC method development and validation, samples of intermediate manufacturing materials containing inactivated virus were taken from production processes of commercial vaccine batches.

Five different materials were sampled: 1. cell culture infection supernatant (CCIS), 2. concentrated materials obtained by polyethylene glycol precipitation procedure (PEG conc), 3. concentrated materials obtained by ultrafiltration procedure (UF conc), 4. aqueous phases before vaccine emulsification process (AqPhase), 5. aqueous phases recovered from vaccines by solvent extraction (AqPhase recov).

These five materials are key steps in FMD vaccine manufacturing processes that had been in regular use for many years at the time of SE-HPLC method development. Therefore, their respective typical antigenic mass content ranges were known from the 140S sucrose density gradient analysis routinely performed for quality control purposes during manufacturing. This previous knowledge was used to guide the selection of appropriate ranges of antigenic mass for the validation study of the SE-HPLC method.

2.3. Samples pretreatment before chromatography

To recover aqueous phase from water-in-oil vaccines, about 20 ml of sample were transferred to a 50 ml centrifuge tube. A volume of 20 ml of chloroform chilled to 2–8 °C was added and thoroughly mixed with a Vortex Genie (Scientific Industries, Inc., Bohemia, NY, U.S.A.) and manual shaking for 5 min. The resulting emulsion was broken by centrifugation at 4000*g* for 5 min, the upper aqueous phase was recovered without disturbing the interface and chloroform extraction was repeated one more time. PEG conc materials were homogenized by mixing thoroughly with a magnetic stirrer for 30 min and then 10 ml were transferred to a flask containing 90 ml of the proprietary buffer used for commercial vaccine formulation. This 1:10 dilution was stirred for 12–16 h at 2–8 °C, to allow for complete resuspension of viral particles once PEG concentration became reduced tenfold. Then a 6 ml aliquot was transferred to a 15 ml centrifuge tube and extracted with an equal volume of chloroform as described for the vaccine. UF conc materials were diluted by adding 0.5 ml of 20 mM Tris-HCl buffer pH 8–1 ml of concentrated material (2:3 dilution factor) in order to reduce the ionic strength of the suspension to facilitate subsequent enzymatic digestion, as Benzonase® endonuclease is inhibited by high concentration of monovalent cations [22]. CCIS and AqPhase materials were nuclease digested without pretreatment.

2.4. Nuclease treatment of samples for cell DNA digestion

For host cell DNA digestion, Benzonase® (Merck KGaA, Darmstadt, Germany, Sigma-Aldrich, St. Louis, MO, U.S.A., E1014-25KU, 250 Units/μL, purity above 90%) was used. The commercial enzyme preparation was kept at −20 °C and a working solution prepared by 200-fold dilution to a final concentration of 1.25 Units/μl with Benzonase® dilution buffer (50 mM Tris, 20 mM NaCl, 2 mM $MgCl_2$). Working solution was kept at 2–8 °C for up to a week. For digestion, 1 ml of pretreated samples were transferred to 1.5 ml microcentrifuge tubes, 20 μl of Benzonase® working solution was added and the tube incubated at 37 °C and 1400 rpm for 1 h in an Eppendorf Thermomixer (Eppendorf AG, Hamburg, Germany). After digestion, the tubes were centrifuged at 16,000*g* and 2–8 °C for 10 min. Finally, 900 μl were transferred to the HPLC vials for chromatographic analysis.

2.5. SE-HPLC chromatography

SE-HPLC runs were performed using an Agilent 1260 Infinity System (Agilent Technologies, Santa Clara, CA, U.S.A.), composed by a G1311C quaternary pump with integrated degasser, a

G1329B standard autosampler with extended volume sample loop, a G1330B thermostat module with Peltier device to control sample temperature in autosampler tray, a G1316A thermostatic column compartment and a G1314F variable wavelength detector. Control and analysis software used was OpenLAB CDS ChemStation Edition Rev. C01.02 14.

The chromatograph was fitted with a TSKgel G4000PWXL column (7.8 mm ID × 30.0 cm L, TOSOH Bioscience LLC, Tokyo, Japan). In order to extend column life, a TSKgel PWXL Guardcol guard column (6.0 mm ID × 4.0 cm L, TOSOH Bioscience) was fitted in tandem upstream of the main column.

Mobile phase used was 400 mM sodium chloride, 30 mM Tris, adjusted to pH 8 with hydrochloric acid.

The column compartment thermostat was set at 20 °C and the autosampler thermostat module to 4 °C. The runs were performed using an isocratic flow of 0.5 ml/min. Injection volume was 100 μl except for CCIS samples which was set to 300 μl injection volume. Detection was performed at 254 nm.

2.6. Antigen mass calculation from virus peak area

Agilent Openlab CDS ChemStation Edition software reports responses as peak areas measured in *mAU*sec*, where peak width units are sec and peak height units are miliAbsorbance Units (*mAU*). Antigen mass was derived from the peak area using the following calculation:

$$\textit{Virus concentration}\ [\mu g/ml] = \frac{\textit{Area}\ [\text{mAU} * \text{sec}] \times 10 \times 1.02}{120 \times 72 \times \textit{Injection volume}\ [\text{ml}]}$$

where

Area: is the value of peak area reported by Openlab CDS ChemStation software in mAU*sec.
Injection volume: is the sample volume injected in the column, in ml.
120: is the number of sec required for 1 ml of mobile phase to flow through the detector cell.
72: is the published [11] specific absorptivity $E^{1\%}_{254nm}$ of the FMDV used in the 140S reference method, for 1 cm optical path cell.
1.02: is a correction factor introduced to correct the small dilution introduced by adding 20 μl of Benzonase® working solution to 1000 μl of sample.
10: is a units conversion factor.

