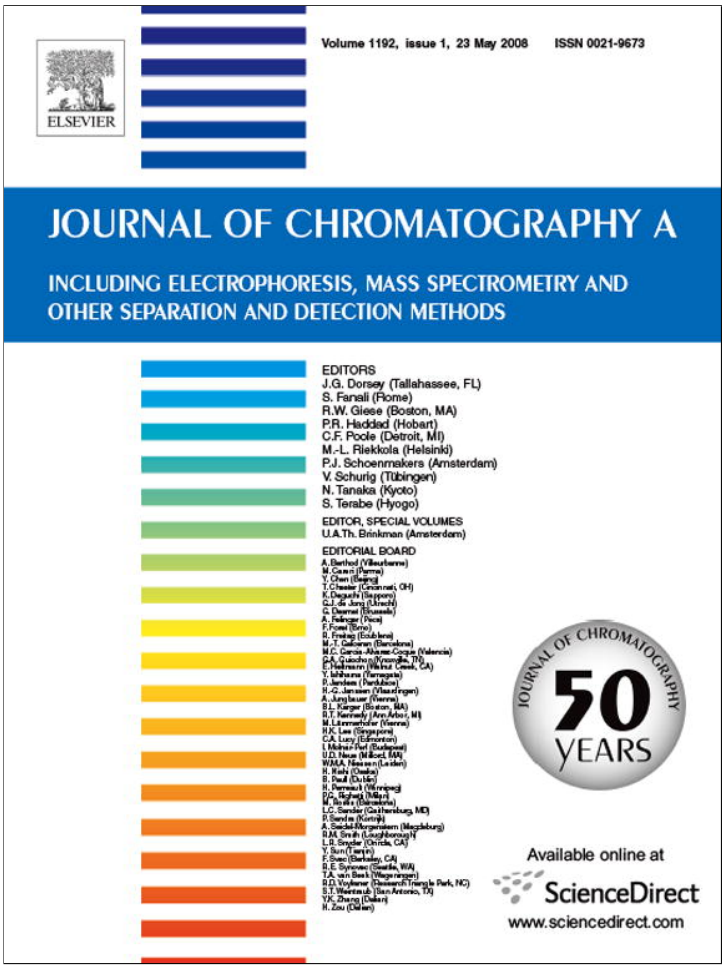

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Sodium dodecyl sulfate-capillary gel electrophoresis analysis of rotavirus-like particles

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ABSTRACT

This work describes the application of a sodium dodecyl sulfate-capillary gel electrophoresis (SDS-CGE) method for the analysis of triple 2/6/7 and double-layered 2/6 rotavirus-like particles (RLPs), candidate vaccines against rotavirus infection. SDS-CGE analysis of RLPs resulted in peaks that could be attributed to the viral proteins (VP2, VP6 and VP7) according to their apparent molecular mass (MW_{app}). Samples containing the glycoprotein VP7 were analysed after deglycosylation with PNGase F. Upon deglycosylation, VP7 MW_{app} decreased 4–7 kDa consistent with a degree of glycosylation of approximately 12–21%. VP2 was eventually detected in the form of more than one related proteins, despite the small areas due to the relative low mass proportion of this protein in the particle (16%). The effect of analytical parameters such as capillary temperature on method performance was evaluated. MW_{app} values estimated by SDS-CGE were compared with values obtained by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. The method described in this work proved to be fast, consistent and reproducible, representing a feasible alternative to the laborious conventional electrophoresis for the characterization of RLPs.

1. Introduction

Virus-like particles (VLPs) are multi-protein structures that mimic the organization and conformation of authentic native viruses but lack the viral genome, potentially yielding a safer and cheaper vaccine candidate. Furthermore, the immunogenic response is higher when the viral proteins are associated into particles than when they exist in linear, soluble form [1]. Hepatitis B vaccines were among the first products to contain recombinant protein-based VLPs synthesized in controlled expression systems [2].

The increasing interest in this type of vaccine can be confirmed by the number of products recently approved or in late development phase. Gardasil (Merck & Co.), produced in *Saccharomyces cerevisiae*, and Cervarix (Medimmune/GlaxoSmithKline), produced in insect cells, are both vaccines based on papillomavirus VLPs designed to prevent cervical cancer.

Rotavirus-like particles (RLPs), vaccine candidates against rotavirus infection, are produced by co-infection of insect cells with baculoviruses coding for some of the viral proteins. They are VLPs composed of three layers: the core (50 nm in diameter) contains 60 dimers of viral protein (VP) 2 (102.5 kDa per monomer) (in the virus native form, the core contains the virus genetic material and the proteins VP1 and VP3); the second layer is formed by 260 trimers of VP6 (44 kDa), and the third layer is formed by 260 trimers of VP7 (37 kDa) (in the virus native form this layer also contains spikes of VP4).

