Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 135–145

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Rational use of stacking principles for signal enhancement in capillary electrophoretic separations of poliovirus samples

Iuliana Oita[a], Hadewych Halewyck[b], Sigrid Pieters[a], Bieke Dejaegher[a], Bert Thys[b], Bart Rombaut[b], Yvan Vander Heyden[a,*]

[a] Vrije Universiteit Brussel-VUB, Center for Pharmaceutical Research-CePhaR, Department of Analytical Chemistry and Pharmaceutical Technology, Laarbeeklaan 103, B-1090, Brussels, Belgium
[b] Vrije Universiteit Brussel-VUB, Center for Pharmaceutical Research-CePhaR, Department of Pharmaceutical Biotechnology & Molecular Biology, Laarbeeklaan 103, B-1090, Brussels, Belgium

ARTICLE INFO

Article history:
Received 16 November 2010
Accepted 16 December 2010
Available online 31 December 2010

Keywords:
Virus analysis
Capillary electrophoresis
Large plug injections
Signal enhancement
Experimental design

ABSTRACT

The use of an earlier developed capillary electrophoresis (CE) method, either to investigate poliovirus (PV) samples with a low viral-purity level or to study the less abundant sub-viral particles, revealed the necessity for an intra-column signal enhancement strategy. Although intra-column signal enhancement is a very popular approach to assay small molecules, it is less straightforward for the analysis of biological macromolecules or particles. A reason could be that, for a proper signal enhancement approach, these samples have to be thoroughly studied to understand the factors affecting the separation process. For the investigated PV samples, a screening design revealed that injecting larger sample plugs significantly enhanced the analytical signal, but also significantly decreased the separation efficiency. A subsequently executed central composite design determined the largest sample plug that can be injected without compromising the separation. Finally, the sample dilution and the length of the injected plug were used for tuning the intensity of the analytical response.

Two combinations of sample dilution and injected plug size, at extreme values, were investigated in detail to define the best procedure for PV analysis using CE. In both situations, PV was effectively separated and quantified in rather complex samples, showing a good repeatability, an acceptable linearity for the PV particles and a decreased limit of detection in comparison with the existing method. In conclusion, intra-column signal enhancement can be successfully applied for viral suspensions, extending the applicability of CE methods to samples with lower virus concentrations, and/or allowing a significant reduction in the minimum required volume of sample. For PV samples, 5 μl of sample is necessary instead of the previous 20 μl, while the analytical signal was enhanced up to 14 times.

The results of this study can provide a basis for the development of routine CE methods for viral particle analysis, especially when rational and reproducible signal enhancement is required.

1. Introduction

Development of standard methods to analyze biological samples is hampered by large sample variability, both in the composition of the sample matrix and the concentration of the target analyte. Moreover, the concentration of the analyte of interest in biologically relevant samples, such as clinical samples or infected cell extracts, is in the nano- or even picomolar range. This would point towards the need for extremely sensitive methods, possibly combined with a sample pretreatment allowing the selective enrichment of the target analyte.

During the last years, virus particles were persistently investigated to find fast and reliable methods for identification, and to systematically study their life-cycle, in order to develop better antivirals and vaccines, or to develop virus-like particles for targeted drug delivery [1]. To explore the intact virus particles, the classical tools in molecular biology are limited to cumbersome tests, such as infectivity tests or the microscopical/functional examination of fractions isolated after sucrose gradient ultracentrifugation. Even less alternatives are available when the intact viruses have to be studied simultaneously with sub-viral particles in complex mixtures containing besides the virus particles, also proteins and/or nucleic acids. The use of separation methods may represent a fast and accurate solution for the analysis of such complex mixtures.

The development of separation methods for viral samples cannot be performed without a careful consideration of the analytical challenges occurring from their intrinsic properties.

* Corresponding author.
E-mail address: yvanvdh@vub.ac.be (Y. Vander Heyden).