2.7. Purified virus standard preparation

In order to prepare a purified, chromatographically homogeneous virus preparation for use in validation assays, concentrated virus material was treated as follows:

- Dialysis against four changes of 4 volumes of low ionic strength buffer (100 mM NaCl, 10 mM MgCl2, 50 mM Tris-HCl, pH 8) for 2 h at 4 °C while mixing with a magnetic stirrer was performed. A final overnight dialysis against 8 volumes of buffer was carried out in the same conditions. The purpose of this dialysis is to lower ionic strength of the virus suspension to allow enzymatic digestion in the next step.
- Dialyzed virus suspension was stirred for 2 h at 37 °C with 25 IU/ml of Benzonase® to digest the host cell DNA into low molecular weight fragments. Any precipitates formed were removed by centrifugation at 9000*g* for 15 min.
- Buffer exchange and elimination of low molecular weight contaminants was carried through a desalting column packed with 150 ml of Sephacryl S-300 GPC media. Alternatively, several runs in an Äkta Purifier UPC-10 chromatography system fitted with a HiPrep 16/60 Sephacryl S-400 HR column (GE Healthcare Life Sciences, Chicago, IL, U.S.A.) were performed, the eluted virus peaks were collected discarding head and tail and the peak middle fractions were pooled.
- Final concentration was achieved by centrifugation at 2000*g* in Centricon PLUS-70 concentrators (Merck KGaA) with 30 kDa cutoff membranes.

2.8. Validation of analytical method

The goal of the validation procedure was to provide documented information indicating that the method consistently produced results adequate for the intended analytical purpose. Validation was conducted following Biogénesis Bagó S.A. standard operation procedures (SOP) and according to FDA guidelines [23]. Acceptance criteria for the different analytical parameters and materials were set forward in validation protocol in advance of the actual measurements. Unless otherwise stated, the acceptance ranges for results were set at plus or minus 15% of the target value. This somewhat wide range of acceptance is SOP for biological entities such as the FMD virus, as they are usually quantified using end-point titrations, bioassays or immunochemical techniques which are inherently less precise than physicochemical methods like SE-HPLC.

2.8.1. Specificity

The purpose of this test was to demonstrate that the HPLC method is able to discriminate and quantify the FMDV when other sample components are present. Heating above 60 °C even for a short time has long been known to completely abolish FMDV infectivity and disassemble the 140S particle into ribonucleic acid and 12S particles [8]. Heat treatment also caused virus peak to disappear in GPC chromatography without substantial changes in chromatographic profile [20]. To assess SE-HPLC method specificity for FMDV, samples of all the five materials listed under *Samples of manufacturing process materials* were subjected to thermal treatment at 60 °C for 2 h and then analyzed by SE-HPLC. Aliquots of the same samples were subsequently spiked with purified virus standard prepared as previously described and analyzed in the same way.

2.8.2. Repeatability

This procedure was performed to evaluate the ability of the method to produce results that closely agree to each other when the measurements are taken by a single person or instrument on the same item, under the same conditions, and in a short period of time. Repeatability was evaluated for each one of the five types of material listed under *Samples of manufacturing process materials*.

2.8.3. Intermediate precision

In this test the repeatability was appraised under more realistic laboratory conditions, i.e. performed by different operators in different days and batches of mobile phase. Materials of three different levels of antigen concentration were tested, i.e. cell culture infection supernatant, aqueous phase before emulsification and concentrated materials obtained by UF procedure.

2.8.4. Accuracy

This assay evaluated how close the results reported by the method were to the true value of the analyzed samples. To assess accuracy, samples of CCIS, PEG conc, AqPhase and AqPhase recov were heated at 60 °C for 2 h to destroy virus peak while keeping the sample matrix composition unaltered, except for the virus disassembly products added. Once cooled down to 2 °C to 8 °C, the heat treated samples were spiked with purified virus standard to a concentration typical of the material being evaluated. The char-

acteristic concentration range of these different virus containing materials was known beforehand from the 140S sucrose density gradient analysis performed for many years while manufacturing vaccine batches.

The concentrated suspensions obtained with the UF procedure were a special case. They had a rather high virus concentration and a less complex matrix than the other sample types, so the virus represented a higher proportion of total components and the virus thermal destruction procedure used for other samples would not have produced a realistic background composition comparable to the one present in real manufacturing samples. Therefore, for UF conc, accuracy was estimated by the *standard addition* procedure [24]. In order to maintain the inactivated virus in a matrix of constant composition, samples of UF conc were diluted with the same proprietary buffer used in the concentration and diafiltration manufacturing procedure.