Like any recombinant therapeutic protein, VLPs must be submitted to rigorous quality control assays and be well characterized prior to commercialization. Typical analytical methods for characterization of VLPs and constitutive VPs are high-performance size-exclusion chromatography (HPSEC), transmission electron microscopy (TEM), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot. However, these methodologies are time-consuming, semi-quantitative and little sensitive. Moreover they require a significant amount of sample and do not provide quantitative information on the particle's stoichiometry, which is one of the most important issues for characterization of VLP yield and quality, since less than 20% of the recombinant proteins produced assemble correctly into VLP [1].

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In many applications, capillary electrophoresis (CE) has proved to be the analytical method of choice to characterize protein variants, since the use of capillaries greatly reduces sample volume and analysis time compared to conventional gel electrophoresis [3]. Capillary zone electrophoresis (CZE) is the most widely used CE technique for analysing recombinant therapeutic proteins (e.g. human erythropoietin, human tissue plasminogen activator) [3], viral capsid protein [4,5] and DNA [6]. However, the useful mobility-to-mass relationship characteristic of SDS-PAGE and Western blot is absent in zone techniques. This drawback may be overcome by SDS-capillary gel electrophoresis (SDS-CGE), since the migration time (t_m) can be directly related to the mass of the protein.

SDS-CGE has been routinely employed for in-process analysis and quality control of purified samples [7,8], particularly in biopharmaceutical companies for analysis of monoclonal antibodies [9–11].

We herein describe the application of SDS-CGE, using a replaceable gel matrix, for the analysis of rotavirus VLPs. The method was optimized and then applied to triple (RLP 2/6/7) and double-layered (RLP 2/6) rotavirus VLP. RLP 2/6/7, which contains VP7, was analysed prior and after deglycosylation with PNGase F. Apparent molecular masses (MW_{app}) were determined based on internal calibration. Quantification and detection limit were determined and RLPs characterized in terms of their VP6/VP7 ratio. Finally, the obtained results were compared with SDS-PAGE and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS).

To our knowledge, this is the first time that a SDS-CGE method is applied to the analysis of proteins from such a complex nanostructure as VLPs.

2. Materials and methods

2.1. RLPs production

RLP 2/6/7 and RLP 2/6 were produced in *Spodoptera frugiperda* (Sf-9) cells obtained from the European Collection of Cell Cultures (ECACC No. 89070101, Salisbury, UK) using the baculovirus expression vector system (BEVS). The baculoviruses, containing bovine rotaviral *vp2*, *vp2-gfp*, *vp6* and *vp7* genes RF strain were kindly provided by Dr. Didier Poncet from Centre National de la Recherche Scientifique (Institut National Recherche Agronomique (INRA), Gif-sur-Yvette, France). The cells were routinely cultured in shake flasks and baculoviruses propagated in 125-mL spinner flasks. These cultures were used as inoculum for 250-mL Wheaton spinner flasks using Gibco Sf-900 II SFM medium (Invitrogen, Paisley, UK). Cells were co-infected with baculoviruses VP2-GFP and VP6 (RLP 2/6) and VP2, VP6 and VP7 (RLP 2/6/7) at multiplicity of infection (MOI) of 2–5 pfu cell^{-1} for each type of the baculoviruses at a cell concentration at infection of 3.0×10^6 cells mL^{-1}. At the time of infection, and every 48 h afterwards, the culture was supplemented with protease inhibitors [1 μg mL^{-1} leupeptin and 1.6 μg mL^{-1} aprotinin (Sigma–Aldrich, München, Germany)]; the bioreaction broth was harvested at 120 h post-infection (hpi).

Cell concentration and viability were assessed by counting using an hemacytometer (Brand, Wertheim, Germany) with cell viability evaluated by 0.4% (v/v) trypan blue exclusion dye (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS).

When cell viability was approximately 50%, corresponding to 120 hpi, purification of RLPs was initiated according to previous report [12]. Briefly, cell bulk was incubated with 1% (v/v) Triton X-100 (Sigma, Saint Louis, MO, USA) in Dulbecco's phosphate-buffered saline (DPBS) (Invitrogen) and then, clarified with a 3-μm depth filter (Sartorius, Goettingen, Germany). The following steps were ultrafiltration with a 750-kDa MWCO hollow fibre membrane (GE Healthcare, Piscataway, NJ, USA) and preparative size-exclusion chromatography (SEC) with Sephacryl S-500 coupled to an AKTA Explorer system (GE Healthcare). The eluted fractions were collected and concentrated by ultracentrifugation. The resulting pellet was resuspended in DPBS and total protein concentration was measured by absorbance at 280 nm in spectrophotometer Beckman DU 530 (Fullerton, CA, USA) using bovine serum albumin (BSA) (96%, Sigma) as standard. Finally, RLPs' purity and integrity were monitored by SDS-PAGE, Western blot and TEM.

2.2. SDS-CGE

The analyses were carried out in a Beckman P/ACE MDQ capillary electrophoresis system (Fullerton, CA, USA) with diode array detection (DAD). A CE-SDS protein kit from Bio-Rad (Hercules, CA, USA) was used, including CE-SDS run buffer, CE-SDS sample buffer, washing solutions and protein standard mixture (lysozyme (14.4 kDa), trypsin inhibitor (21.5 kDa), carbonic anhydrase (31 kDa), ovalbumin (45 kDa), BSA (66.2 kDa), phosphorylase B (97 kDa), β-galactosidase (116 kDa) and myosin (200 kDa)).