0731-7085/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2010.12.032

Special features of the analyte, such as its colloidal nature, large molecular size, amphoteric character, aggregation tendency and sensitivity to environmental changes (pH, ionic strength, solvents and surfactants) require a different approach compared to small chemicals [2–5]. Usually, the intact virus particle (the virion), is just a minor component of the sample, compared to the other components present in the sample matrix [2–5]. The concentration of the virions in the sample can be hardly controlled and depends on several factors, including both the growth yield, and the number and yield of the steps used during purification. In some cases, the virions are available in interfering matrices; therefore they should be separated also from the matrix compounds and not only from sub-viral particles. Moreover, only low volumes of the sample are available, while reference materials are usually unavailable. Usually, a highly purified viral suspension, needed as working standard for method development, is available in volumes of a few milliliters, only after several months of collecting infected cell growths.

Capillary electrophoresis (CE) appears to be the separation technique of choice for these samples, as it is able to perform effective separations of complex samples and to manage most of the aforementioned challenges. However, the drawback for using CE is most of the time the low signal intensity, especially when UV detection is employed [6]. Over the years, several approaches for an appropriate signal enhancement were described in the literature, and represented also the topic of some reviews [7–12]. To our knowledge, none of these works concerns virus particle samples. Moreover, the number of the research groups performing separations of intact virus particles is limited, while the investigation of the most significant factors for the separation is far from trivial. Although an effective application for biologically relevant samples is desired as soon as possible after method development, progress cannot be achieved without an initial systematic study of samples with a higher viral purity. In this way, the separation particularities specific to the analyte itself can be isolated from those specific to the matrix and the contaminants. Only in this way, a reliable and consistent separation method can be designed, turning into reality daring goals such the analysis of one-cell components or meaningful *in vitro* vaccine characterization.

Intra-capillary signal enhancement includes a wide range of approaches, from the simplest and most frequently used, sample stacking [9,11–13] to acetonitrile stacking [14], pH mediated stacking [15–17], transient isotacophoresis [5,12,18] and sweeping [8,19], each associated with advantages and drawbacks. Although, the principles behind these approaches are better and better understood each day, there is no universal recipe for a successful application. The application of sample stacking is even much more complex for samples with biological origin, such as viral particle suspensions extracted from infected cells, where the intrinsic sample properties previously mentioned cannot be ignored, and a methodical investigation of the factors relevant for the separation is required [5,12,20]. For small, commercially available molecules, the investigation and development of a successful procedure can be easily performed, since series of samples, with different concentrations and various conductivities relative to the background electrolyte (BGE) can be obtained and explored with few efforts. For viral samples, the conductivity is usually too high to immediately apply sample stacking. In this case, an adequate decrease in sample conductivity without a decrease in the bio-analyte concentration is desired, but mostly unachievable. A larger injection plug could compensate for the concentration decrease, but the separation conditions should be tuned to avoid excessive broadening occurring from a significant difference in electro-osmotic flow (EOF) magnitude between sample and BGE [13].

Earlier, using highly purified samples, collected after a sucrose gradient ultracentrifugation, poliovirus (PV) particles were detected and separated from the matrix components applying a simple capillary electrophoresis method [21]. At the applied separation conditions, the PV particles were reaching the detector after ~6.5 min, showing a low net electrophoretic mobility of $\sim 12 \times 10^{-9}\ m^2\ V^{-1}\ s^{-1}$. The origin for the electrophoretical mobility of the PV comes from the superficial charge of the proteinaceous capsid of the virus particles in aqueous media [22]. The p*I* of the PV was estimated to be within the 7–8.2 range [23], conferring a slightly negative net charge to the viral capsid at the pH 8.3 of the used BGE. The choice of the pH of the BGE is restricted to values below 9 because of the low stability of the virus in alkaline conditions [24]. Another important factor affecting the net mobility of the virus is the presence of sodium dodecyl sulphate (SDS) in the BGE. To extend the applicability of our CE method, the SDS concentration in the BGE was maintained at the lowest possible level in order to be able to apply the method for empty capsids, i.e. large protein assemblies which are possibly unstable in the presence of SDS [25]. During method development in [21], an experimental design was used to identify the factors having the largest influence on the separation of PV particles. The effects of buffer concentration, surfactant concentration, temperature, voltage, pH, injection volume, percent of organic modifier, and inorganic additive added to the sample, were simultaneously screened using a two-level 12-experiments Plackett–Burman design [21]. None of the investigated factors were found to be significant on the effective mobility of the PV.