2.8.5. Linearity

To probe the proportionality of area responses to injected antigen mass, linearity was evaluated for all five materials listed under the *Sample* paragraph. Each linearity curve was constructed at 5 levels. For each level, 2 independently prepared samples were run. Levels were prepared by spiking different amounts of purified virus standard into the respective materials depleted of virus as described before, except for UF conc materials. For CCIS, levels of 50%, 100%, 125%, 150% and 200% of the typical 5–7 μg/ml were used. For PEG conc, AqPhase and AqPhase recov, levels were prepared at 25%, 50%, 100% and 200% of the typical concentrations in manufacturing process. UF conc samples were injected without modification for the 100% level, diluted 1:2 and 1:4 with final diafiltration buffer for 50% and 25% levels respectively. For 125% and 150% levels, injections of 125 μl and 150 μl were performed. Acceptance criteria were: (1) that r^2 must be equal or above 0.99, (2) slope must be in the range of ±10% of the theoretical[2] value (254.1 *mAU**sec/μg/ml for 300 μl injection volume, 84.7 *mAU**sec/μg/ml for 100 μl injection volume and 56.5 *mAU**sec/μg/ml for 100 μl injection volume of UF conc diluted 2:3), and (3) X-intercept value should not differ from origin in more than 10% of the average of lowest and highest values in the tested range.

2.8.6. Limit of quantitation (LoQ) and limit of detection (LoD).

These limit assays inform about the minimum concentration that can be measured with accuracy and precision (LoQ) or that can be just detected without necessarily being possible to quantify it (LoD). These parameters were evaluated for the lower concentration material, i.e. CCIS. A sample of this material, heat treated as described before was spiked at 50% and 15% of the typical concentrations found in production cycle to determine LoQ and LoD respectively by the signal-to-noise (S/N) method as implemented in the OpenLAB CDS ChemStation Edition software, with S/N > 10 for LoQ and S/N > 3 for LoD.

2.8.7. Robustness

This procedure was intended to gauge the tolerance of the method to slight variations in operating conditions and mobile phase composition that might arise during normal day-to-day use. Three concentration levels of concentration in CCIS, AqPhase and UF conc were analyzed. The modified parameters were: (1) Concentration of NaCl in mobile phase ±5%, (2) pH of mobile phase ±0.5, (3) Change of the thermostatic column compartment temperature from 20 °C to 25 °C, (4) Change of thermostat module temperature for holding vials in autosampler queue from 4 °C to 8 °C and (5) Mobile phase age from freshly prepared to 7 days.

2.8.8. Analytical sample stability

In order to address possible issues with the stability of samples while they are waiting in the autosampler queue, pretreated samples of all five aforementioned materials were injected in duplicates, either immediately after preparation or after at least 24 h spent in the refrigerated autosampler tray.

2.8.9. Concordance analysis with ultracentrifugation 140S results

Results obtained with the HPLC method were compared to the hitherto standard 140S ultracentrifugation method for FMDV antigen content determination. A total of 479 samples comprising CCIS, PEG conc, AqPhase and AqPhase recov were analyzed both by HPLC and by the 140S method in a period from 2012 to 2013. The four FMDV strains listed in the *Viruses* section above were represented in each material. Concentrated materials obtained by the UF procedure were not part of this study, as they were not in wide use in Biogénesis Bagó S.A. manufacturing facility at the time. The ordinary least-squares regression method is unsuitable to compare results from two analytical methods [25] because it requires that the *X*-axis values are fixed by experimental design whereas in method comparison both *X*-axis and *Y*-axis values are free to vary and are subject to error. Therefore, the Concordance Correlation Coefficient method [26,27] was used for this study, using MedCalc ®version 11.6 (MedCalc Software bvba) for data analysis. Further, in order to facilitate comparisons between results of the two methods, ratios of results from the SE-HPLC method to the ultracentrifugation 140S method (HPLC/140S) were determined for each material and virus strain and medians and arithmetic means were calculated.

3. Results

3.1. SE-HPLC chromatography

With adequate sample pretreatment, the chromatographic conditions described produced a Gaussian shaped peak with an elution volume from about 15 to 16 min (i.e. 7.5 to 8 ml) and a peak base from 14 to 18.5 min (Fig. 1A). UV absorbing low molecular weight components in the sample eluted after 19 min so they were completely resolved from the virus peak.

3.2. Purified virus standard preparation

The purified virus standard produced a concentration of 374 μg/ml when measured spectrophotometrically. This purified virus standard was chromatographically homogeneous as it produced a single peak both in the HPLC run (Fig. 1B) and in the FPLC system as described elsewhere [14]. Chromatographic measurements differed from UV spectrophotometric determinations by less than 1% (data not shown).

3.3. Validation of analytical method

3.3.1. Specificity

The preset acceptance criteria for this parameter were lack of any peaks at the virus elution volume in the heat treated samples and absence of any interfering peaks in the spiked samples. No peaks were detected at the elution time of FMDV peak in placebos prepared by thermally destroying FMDV. Virus peaks in spiked placebos were well resolved from nearby matrix and from degradation products peaks. Fig. 2 shows an example of compared chromatographic profiles of placebo (Fig. 2A) and virus spiked placebo

[2] Theoretical values for slopes were calculated by replacing the appropriate volume for *Injection volume* [ml] in the equation given in the section *Antigen mass calculation from virus peak area* and solving for the slope, i.e. the ratio Area [*mAU**sec]/Virus concentration [μg/ml].