Non-deglycosylated samples and standards (5 μl) for SDS-CGE analysis were prepared by dilution with phosphate buffer pH 7.5 (5 μl) and CE-SDS sample buffer containing 5% (v/v) β-mercaptoethanol (Sigma) (10 μl), and incubation at 90 °C for 10 min followed by centrifugation. Deglycosylated samples were previously denatured (2.5 μl) with a solution of 0.02% (w/v) SDS and 10 mM of β-mercaptoethanol in 50 mM phosphate buffer pH 7.5 followed by incubation at 100 °C in a water bath for 10 min. After cooling, 2 U of PNGase F (5 μl) (from *Elizabethkingia* (*Chryseobacterium*/*Flavobacterium*) *meningosepticum*, Sigma) were added to each denatured sample and then incubated for 2 h at 37 °C. Complexation with SDS was performed as described for non-deglycosylated samples with 10 μl of CE-SDS sample buffer containing 5% (v/v) β-mercaptoethanol.

For MW_{app} determination and protein quantification, samples were further mixed with standard solutions of lysozyme (from hen egg white, Fluka, Seelze, Germany) and BSA (96%, Sigma) prior to injection.

Non- and deglycosylated RLP 2/6/7 and RLP 2/6 and standards were injected at 34.5 kPa for 50 s. Fused-silica uncoated capillaries of 30 cm total length and 75 μm I.D. from Agilent (Santa Clara, CA, USA) were used. Separations were performed at 15 kV constant voltage and monitored at 220 nm for up to 20 min. Prior to each injection, the capillary was rinsed with 0.1 M NaOH, 0.1 M HCl and CE-SDS run buffer, for 120, 90, and 240 s, respectively. Sample temperature was kept at 10 °C, whereas capillary temperature was maintained at 20, 30 or 40 °C for evaluation of the effect of this parameter upon relative migration time (RMT) and MW_{app}. Lysozyme was used as reference marker for RMT calculation and MW_{app} was estimated based on the calibration curve of internal standards (1/RMT × log MW).

Duplicates or triplicates of each sample were injected three times each for evaluation of method precision.

2.3. SDS-PAGE and Western blot

Sample preparation for SDS-PAGE and Western blot followed the same steps as for SDS-CGE, except that NuPAGE LDS sample buffer (Invitrogen) was used. Electrophoretic separation was carried out on precast NuPAGE Tris-Glycine gels (Invitrogen) at 125 V according to manufacturer instructions. Molecular weight markers were BenchMark pre-stained standard proteins (Invitrogen) or low molecular weight (LMW) from GE Healthcare for MW_{app} estimation since the calibration curve built with LMW unstained proteins gave better mass accuracy. The gels were either stained

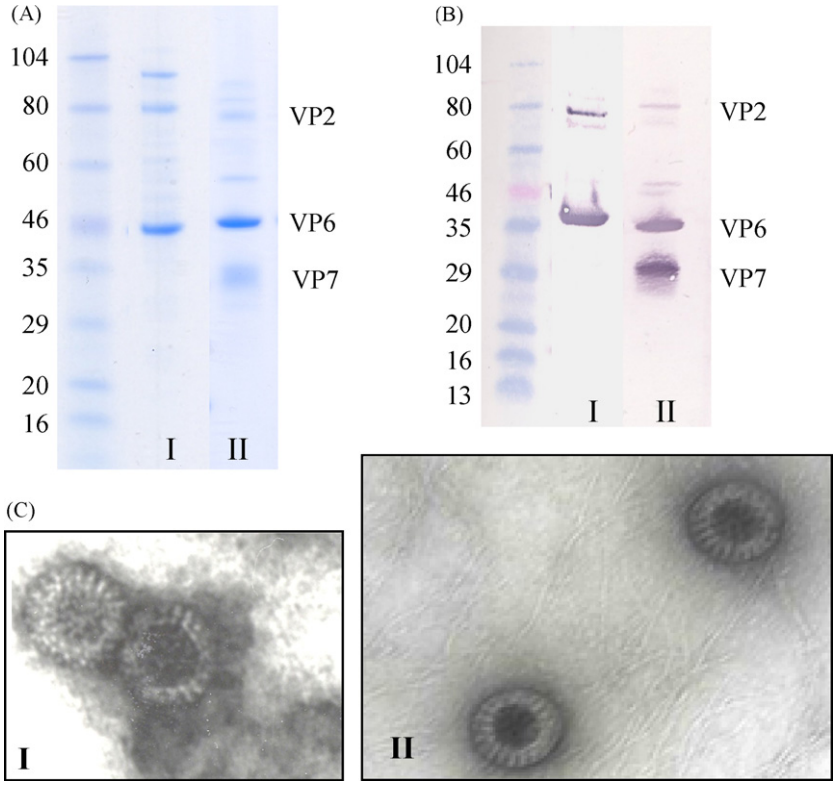

Fig. 1. Analysis of purified RLP 2/6 (I) and RLP 2/6/7 (II) by SDS-PAGE (A), Western blot (B), and TEM (C) with magnification of 160,000× (I) and 100,000× (II). Molecular weight markers were from BenchMark pre-stained kit calibrated with LMW. Electrophoretic conditions as in Section 2.3.