Although the method of reference [21] performs well for highly purified samples, there is a strong interest to adopt it for less purified samples and for the simultaneous analysis of PV and subviral particles. The first goal of this study consists in defining the main factors that contribute to signal enhancement. Taking into account the sample particularities, the injection of larger sample plugs appeared to be the simplest approach. However, most large plug injections are associated with a certain loss of separation [13]. The effect of the large plug injection of a PV suspension was investigated simultaneously with sample treatment and separation factors, using the set-up of a Plackett–Burman design. The second goal of this research was to study in more detail the impact of larger sample plug injections and to identify the separation conditions that allow injection of larger sample plugs without a loss of separation.

A successful signal enhancement approach would allow the analysis of samples with lower concentrations than the highly purified samples, without supplementary sample pre-treatment for PV enrichment, and a reduction in the volume of the required reference sample. Moreover, a reliable and consistent signal enhancement could contribute to the development of a sensitive standard method, applicable on a wide array of sample matrices.

2. Materials and methods

2.1. Chemicals and reagents

Sodium dodecyl sulphate (98.5%) was purchased from Sigma (Steinheim, Germany), *o*-phtalic acid (*puriss*, >99.5%) from Fluka (Steinheim, Germany), and sodium hydroxide (NaOH) 1 M from Fisher Scientific (Leicestershire, UK). All other chemicals were purchased from Merck (Darmstadt, Germany). All chemicals were used as bought.

2.2. Solutions

All solutions were prepared using ultra pure water, produced in-house by a Nanopure Diamond water purification system (Barnstead, Dubuque, IA). Buffers were prepared by dissolving the necessary amounts of boric acid and SDS in water and adjusting the pH using 1 M NaOH before bringing to volume.

Reticulocyte standard buffer (RSB) pH 7.3 was prepared using the following recipe: 0.01 M tris(hydroxymethyl)aminomethane (Tris), 0.01 M sodium chloride (NaCl) and 0.0015 M magnesium chloride ($MgCl_2$).

The pH measurements were performed using a pH-meter Orion 520 A (Orion Research, Boston, MA). Solutions were degassed by ultrasonication for 20 min in an ultrasonic bath (Branson Ultrasonic Corporation, CT) and filtered through a polypropylene membrane with 0.2-μm pore size (VWR, Leuven, Belgium) prior to CE analysis.

2.3. Instrumentation

CE experiments were performed on a Beckman P/ACE MDQ CE system (Fullerton, CA), equipped with a diode array detector (190–600 nm) placed at the cathodic side of the capillary. Untreated fused-silica capillaries with a total length of 50.2 cm (effective length was 40 cm), and inner diameters of 50 μm were purchased from Composite Metal Services (Ilkley, UK). Samples were injected hydrodynamically from the inlet vial. The pressures and injection times needed for a prescribed injection plug, expressed as percentage of the length to the detection window, were calculated using Beckman "CE Expert" software.

New capillaries were conditioned by flushing with 100 mM hydrochloric acid, followed by water, 1 M sodium hydroxide, and again water, each time for 10 min, using a 20 psi pressure. Prior to each measurement the capillary was rinsed with 0.1 M NaOH, water, and BGE for 2 min each by applying a 14 psi pressure.