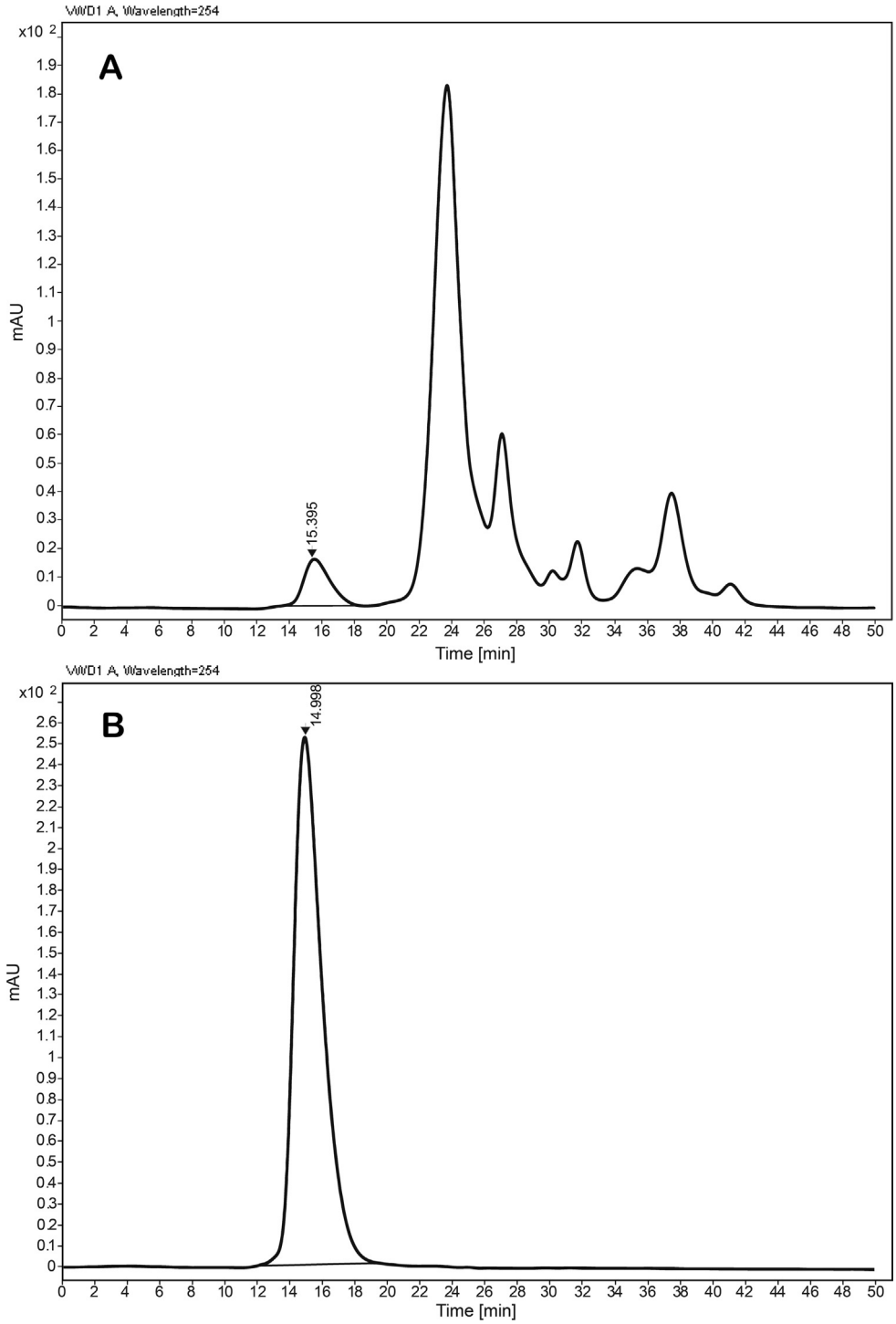

Fig. 1. (A) Chromatogram of a concentrated material obtained by PEG precipitation procedure. The concentrated material was diluted 1:10, stirred overnight and nuclease digested as described in Materials and Methods. (B) Chromatogram of a purified standard of FMDV showing a single virus peak.

(Fig. 2B) of an aqueous phase, sampled before its use in the vaccine emulsification process.

3.3.2. Repeatability

For each one of the five types of materials intended to be measured by SE-HPLC, five independent sample pretreatments and nuclease digestions were performed. Each pretreated and digested sample was run in duplicate in SE-HPLC. With the antigen mass results acquired in this way, the usual statistics of mean, standard deviation and percent relative standard deviation (%RSD) were calculated. Acceptance criterion was preset in the validation protocol as %RSD should be not greater than 10% for every material evaluated.