with Coomassie SimplyBlue SafeStain (Invitrogen) or transferred to nitrocellulose membranes. Immunochemical staining for detection of VP2, VP2-GFP, VP6 and VP7 was carried out with a goat polyclonal anti-rotavirus antibody (Abcam, Cambridge, UK). Blots were developed after incubation with an alkaline phosphatase conjugated anti-goat IgG antibody (Sigma) using 1-stepTM NBT/BCIP blotting detection reagent (Pierce, Rockford, IL, USA). The area and intensity of bands were quantified by densitometry analysis (ScionImage, NIH, Bethesda, MD, USA).

2.4. TEM

To observe the integrity and morphology (shape, size) of RLPs, electron microscopy was performed as follows: a drop (3 μl) of sample was adsorbed onto a formvar coated 400 mesh copper grid (Electron Microscopy Sciences, Ft. Washington, PA, USA) for 60 s. Afterwards the grid was soaked in 1% uranyl acetate for 30 s, dried in air at room temperature (22 °C) and examined with a JEM 200 Ex electron microscope (JEOL, Tokyo, Japan).

2.5. MALDI-TOF-MS

The RLPs were spotted on MALDI plate using 20 mg mL^{-1} DHAP in 70% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid as matrix. Spectra were acquired with a PerSeptive Biosystems MALDI-TOF-MS Voyager-DE STR (Framingham, MA, USA) equipped with delayed extraction, and a 337-nm N_2 laser, in linear mode using optimized laser intensity. A mass range of 20–110 kDa was acquired in order to determine VPs' molecular masses and to investigate the existence of aggregates smaller than 100 kDa. External mass calibration of the spectra was performed using a standard mixture of proteins, Protein calibration Mix3 (LaserBio Labs, Sophia-Antipolis, France). The individual spectra were processed by Data Explorer 4.2 software (Applied Biosystems, Foster City, CA, USA) used for baseline correction, noise removal and peak detection.

3. Results and discussion

3.1. RLPs characterization

In order to assess the purity and integrity of RLPs to be used in this work, SDS-PAGE, Western blot and TEM analysis were carried out (Fig. 1A to C). Minor contaminant bands (e.g. remaining baculovirus proteins), which are commonly found in purified RLPs [12,13] and other VLPs produced with insect cells/baculovirus system [14], can be seen between VP2 and VP6, however, the bands close to VP2 have been previously attributed to VP2-related proteins with MW_{app} of 94, 85 and 77 kDa that appear at considerably low concentration [15]. The electron microscopy (Fig. 1C) of RLP 2/6 and RLP 2/6/7 show, in both cases that RLPs are intact and had a similar morphology to that previously reported [16,17].

Total protein concentration of purified solution was estimated to be approximately 695 (RLP 2/6) and 433 μg mL^{-1} (RLP 2/6/7).

3.2. Application of SDS-CGE for RLPs analysis

3.2.1. Method optimization

Using the experimental procedure suggested by the CE-SDS kit manufacturer did not allow detection of VPs in RLPs samples. Thus, injection parameters such as injection mode (electrokinetic or pressure injection), value of pressure and injection time were optimized. The protein standard solution provided by the BioRad CE-SDS kit was used with this purpose. Electrokinetic (5 kV) and pressure injection (34.5–165.6 kPa) were tested for 5–90 s. Pressure injection usually resulted in sharper peaks and larger areas for the same injection time, even at the lowest

Table 1
Effect of capillary temperature on estimated apparent molecular mass (MW_{app}), in kDa

Samples	T = 20 °C	T = 30 °C	T = 40 °C
VP6 (RLP 2/6/7)	45	44	42
VP6 (deglycosylated RLP 2/6/7)	45	46	43
VP6 (RLP 2/6)	45	45	43
VP7 (RLP 2/6/7)	35	35	34
VP7 (deglycosylated RLP 2/6/7)	29	28	27

pressure tested (34.5 kPa) (data not shown). Pressures higher than 34.5 kPa had a detrimental effect on peak shape while injection times longer than 50 s affected resolution negatively. Based on this study, an optimal injection time and pressure of 50 s and 34.5 kPa, respectively, were used for sample analysis.

In order to evaluate the migration time (t_m) repeatability, multiple SDS-CGE runs were performed with the standard solution kit. Average t_m and RSD (n = 10) for representative proteins were 15.4 min and 4.6% (galactosidase), 13.4 min and 5.2% (BSA) and 10.3 min and 3.7% (trypsin inhibitor). Since run-to-run variation of migration time was high and could compromise MW_{app} determination by external calibration, an internal mass calibration by addition of lysozyme and BSA to each sample for MW_{app} estimation was used during the subsequent experiments.