2.4. Samples

Several batches of PV Sabin strain (type 1) were grown, collected and purified as described in [26]. Briefly, after HeLa cell suspension cultures are infected with a low multiplicity of infection, the cell cultures are further incubated until a complete cytopathic effect is observed. The cells are freeze-thawed three times and cellular debris is removed by low-speed centrifugation. The supernatant is concentrated by evaporation to ca. 1/200th of the original volume. To remove the high salt concentrations, the concentrate is dialyzed against phosphate buffer 0.02 M pH 7.3. The virus is further purified by anion-exchange chromatography by applying it to a home-made column containing epichlorohydrin triethanolamine (ecteola) as stationary phase. The virus is eluted from the column with a phosphate buffer 0.02 M pH 7.3. The columns were prepared according to [27]. Optionally the virus can be further purified by a sucrose gradient ultracentrifugation. After ultracentrifugation, the sucrose gradient is fractionated and the fractions containing PV are pooled. This represents the sucrose gradient purified virus (SGPV) sample.

Four different PV batches were used throughout this study. The concentration of the virus was determined spectrophotometrically for all tested samples [28]. Only one viral sample was purified using also sucrose gradient ultracentrifugation, and the concentration of the virus was found to be 140 μg/ml. The other three samples were collected from anion-exchange chromatography and had the following concentrations: 490 μg/ml, 1360 μg/ml and 910 μg/ml. To the samples injected as small plugs, 0.003% dimethylformamide, as EOF marker, and 20 μg/ml *o*-phtalic acid, as internal standard, were added. To the samples injected as large plugs, 0.00044% dimethylformamide, as EOF marker, and 6 μg/ml *o*-phtalic acid, as internal standard, were added.

The dialysis of the viral samples was performed using Slide-A-Lyzer MINI Dialysis Units from Pierce Biotechnology (Rockford, IL). 30 μl of virus suspension was dialyzed four times for 20 min against the prescribed buffer.

2.5. Experimental designs and calculations

2.5.1. Screening design

Five factors with a potential influence on the signal intensity and the quality of the PV separation, i.e. the dilution of the RSB used for dialysis, the sample plug size, the temperature, the presence of a water plug injected in front of PV suspension and the concentration of the separation buffer, were selected for investigation. The factors were examined at two levels −1 and +1 (Table 1) using an eight-experiments Plackett–Burman design (Table 2). To complete the 7 factors in the design, two dummy factors are added. The experiments were performed in random order. An experiment with all factors at central level (0) was performed at the beginning and after each fourth design experiment.

The effect of each factor was calculated as:

$$E_X = \frac{\sum Y(+1) - \sum Y(-1)}{N/2} \quad (1)$$

where E_X is the effect of factor X, $\sum Y(+1)$ and $\sum Y(-1)$ are the sums of the responses where X is at (+1) or (−1) level, respectively, and N is the number of design experiments [29].

The significance of an effect was assessed both graphically and statistically by means of a half-normal probability plot (not shown) and a two-sided *t*-test, respectively [30]. Each effect was compared with a critical effect (E_{critical}):

$$E_{\text{critical}} = t_{(\alpha,\text{d.f.})} \times \text{SE}_{(e)} \quad (2)$$

where $\text{SE}_{(e)}$ is the standard error of the effect, t the tabulated t-value, with significance level $\alpha = 0.05$ and d.f. the degrees of freedom associated with the $\text{SE}_{(e)}$ calculation [31].

$\text{SE}_{(e)}$ was estimated using the algorithm of Dong:

$$s_0 = 1.5 \times \text{median}|E_x| \quad (3)$$

$$\text{SE}_{(e)} = s_1 = \sqrt{m^{-1} \sum E_j^2} \quad (4)$$

where E_j is an effect that, in absolute value is smaller than or equal to $2.5 \times s_0$, m is the number of such effects, and also d.f. for E_{critical}

Table 1
Factors and levels investigated in the experimental designs.