The repeatability results observed were satisfactory, with %RSD ranging from 0.38% for the pre-emulsion aqueous phase to 2.20% for the cell culture infection supernatant. The CCIS is the most challenging material for precise measurements, because it has the lowest mean antigen content (6.06 μg/ml in this case). Therefore, the

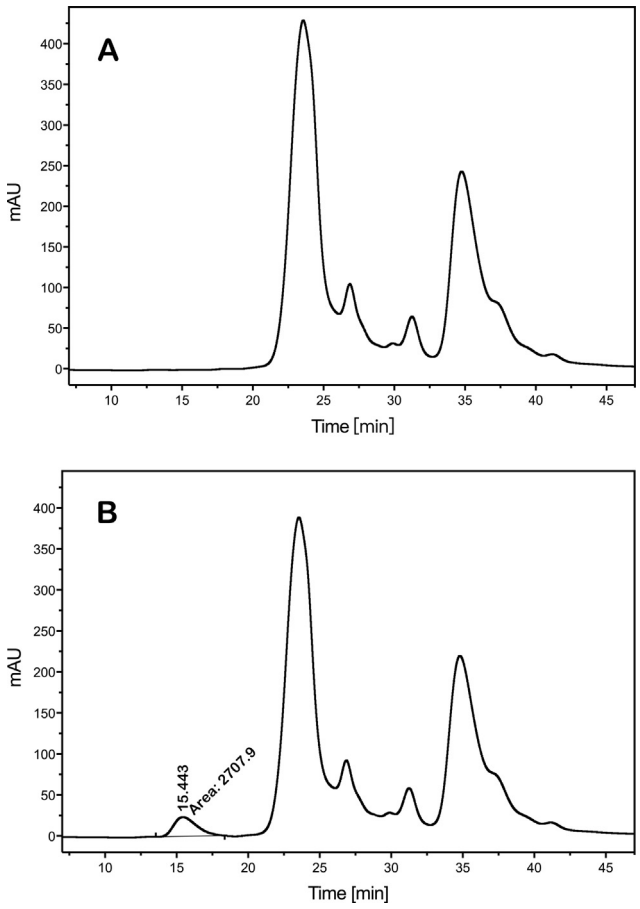

Fig. 2. Compared chromatographic profiles of placebo before (A) and after (B) spiking with purified virus standard. The placebo was prepared by thermally destroying the FMD for 2 h at 60 °C in an aqueous phase sample. The spiked virus peak is well separated and elutes ahead of other sample components.

acceptance criterion of %RSD < 10% was easily met for all five materials analyzed.

3.3.3. Intermediate precision

For each material analyzed, two operators in different days performed five independent sample pretreatments. Each pretreated sample was analyzed with duplicate injections into the chromatography system. The acceptance criteria for this parameter were set as that the difference in average between the two operators must be not greater than 10% of value and variances should not be significantly different in the Snedecor *F*-test (P > 0.05). The results shown in Table 1 indicate that the SE-HPLC method is capable of producing results that are not influenced by the analyst performing the measurements or when repeated measures were carried out in different days, or using different batches of mobile phase. The small differences observed between operators or workdays would be of no practical significance for manufacturing or formulation decision making processes.

3.3.4. Accuracy

As there are no inactivated FMDV standards available from authoritative sources, accuracy was assessed using the purified, chromatographically homogeneous virus preparation described above for sample spiking. For CCIS, PEG conc, AqPhase and AqPhase recov, five independent pretreatments and nuclease digestion procedures were performed to samples of each material spiked to a concentration within the characteristic range for the material during the manufacturing cycle, as described in *Materials and methods*. For each sample treatment, duplicate injections were performed and recovery values calculated as % of the spiked concentration. Acceptance criterion was that the % recovery must be within 85–115% of the value calculated from the spiked amount of purified virus standard. For concentrated suspensions produced by the UF procedure, samples were brought to a concentration of about 100 μg/ml by dilution with the proprietary diafiltration buffer, spiked to 125%, 150% and 175% of the nominal value and run in duplicates. The acceptance criteria were that %recovery for each spiking level must be in the 85–115% range and the value measured for the 100% level must also not differ from the X-intercept of the *standard addition curve* [24] in more than ±15%.

Both recovery calculation and standard addition assays demonstrated that the area responses obtained produced accurate quantitative estimates of the FMDV content of the analyzed samples. The results are listed in Table 2, section (a) for CCIS, PEG conc, AqPhase and AqPhase recov, and section (b) for UF conc.

Table 1
Intermediate precision results. Comparison of results attained by two different operators in different workdays. Five independent sample pretreatments were performed and injected in duplicates. The differences between operators were below 2% for all the three types of samples tested and the differences in variance were not significant ($p > 0.05$).

	CCIS	***UF conc***	***AqPhase***
$\bar{x}$ **(μg/ml) Operator 1**	6.07	430.42	27.00
$\bar{x}$ **(μg/ml) Operator 2**	6.09	427.80	27.52
% difference	0.33	0.61	1.91
s **(μg/ml) Operator 1**	0.13	2.70	0.19
s **(μg/ml) Operator 2**	0.09	6.88	0.08
F **test**	$p = 0.845$	$p = 0.204$	$p = 0.760$

$\bar{x}$: mean, ***s***: standard deviation, FMDV containing materials: ***CCIS***: Cell culture infection supernatant, ***UF* conc**: Concentrated materials obtained by the UF procedure, ***AqPhase***: Aqueous phases before vaccine emulsification process.