3.2.2. *Effect of capillary temperature on MW_{app} determination*

The effect of capillary temperature (20, 30 and 40 °C) on migration time and on MW_{app} was evaluated (Table 1). The increase on capillary temperature generally allowed better current stabilization despite a slight decrease in resolution. Overall, MW_{app} values are similar, despite varying approximately 1–3 kDa between 30 and 40 °C. As shown in Table 1, the shift in VP6 MW_{app} from 20 to 30 °C was below 1 kDa, whereas at 40 °C these values decreased by almost 14%. Apparently, the use of internal standards was not able to completely prevent the differences in MW_{app} from one temperature to the other. Since VP7 usually consisted of a band with more than one peak, MW_{app} was calculated based on the t_m corresponding to the most intense peak. Its MW_{app} was less affected by capillary temperature than that of VP6 in all samples.

The differences in migration time and, consequently, in MW_{app} estimates, can be explained by the decrease in run buffer viscosity and sieving capacity and consequent increase of electrophoretic mobility with capillary temperature rise [18–20]. For this reason 20 °C was chosen for subsequent analyses. Typical electropherograms for RLP 2/6 and RLP 2/6/7 at this temperature are shown in Fig. 2.

3.2.3. *Analysis of VPs' MW_{app} accuracy*

The MW_{app} values obtained with RLP 2/6/7 and RLP 2/7 at 20 °C were as expected for the respective VPs. The VP6 MW_{app} were 45 kDa in RLP 2/6 and 43 kDa in RLP 2/6/7 (Table 2), whereas the theoretical MW (from amino acid sequence) is 44.3 kDa. Differences of this magnitude between theoretical MW and MW_{app} estimation by SDS-CGE have been reported in literature [21,22]. In the particular case of recombinant rotavirus VP6, it has been reported a MW_{app} estimation by SDS-PAGE of 41 [23] and 43 kDa [24] for SA11 and Cowden strains, respectively. The molecular mass of VP7, based on the amino acid sequence, is predicted to be 37.4 kDa [25]. A hypothesis for the difference of the observed value of 34 kDa is the lack of the signal peptide in the baculovirus construction [26]. Indeed, it has been published that recombinant VP7 MW_{app} estimation by SDS-PAGE was 34 kDa [27]. On the other hand, VP7 peak is rather broad probably as a result of heterogeneity due to different isoforms.

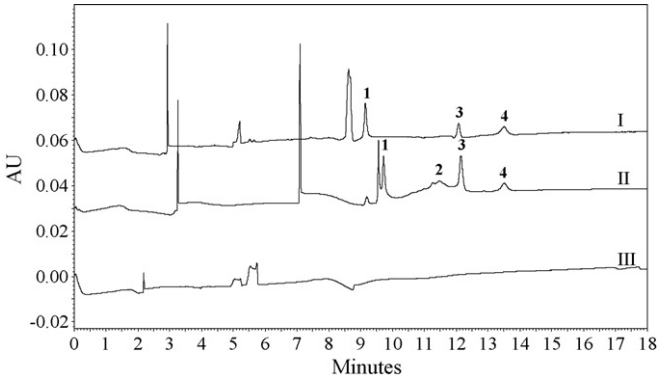

Fig. 2. Electropherogram of RLP 2/6 (I), RLP 2/6/7 (II) and negative control (sample buffer) (III). Numbered peaks correspond to: lysozyme (1), VP7 (2), VP6 (3) and BSA (4) at 20 °C (other conditions as in Section 2.2).