Design characterization		Factors investigated		Units	Tested levels				
Goal	Type					−1	0	+1	
Screening	Eight-experiments Plackett–Burman design	X_1	RSB concentration	Ratio of initial concentration		1/10	5.5/10	1/1	
		X_2	Sample plug size	% capillary length		5	17.5	30	
		X_3	Capillary Temperature	°C		19	22	25	
		X_4	Water plug size	% capillary length		0	1	2	
		X_5	Separation buffer concentration	mM		50	75	100	
					$-\alpha$				$+\alpha$
					−1.414	-1	0	+1	+1.414
Optimization	Central composite design	X_1	Voltage	kV	10	11.5	15	18.5	20
		X_2	Sample plug length	% capillary length	5	6.5	10	13.5	15

Table 2
The eight-experiments Plackett–Burman design (A) and responses (B) to evaluate the effects (C) of five experimental factors on the responses H, R_s, and w_s. Factors X_i and levels, see Table 1 d1, d2 = dummy factors.

(A)

Exp	Factors						
	X_1	X_2	X3	X_4	X_5	d1	d2
1	+1	+1	+1	−1	+1	−1	−1
2	−1	+1	+1	+1	−1	+1	−1
3	−1	−1	+1	+1	+1	−1	+1
4	+1	−1	−1	+1	+1	+1	−1
5	−1	+1	−1	−1	+1	+1	+1
6	+1	−1	+1	−1	−1	+1	+1
7	+1	+1	−1	+1	−1	−1	+1
8	−1	−1	−1	−1	−1	−1	−1

(B)

Responses		
H	R_s	w_s
34746	0	7.02
72486	1.43	8.62
19222	1.95	3.65
19846	1.50	4.62
71922	1.16	7.00
15408	1.25	5.86
61538	0.00	8.04
32880	2.23	5.58
Average responses		
41006	1.31	6.30

(C)

Effects			
Factors	H	R_s	w_s
X_1	−16243	−0.76	0.17
X_2	38334	−0.84	2.74
$X3$	−11081	0.18	−0.03
X_4	4534	−0.18	−0.13
X_5	−9144	0.17	−1.45
d1	7819	0.05	0.45
d2	2033	−0.44	−0.32
Critical effects			
	23557	0.62	0.69

estimation. $|E_x|$ larger or equal to $E_{critical}$ are considered significant [30].

2.5.2. Central composite design

A central composite design (CCD) setup (Table 3) was applied to establish the experimental conditions that represent the best compromise between signal increase and separation efficiency. The experiments were again performed in a random order. For each response, a quadratic model was fitted to the data:

$$\hat{y} = b_0 + b_1x_1 + b_2x_2 + b_{11}x_1^2 + b_{22}x_2^2 + b_{12}x_1x_2 + \varepsilon \quad (5)$$

where $\hat{y}$ represents the predicted response, b the coefficients of the model estimated by least squares regression, ε the residual, and x_1 and x_2 the factors voltage and sample plug size, respectively (Table 1).

The response surfaces representing Eq. (5) were determined using Matlab version 7.01 (The MathWorks, Natick, MA). The optimal experimental conditions were selected based on the visual analysis of the response surfaces [29].

2.6. Responses

Three responses were considered throughout this study: the PV peak height (H) to express the signal intensity, the PV peak spatial width (w_s) to account for peak broadening, and the resolution between the PV peak and the neighboring peak (R_s) for the separation quality.

The height and the width of the PV peak were computed from electropherograms using the Beckman software 32 Karat 8.0. The PV peak width was corrected for the peak's velocity and the finite size of the detector zone as in [32].

Table 3
Experimental matrix, responses and coefficients for CCD. Factors and levels see Table 1.