3.3.5. Linearity

Validation results showed that the area responses exhibited an excellent linear response in all three concentration ranges of interest in vaccine manufacturing, i.e. the low level in virus crude supernatants of infected cell cultures, the intermediate concentration of aqueous phases used in vaccine formulation and the highly concentrated stocks (with concentrations above 600 μg/ml) prepared by ultrafiltration for antigen handling and storage. The linearity results are outlined in Table 3 and the results for vaccine aqueous phases are plotted in Fig. 3.

3.3.6. Limit of quantitation (LoQ) and limit of detection (LoD)

A LoQ value of 2.70 μg/ml was obtained with a S/N ratio of 154, i.e. much higher than the acceptance criteria of S/N > 10. The LoD was of 0.6 μg/ml with a S/N of 37, again much higher of the S/N > 3 required for acceptance.

The LoD and LoQ determined in the validation show that the method is sensitive enough for quantitative analysis of FMDV in all materials of practical interest in vaccine manufacturing and control. The culture medium after cell infection is the material with the lowest virus concentration along the production process, with values ranging routinely from 5 to 8 μg/ml as measured by the 140S sucrose density gradient analysis, i.e. about two to three times above the LoQ determined in the validation study.

3.3.7. Robustness

Some deviations in operational conditions and changes in workloads are unavoidable in the laboratories of manufacturing facilities and regulatory agencies in charge of vaccine monitoring and approval. To appraise the possible impact of these factors, small

Table 2
Accuracy results.

(a) Recovery of virus spiked into thermally generated placebo was measured in five independent treatments of each sample type.

	CCIS	*PEG conc*	*AqPhase*	*AqPhase recov*
Spike (μg/ml)	**6.4**	**19.9**	**32.5**	**34.2**
% recovery #1	89.2	90.3	93.8	88.8
% recovery #2	89.0	91.3	94.0	89.0
% recovery #3	88.7	90.7	94.7	88.7
% recovery #4	88.7	91.1	94.6	89.3
% recovery #5	92.7	91.1	98.5	88.9

(b). Accuracy assessment in concentrated material produced by ultrafiltration using the method of *standard addition* [23]. For the 125–175% spiking levels, recoveries were measured directly. For the 100% level, % recovery was calculated as 100 times the ratio of direct measurement $\bar{x}$ to the X-intercept of the *standard addition* plot.

UF conc

Level (*)	**Spike (μg/ml)**	**Measured spike (μg/ml)**	**% recovery**
100%	0.00	0.00	–
125%	32.2	29.9	92.8
150%	64.3	60.8	94.5
175%	96.2	94.9	98.7
Level (*)	**$\bar{x}$ (μg/ml)**	**X-intercept (μg/ml)**	**% recovery**
100%	91.9	87.4	105.2

$\bar{x}$: mean. FMDV containing materials: ***CCIS***: Cell culture infection supernatant. ***PEG conc***: Concentrated materials obtained by the PEG precipitation procedure. ***UF conc***: Concentrated materials obtained by the UF procedure. ***AqPhase***: Aqueous phases before vaccine emulsification process. ***AqPhase recov***: aqueous phases recovered from vaccines by solvent extraction. (*) From standard addition curve.

intentional modifications were introduced in the conditions of the analytical procedure. The results acquired in these slightly different conditions were compared to the results obtained when the same samples were analyzed in the standard ('datum') conditions specified for the method of analysis. For each material type and modified parameter analyzed, duplicate injections were performed. Acceptance criteria were that recovery for each modified condition should be in the 85–115% of the average in datum conditions and %RSD should not be more than 10% in any of the modified conditions.

Results for *Robustness* are presented in Table 4 showing that neither changes in pH, composition or age of mobile phase nor the temperature of the column oven produced results exceeding the acceptance range or had significant impacts on result means or recoveries. These results indicated that the method is tolerant enough of these variations to be of practical application in vaccine control or manufacturing contexts.

3.3.8. Analytical sample stability

Albeit the nominal queue time of a sample waiting for injection was preset at 24 h in validation protocol, for practical and logistical reasons actual waiting times for samples in performed experiments were somewhat longer, ranging from 26.5 to 29 h, i.e. much longer than the typical delays in a laboratory workday. The criteria set forward to accept sample stability were that average recovery after 24 h or more should be in the 85–115% range of the average for immediately injected samples. Even with these longer waiting periods, results were all well within acceptance range, with values varying from 99.3% recovery for aqueous phase to 107.7% recovery for concentrates obtained by the PEG procedure.

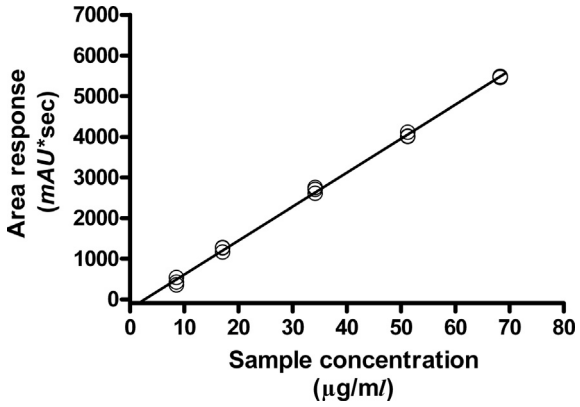

Fig. 3. Linear response of SE-HPLC analysis of aqueous phase recovered from vaccine by chloroform extraction.