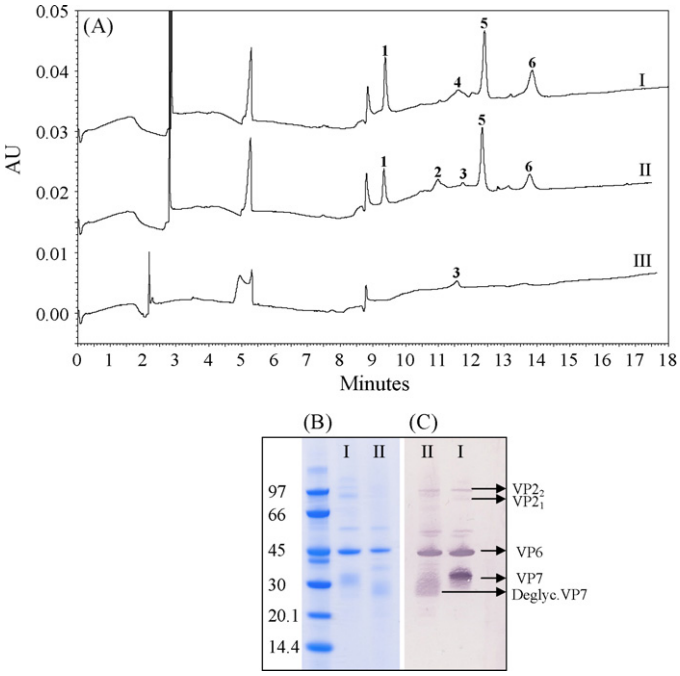

Fig. 3. Comparison of deglycosylated and non-deglycosylated RLP 2/6/7 by SDS-CGE, SDS-PAGE and Western blot: (A) electropherogram of RLP 2/6/7 (I), deglycosylated RLP 2/6/7 (II) and negative control (sample buffer with PNGase) (III); (B) SDS-PAGE and (C) Western blot of RLP 2/6/7 (I) and deglycosylated RLP 2/6/7 (II). Molecular weight markers were from LMW kit at 20 °C (other conditions as in Sections 2.2 and 2.3). Numbered peaks correspond to: lysozyme (1), deglycosylated VP7 (2), PNGase (3), VP7 (4), VP6 (5) and BSA (6).

Upon deglycosylation with PNGase F there is a decrease in the MW_{app} of VP7 of 4–7 Da, which is in agreement with published works on SDS-PAGE analysis of VP7 lacking the oligosacharides [28,29]. The peak observed after VP7 deglycosylation is also sharper but smaller than the peak of the glycosylated form whereas VP6 remains unaltered. In Fig. 3A it is possible to observe the shift in VP7 peak after RLP 2/6/7 deglycosylation and in Fig. 3B and C the difference in VP7 band by SDS-PAGE and Western blot, respectively.

The glycosylation of a therapeutic protein is, in most cases, essential to its biological activity and that is the reason why the proposed method must clearly discriminate glycosylated from non-glycosylated VP7.

Table 2
Repeatability of the SDS-CGE method

Samples	Average RMT	RSD of RMT (%)	Average MW_{app} (kDa)	RSD of MW_{app} (%)	Average peak area	RSD of peak area (%)
VP6 (RLP 2/6/7), $n=9$	1.27	1.8	43	1.9	7.7×10^5	8.9
VP7 (RLP 2/6/7), $n=9$	1.20	1.5	34	2.2	5.9×10^5	10.1
VP6 (RLP 2/6), $n=6$	1.37	1.0	45	0.25	3.5×10^5	6.8

Precision data for three parameters: RMT, MW_{app} and peak area.

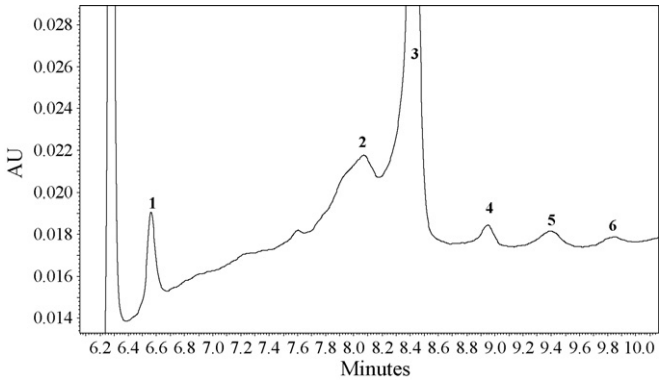

Fig. 4. Detection of VP2 in one of the electropherogram of RLP 2/6/7 at capillary temperature of 30 °C. Numbered peaks correspond to: (1) lysozyme, (2) VP7, (3) VP6, (4) BSA, (5) $VP2_1$ (85 kDa) and (6) $VP2_2$ (94 kDa). Electrophoretic conditions as in Section 2.2.

3.2.4. Precision evaluation of MW_{app} estimation

In order to evaluate the repeatability of the proposed SDS-CGE method, three preparations of RLPs samples were each one analysed at least twice. Slightly shifts in migration time in different runs were observed and that is the reason why internal calibration was used. Table 2 summarizes the results for RMT, MW_{app}, peak areas and respective relative standard deviations (RSD, $n=6$).

All RMT peaks met the criteria of RSD < 2%. The RSD of the average MW_{app} for each sample are also below 2%, except for VP7. Repeatability for quantitative analysis was evaluated by the RSD of the peak area for each sample (Table 2).

3.2.5. Detection of VP2

VP2 was not detected in all electropherograms of RLP 2/6/7. Some authors have referred anomalous interaction of high-MW proteins with SDS at high temperature [11]. Eventually, two peaks were detected in RLP 2/6/7 ($VP2_1$ and $VP2_2$) corresponding to MW_{app} of 86 and 97 kDa (Fig. 4). Recombinant rotavirus VP2 from bovine RF strain is expected to have a MW_{app} of 94 kDa [30], although its theoretical MW is 102.4 kDa [25]. VP2-related proteins with MW_{app} of 94, 85 and 77 kDa at considerably low concentrations have been previously reported [15], and this may affect VP2 limit of detection. Moreover, VP2 mass represents only a small fraction (16%) of the whole RLP 2/6/7, which is an added difficulty to its detection by either SDS-PAGE (Fig. 1A) or SDS-CGE.