CCD	Factors		Responses		
Exp	X_1	X_2	*Height*	R_s	w_s
0	0	0	85237	1.16	5.97
1	−1	−1	66392	1.50	5.10
2	1	−1	62914	1.36	5.61
3	−1	1	121807	1.04	5.75
4	1	1	102874	0.97	6.50
0	0	0	86734	1.17	5.87
5	−1.414	0	90667	1.12	6.10
6	1.414	0	77686	1.23	6.03
7	0	−1.414	56248	1.59	4.64
8	0	1.414	99639	0.82	6.01
0	0	0	84438	1.22	6.11

	Coefficients			
Responses		*B*	*t*	*p*
PV peak height	b_0	85470	20.53	
	b_1	−5096	−1.99	0.1021
	b_2	19592	7.68	0.0006
	b_{12}	−3863	−1.07	0.3328
	b_{11}	1212	0.40	0.7059
	b_{22}	−1904	−0.63	0.5579
Resolution R_s	b_0	1.179	27.96	
	b_1	−0.007	−0.30	0.7742
	b_2	−0.241	−9.35	0.0002
	b_{12}	0.018	0.48	0.6484
	b_{11}	0.005	0.16	0.8822
	b_{22}	0.018	0.58	0.5846
Broadening (w_s)	b_0	5.982	43.59	
	b_1	0.146	1.73	0.1438
	b_2	0.435	5.18	0.0035
	b_{12}	0.057	0.48	0.6539
	b_{11}	0.052	0.53	0.6234
	b_{22}	−0.317	−3.17	0.0249

3. Results and discussions

3.1. Preliminary investigations

The CE method for PV separation was developed using samples with extremely high viral purity (SGPV samples), assumed to contain only the intact PV particles. Hence, extremely clean electropherograms are obtained (Fig. 1A trace (a)). The use of SGPV samples for method development was necessary to simplify the peak identification, which is difficult in the absence of reference materials for PV [33]. However, for this method, the possible applications are limited to PV identification and quantification, after extensive purification of the original samples. The injection of less pure samples was expected to reveal peaks of both PV particles and subviral particles or contaminants. In this way, the CE methods could be used to study the purification of PV samples or even replication steps not fully explained or linked yet [34–36].

When a PV sample with a concentration of about 140 μg/ml was injected, the peak height was below 5 mAU (Fig. 1A trace (a)). Compared to real life virological samples, this is still a high concentration, and it was expected that this method will not be useful to study PV from cell extracts, where much lower concentrations may

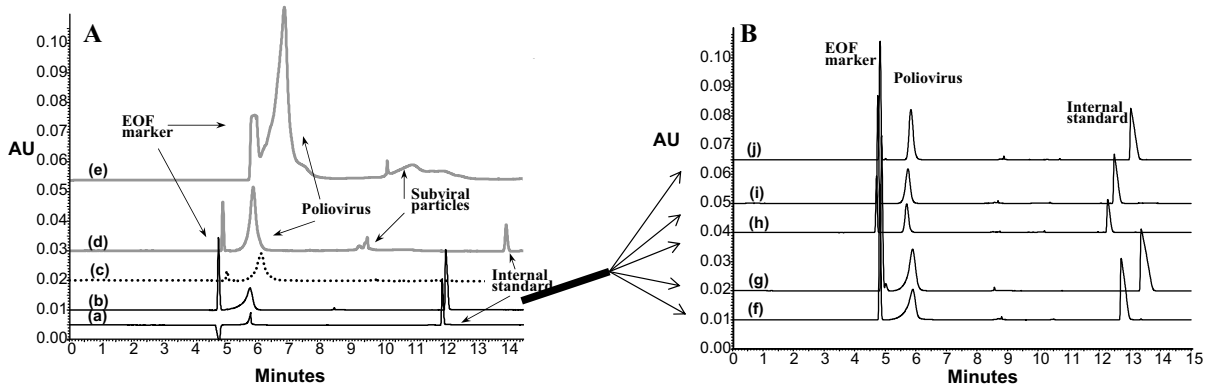

Fig. 1. Separation of various types of PV samples using short plug injections. Separation conditions as in [21], except for A-(e) when separation was performed at 20 °C instead of 30 °C. (A) Non-dialyzed samples: (a) SGPV sample injected as 1% plug; (b)–(d) different batches of AEX samples injected as 1% plug; PV concentrations were 490 (b), 910 (c) and 1360 μg/ml for (d); (e) AEX sample as from trace (d) injected as 5% plug. (B) Dialyzed 690 μg/ml AEX samples (sample as for trace (b)) against: (f) borate buffer 50 mM pH 8.3; (g) RSB buffer; (h) purified water; (i) borate buffer 5 mM pH 8.3; (j) RSB buffer diluted 1:10 with purified water.