3.3.9. Concordance analysis with ultracentrifugation 140S results

Concordance analysis showed good agreement between HPLC and ultracentrifugation 140S method (Fig. 4). The concordance correlation coefficient ($\rho_c = \rho\ C_b$) for all the samples considered together was 0.9725, with a 95% confidence interval of 0.9681–0.9763. The Pearson ρ_c (precision) factor was 0.9848, while the bias correction factor C_b (accuracy) was 0.9875. The value of ρ_c was within the "substantial" range in the McBride strength of agreement scale [28]. The concordance coefficients for individual materials and strains were lower, particularly when the number of samples for a certain strain and material was low and for materials with high 140S uncertainty, like PEG concentrates. Plus, in order to compare results from both methods in an easy to understand form, ratios of results obtained with SE-HPLC to results acquired with the 140S method for different materials were calculated and averaged. This ratio would be 1 when both methods produced the same result, above 1 when SE-HPLC produced a higher result and below 1 if the opposite was true. The actual ratios observed are given in Table 5.

4. Discussion

This validation study presents a novel SE-HPLC method for measuring the antigenic mass of FMDV in intermediate materials along

Table 3
Linearity results. Linear response of peak area to variable FMDV concentrations in samples. Slope, X-intercept and coefficient of determination for each material are shown. Acceptance ranges for slopes were set at ±10% of the theoretical slope values once injection volume and dilution factors were taken into account. For X-intercept, acceptance ranges were set as ±10% of the average between the minimum and maximum values tested in the respective concentration range of each sample type.

	Slope *mAU*sec/μg/ml*	**Slope acceptance range *mAU*sec/μg/ml***	**X-intercept μg/ml**	**X-intercept acceptance range μg/ml**	**r^2**
CCIS	254.00	228.7–279.5	0.674	± 0.746	0.9977
PEG conc	79.07	76.2–93.2	0.779	± 2.479	0.9999
UF conc	57.85	50.8–62.1	5.756	± 25.110	0.9999
AqPhase	83.77	76.2–93.2	0.655	± 3.842	0.9999
AqPhase recov	83.54	76.2–93.2	2.663	± 3.842	0.9987

FMDV containing materials: ***CCIS***: Cell culture infection supernatant, ***PEG conc***: Concentrated materials obtained by the PEG precipitation procedure, ***UF conc***: Concentrated materials obtained by the UF procedure, ***AqPhase***: Aqueous phases before vaccine emulsification process, ***AqPhase recov***: aqueous phases recovered from vaccines by solvent extraction.

Table 4
Robustness results. Mean, standard deviation and recoveries obtained when small controlled deviations from *datum* conditions were introduced, i.e. ±0.5 pH units or ±5% change in NaCl concentration in mobile phase, use of mobile phase after a week of being prepared, a + 5 °C increase in column temperature or a + 4 °C increase in sample tray temperature.

		datum	Mobile phase 7-day	Mobile phase pH 7.5	Mobile phase pH 8.5	Mobile phase 95% NaCl	Mobile Phase 105% NaCl	Column thermostat 25 °C	Sample temp. 8 °C
CCIS	$\bar{x}$	1857	1792	1911	1747	1843	1806	1658	1759
	s	19.2	47.2	11.2	100.3	40.9	29.9	36.3	96.6
	%RSD	1.03	2.63	0.58	5.74	2.22	1.66	2.19	5.49
	%Recov	–	*96.5*	*102.9*	*94.1*	*99.3*	*97.3*	*89.3*	*94.7*
***UF* conc**	$\bar{x}$	24,542	23,815	23,509	23,003	23,064	23,134	23,557	23,569
	s	39.4	135.1	262.5	353.1	457.4	457.6	65.7	93.5
	%RSD	0.16	0.57	1.12	1.54	1.98	1.98	0.28	0.40
	%Recov	–	*97.0*	*95.8*	*93.7*	*94.0*	*94.3*	*96.0*	*96.0*
AqPhase	$\bar{x}$	2332	2184	2292	2289	2311	2313	2223	2242
	s	6.1	54.0	0.1	8.1	2.0	1.7	8.3	11.9
	%RSD	0.26	2.47	0.01	0.36	0.09	0.07	0.38	0.53
	%Recov	–	*93.6*	*98.3*	*98.1*	*99.1*	*99.2*	*95.3*	*96.1*

$\bar{x}$: mean, ***s***: standard deviation, **%RSD**: relative standard deviation,**%Recov**: percent recovery, FMDV containing materials: ***CCIS***: Cell culture infection supernatant, ***UF* conc**: Concentrated materials obtained by the UF procedure, ***AqPhase***: Aqueous phases before vaccine emulsification process.