In the case of RLP 2/6, VP2 was fused to GFP. Nevertheless, VP2-GFP was eventually detected as a small peak at a MW_{app} corresponding to 106 kDa, which may be attributed to the sum of one of VP2-related protein MW with that of GFP (data not shown). Accordingly, in SDS-PAGE analysis of RLP 2/6 (Fig. 1A, lane I), VP2-GFP MW_{app} was estimated to be 100 kDa.

3.2.6. Quantification of RLP 2/6/7 and method's limit of detection

Quantification of RLP 2/6/7 was obtained by the sum of VP6 and VP7 peak areas in the electropherogram at 280 nm and their correlation with BSA peak area, quantitatively added to each sample. Previously, a calibration curve was drawn for BSA in the range of 25–200 μg/ml for linearity evaluation ($r=0.993$). RLP 2/6/7 concentration by absorbance at 280 nm (measured in spectrophotometer) was 433 $\mu g\,mL^{-1}$ whereas by SDS-CGE this value was 330 $\mu g\,mL^{-1}$, taking into account the peak areas of VP6 and VP7. Considering that the concentration by SDS-CGE excluded VP2's peak contribution, the values are quite similar. The comparison of SDS-CGE with other techniques for quantification of proteins has been previously reported [22,31]. In both works differences in protein concentrations, up to 50% [31], were found according to the applied method. A common solution to improve the quantification accuracy usually relies on the use of standard solutions with composition similar to the target protein [22].

The correct stoichiometry of RLP 2/6/7 should be as close as possible to native rotavirus in order to use it as a vaccine. Based on proteins' peak areas, the ratio VP6/VP7 can be determined. The ratio obtained with SDS-CGE (1.3) was close to the theoretical (1.2) (Table 3). The ratio VP6/VP7 in deglycosylated RLP 2/6/7 (2.7) was higher than in RLP 2/6/7. This difference, which was also observed by Western blot, where the ratio had a 2.4-fold increase after deglycosylation, may be explained by the decrease in VP7's peak area due to incomplete VP7 deglycosylation.

In order to assess the limit of detection (L_d) of the proposed method, the following equation was used:

$$L_d = \frac{3.3 \cdot \overline{A_n} \cdot [\text{protein}]}{\overline{A_{\text{protein}}}}$$

where $\overline{A_n}$ is the average noise amplitude at negative control, [protein] is BSA or lysozyme at low concentration (5 $\mu g\,mL^{-1}$) and

Table 3
Comparison of MW_{app} (kDa) and VP6/VP7 ratio by SDS-CGE, SDS-PAGE and MALDI-TOF-MS

Proteins	Samples	SDS-CGE	SDS-PAGE	MALDI-TOF-MS	Theoretical [25]
VP6	RLP 2/6/7	43	45	44.9	44.3
	Deglycosylated RLP 2/6/7	45	45	n.d.	
	RLP 2/6	45	42	44.5	
VP7	RLP 2/6/7	34	31	32.7	37.4 or 31.2[a]
	Deglycosylated RLP 2/6/7	28	27	n.d.	–
Ratio VP6/VP7[b]	RLP 2/6/7	1.3	0.81	n.d.	1.2
	Deglycosylated RLP 2/6/7	2.7	1.97	n.d.	

[a] MW based on the amino acid sequence with and without the signal peptide, respectively.
[b] Calculated from the average peak area from Table 2 (SDS-CGE) and band intensity (Western blot).

$\overline{A_{protein}}$ is the average peak height of BSA or lysozyme at this concentration. L_d was 0.35 and 0.1 μg mL^{-1}, respectively, for BSA and lysozyme. The difference in the limit of detection for both proteins may explain some results found in this work, especially concerning VP2 detection.

L_d was evaluated qualitatively by analysing RLP 2/6/7 at three different concentrations (based on spectrophotometric determinations): 144, 36 and 9 μg mL^{-1}. Considering that VP6 accounts for approximately 47% of the whole RLP 2/6/7, its concentration in this last sample would be approximately 4.2 μg mL^{-1}. VP6 peak was still visible at this concentration (data not shown), which is in agreement with the calculated L_d. Taking into account the minimum 3-fold dilution necessary for sample preparation due to the addition of sample buffer and internal standards and considering the expected relative proportion of the different VPs, it can be estimated that the detection limit of the method for both VP6 and VP7 detection is higher than 36 μg mL^{-1} total protein, as VP7 peak was not detected at this concentration.