occur. Also, when highly concentrated PV samples are degraded upon heating, or viral-assembly particles are injected, the low level of the signal will make the investigation of those sub-viral particles extremely difficult [33]. Therefore a signal-increasing approach, also applicable to less pure PV samples, was necessary as an elegant solution to the problems mentioned above.

In a first step, the injection of PV sample with lower viral purity was investigated to evaluate if separation of the PV peak is still maintained. Samples collected immediately after the anion-exchange chromatography (AEX) were analyzed using the method described in [21]. Further, larger plugs of AEX samples were hydrodynamically injected to investigate problems associated with large-plug injections and to identify possible solutions.

When the AEX sample, corresponding to the same PV batch as the SGPV sample, was injected, the relative area of the PV peak increased four times (Fig. 1A traces (a) and (b)). This increase can be explained as being the consequence of the reasons: (i) each purification step has its own yield, causing a decrease in the final concentration of the purified analyte and (ii) SGPV samples are extremely viscous and thus a smaller volume than from an AEX sample is probably injected. On the electropherograms of the AEX samples, several other peaks were also revealed. They were attributed to contaminants present in the AEX sample, probably with cellular or viral origin, from viral assembly or degradation. Unfortunately, the signals generated by these contaminants were too modest for a thorough investigation, underlining the necessity of a signal increase.

The electropherograms of two other AEX samples, used throughout this study, are also presented (Fig. 1 traces (c) and (d)). The AEX sample (Fig. 1A trace (d)), originating from the highest viral yield available, revealed a symmetrical PV peak. It seems that the variability of the AEX samples, besides considerably different PV concentrations, includes also variability in the composition of the sample matrix. Apparently, the height of sub-viral particles/contaminant peaks also seems to be higher for samples with higher PV concentration.

When the injected sample plug was increased to 5%, the separation was significantly degraded (Fig. 1A trace (e)). The peaks corresponding to PV and contaminants were broadened, and PV overlapped with the EOF marker. Another interesting phenomenon was the change in the shape of the EOF marker peak (Fig. 1A trace (d) vs (e)). In the short plug injection (Fig. 1A trace (d)) the marker migrates as a sharp peak while for the larger plug it tends to take a rectangular shape. The matrix of the AEX samples is phosphate buffer 0.02 M pH 7.3. Therefore the mismatch between sample and BGE conductivities will generate a heterogeneous electrical field inside the capillary, responsible for both above described observations. The field intensity is lower in the sample plug, reducing the velocity of the entities present in the sample. The slowing-down effect was stronger for the EOF marker, which has no own mobility and for PV, which has a low mobility. The internal standard, which has an effective mobility almost four times higher than PV, was able to leave the sample zone and was less affected by this effect.

These experiments highlighted that injection of AEX sample can result in higher PV peaks and even peaks corresponding to sub-viral particles or contaminants. The separation from the EOF marker was well maintained for samples injected as 1% plugs. Larger sample plugs lead to higher signals, but a reduction in sample conductivity seems necessary to maintain the separation.

3.2. Dialysis of PV samples

The sample conductivity should be decreased in a manner that conserves the PV concentration. Ideally, the procedure should be applicable to microliters of sample. In theory, dialysis appears to manage all mentioned problems. Therefore its ability to reduce the conductivity of AEX samples and to minimize the electrodispersion effects was assessed.