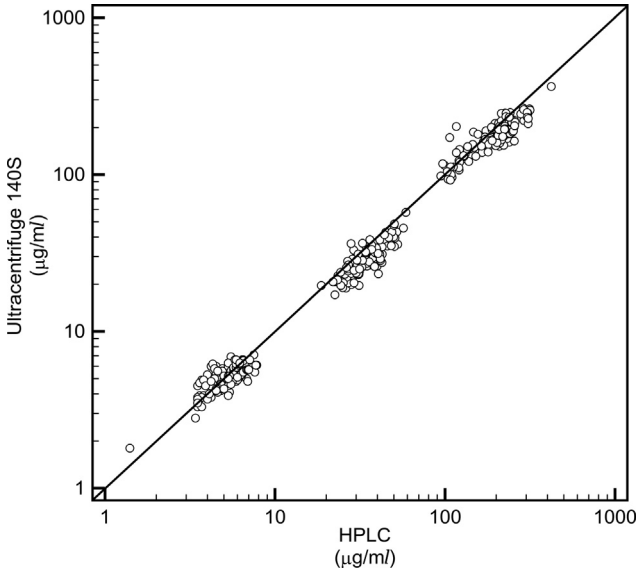

Fig. 4. Concordance correlation plot for SE-HPLC and 140S results for method comparison [26,27].

the manufacturing process and final vaccine product. The validation results show that the procedure is precise, accurate and robust, making it a suitable replacement for the commonly used 140S ultracentrifugation in sucrose concentration gradients. The 140S method has been in use since its inception in 1974 [11] and is regarded as the 'gold standard' for antigenic mass determination of FMDV. While highly valuable, this method requires the preparation of a sucrose concentration gradient for each sample, manual loading of the sample atop the gradient, setting of the tube into the ultracentrifuge rotor by hand, a lengthy ultracentrifugation and final attachment of the tube and pumping of the gradient through a stand-alone U.V. monitor. These manual operations make the 140S method highly operator dependent, of low throughput and difficult to automate and standardize [15].

The SE-HPLC method presented hereby uses off-the-shelf HPLC equipment and is completely automated after the samples are loaded in the autosampler tray, affording a throughput of about 20 samples a day, working mostly unattended.

SE-HPLC separates components based on their molecular size. Host cell DNA is the main interference in FMDV analysis because its apparent hydrodynamic radius is similar to the virus, causing the column to only partially resolve virus and nucleic acid peaks. In the method described here, this interference is fully eliminated

Table 5
Average ratio of results SE-HPLC/140S for method comparison. A value of 1 indicates that both methods produced, on average, the same antigen mass results. Ratios above 1 point to SE-HPLC producing results higher than the ones obtained with the 140S method, while the opposite is true for values below 1.

	Median	Mean ($\bar{x}$)	Standard deviation (*s*)	Confidence interval 95%
Cell culture infection supernatants				
O1 Campos	1.05	1.07	0.12	1.03–1.10
A24 Cruzeiro	1.08	1.05	0.16	1.00–1.09
A2001 Argentina	0.99	0.98	0.12	0.93–1.03
C3 Indaial	1.07	1.08	0.10	1.04–1.13
Concentrated materials obtained by PEG precipitation procedure				
O1 Campos	1.07	1.08	0.16	1.03–1.12
A24 Cruzeiro	1.15	1.14	0.14	1.10–1.18
A2001 Argentina	0.99	1.00	0.14	0.94–1.06
C3 Indaial	1.18	1.19	0.10	1.16–1.22
Vaccines aqueous phases before emulsification process				
	1.21	1.22	0.15	1.18–1.26
Aqueous phases recovered from vaccines by solvent extraction				
	1.15	1.16	0.15	1.13–1.20

in advance of chromatographic separation by digestion of samples with Benzonase®.

The SE-HPLC shows good correlation with the classical 140S method at different antigen mass ranges (Fig. 4, Table 5). A slight increase (~20%) observed in the results for vaccine aqueous phases can be attributed to the spread of some virus content along the sucrose gradient, as detected by Western blot (WB) of viral proteins in gradient fractions, while HPLC eluates show WB signal only in virus peak (data not shown).

While we report the exact brand and model of the chromatography equipment used, any similarly configured HPLC chromatograph should produce analogous results if identical columns, flow, mobile phase composition and injection volumes are used. The cost of the equipment required is similar to the combined cost of the ultracentrifuge, gradients formers and U.V. monitors used for the 140S method. The per-sample costs are also similar, because while SE-HPLC requires Benzonase® for DNA digestion, it sidesteps the need for the analytical grade sucrose consumed in the 140S method. The HPLC equipment is easily decontaminated as the only parts in contact with the sample are the injector needle, the inner surfaces of the flow path and the column packing, all of them unaffected by the mildly acidic conditions required to inactivate the FMDV.

5. Conclusion

The described procedure for virus standard purification allows any FMD vaccine manufacturing facility to prepare *in situ* homogeneous, pure standards for calibration, validation or qualification purposes. We contend that this validated SE-HPLC method is a suitable replacement for the ultracentrifugation 140S procedures for both in-process control of FMDV vaccine manufacturing and the final vaccine control by research institutions and regulatory agencies, with substantial advantages regarding accuracy, precision and automation capabilities.

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Declaration of Competing Interest

This study was partly funded and largely conducted by Biogénesis Bagó S.A., Buenos Aires, Argentina. All authors, with the exception of M-GS and S-JY are (or were) employees of Biogénesis Bagó S.A. M-GS and S-JY have no conflicts of interest to declare.

ICMJE statement

All authors attest they meet the ICMJE criteria for authorship.

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