3.3. Comparison of SDS-CGE with SDS-PAGE and MALDI-TOF-MS

In order to evaluate the proposed SDS-CGE method, the results for MW_{app} were firstly compared with those obtained by SDS-PAGE (Table 3). Generally, both methods resulted in similar MW_{app} values. The reason why VP7 MW_{app} estimated by both techniques differs from the theoretical may be related to the amino acid sequence, the glycosylation level or method accuracy. Previous work from Dormitzer et al. on the characterization of purified recombinant VP7 monomers from RRV strain showed that, depending on the analytical method, MW_{app} differed from the predicted mass [32]. MALDI-TOF-MS, analytical ultracentrifugation and HPSEC resulted in MW_{app} of 32, 34 and 46 kDa, respectively. Furthermore, the glycosylation level, which varies depending on the rotavirus strain, may also affect the MW_{app} estimates. Two different approaches have been reported for deglycosylation of VP7: enzymatic digestion with PNGase [29] and expression of a VP7 mutant lacking the glycan moiety [28]. In both cases, a shift of 4–7 kDa was found between glycosylated and deglycosylated VP7, which is in agreement with the current work.

The molecular masses of VP2, VP6 and VP7 where determined by MALDI-TOF-MS using an optimized disassembly procedure (unpublished work).

The mass spectra for RLP 2/6/7 and RLP 2/6 are shown in Fig. 5A and B, respectively.

In Fig. 5A, it is possible to observe the three proteins in RLP 2/6/7. VP2 (102.5 kDa) and two VP2-related proteins (77.8 and 89.8 kDa) were also detected. As also summarized in Table 3, VP6 molecular mass determined by MALDI is closer to theoretical than the MW_{app} estimated by SDS-CGE. This difference has been observed by other groups and seems to increase for glycosylated proteins as expected [33]. If VP7 signal peptide lacks in the amino acid sequence [26], the theoretical MW should be 31.2 kDa [25]. In this case, VP7 mass by MALDI-TOF-MS (32.6 kDa) was, obviously, more accurate than the MW_{app} estimated by SDS-CGE.

In RLP 2/6 analysis by MALDI only VP6 could be detected (Fig. 5B). Eventually, the low proportion of VP2 in the particle may explain the lack of its peak in this spectrum.

Mass spectrometry gives accurate information on molecular mass, but quantification with this technique is limited due to the heterogeneous co-crystallization of the sample with the matrix on the MALDI plate and, in this particular study, also due to the absence of adequate standards.

One could say that both techniques are complementary and should, ideally, be hyphenated, however, difficulties inherent to gel techniques may be a limitation.

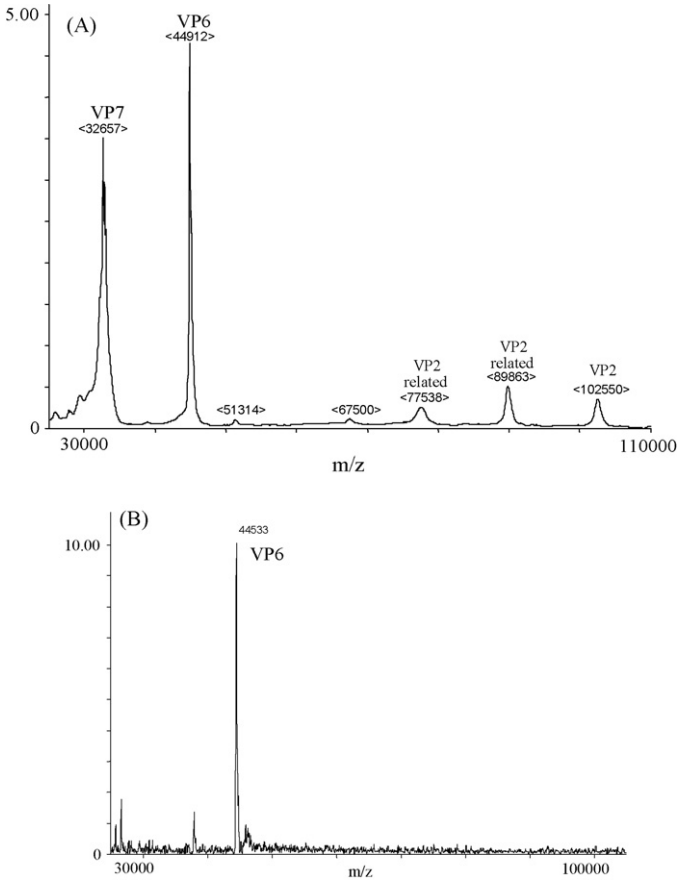

Fig. 5. MALDI-TOF mass spectrum of (A) RLP 2/6/7 and (B) RLP 2/6.

4. Concluding remarks

This work describes for the first time a SDS-CGE technique for the analysis of recombinant rotavirus structural proteins VP2, VP6 and VP7. The final aim of the proposed method was the quantitative and qualitative characterization of RLPs in terms of presence and relative proportion of these proteins for quality control purpose. The method demonstrated to be accurate for MW_{app} determination and allows qualitative evaluation of the degree of glycosylation. Its precision allows for determination of VP6/VP7 ratio differences between different samples as well as for protein quantification. The method is comparable to both SDS-PAGE and MALDI-TOF-MS in terms of sensitivity although injection time and pressure as well as complexation temperature were determinant for achieving it. The electrophoretic method is fast (below 20 min per sample) and can be applied to multiple samples in an automated fashion, but the major advantage is the relative small amount of sample needed in comparison to traditional SDS-PAGE.

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