Aliquots of AEX samples presented in Fig. 1A trace (b) were dialyzed against five different media with conductivities equal or below that of BGE, i.e. (i) borate buffer 50 mM pH 8.3, (ii) RSB, (iii) water, (iv) borate buffer 5 mM pH 8.3, and (v) RSB 10-fold diluted with water. 20 μl liquid present on the upper side of the Slide-A-Lyzer membrane was collected and supplemented with EOF marker and internal standard and further analyzed using the CE method from [21].

Dialysis is a simple and easy-to-use technique, but different volumes were obtained. As expected, the highest volume was found for the sample dialyzed against water. An improvement in the PV peak shape was noticed for all dialyzed samples (Fig. 1B traces (f)–(j)). The samples dialyzed against water (h), borate buffer 5 mM pH 8.3 (i), and diluted RSB revealed quasi Gaussian peaks. The sharpest peaks were obtained for the samples dialyzed against diluted borate buffer (Fig. 1B trace (i)), while the highest signals were measured for the samples dialyzed against RSB (Fig. 1B traces (g) and (j)). In both latter samples, a peak was detected between the EOF marker and PV peaks, originating from RSB itself.

The CE method showed to be extremely sensitive to differences in sample conductivity arising from dialysis against the same buffer, but in different concentrations (see Fig. 1 traces (f) vs (i) and (g) vs (j)). Therefore, from dialysis against different buffer concentrations,

a series of samples with controlled conductivity can be obtained, if needed. This set of experiments showed that dialysis could be an effective approach to reduce electrodispersion effects for AEX samples. The best results were obtained when the AEX samples were dialyzed against the RSB 10-fold diluted with water. Still, at least three major inconveniences of the dialysis procedure were also noticed, i.e. large sample variability, difficulty to control for quantitative approaches, and rather large sample volumes required (relative to the total volume of available sample).

3.3. Screening for the most influencing factors on signal intensity and broadening

A screening design was performed to study the dispersive effects associated with the injection of larger sample plugs, noticed during the above experiments (Fig. 1A traces (d) and (e)). This knowledge was expected to facilitate the identification of CE separation conditions for PV where the analytical signal and resolution are maximized while peak broadening is minimized.

Five factors were investigated simultaneously using a two-level, eight-experiments Plackett–Burman design (Tables 1 and 2). The first factor was the dilution of the RSB used for dialysis. Its investigation was expected to provide an indication about the effect of the sample conductivity on the separation when injecting larger sample plugs. The second factor was the length of the injected plug, examined in order to estimate the balance between the signal intensity and the strength of the dispersive phenomena. The screening design results were expected to give an indication of a threshold for the injected plug size, which, once exceeded, compromises the separation. The third factor was the capillary temperature. It was selected based on previous data, for short plug injections, that indicated temperature as a critical factor for PV resolution [21]. Compared to the conditions described in [21], i.e. 30 °C, the temperature was decreased (Table 1) in order to limit the EOF increase, expected to occur as an effect of the enlarged sample volume injected.

As a fourth factor, the length of a water-plug, injected in front of the PV plug, was studied. This factor was chosen to compensate for the non-ideal conditions for sample stacking. Usually, a successful sample stacking requires a sample at least 10 times less conductive than the BGE [9]. For the PV samples, even reducing the conductivity in a well controlled manner, it was still a problem. In such cases, injection of a small water plug in front of the sample might be able to compensate for the non-ideal conductivity difference. Moreover, the injection of such small water plug was reported to increase sensitivity and reproducibility [37]. The fifth factor was the concentration of the BGE. Similarly to the capillary temperature, the BGE concentration (Table 1) was increased compared to [21]. It was expected that the use of a more concentrated buffer would promote sample stacking.

The following responses allowing a stringent characterization of the separation were selected: H for signal intensity; R_s for separation quality and w_s for peak broadening to characterize the separation efficiency [32,38]. The occurrence of response drifting was investigated by plotting the responses recorded at central levels as a function of time. No drift in the responses was seen (data not shown), and thus no correction of the responses prior to effect estimations was needed [30].

The use of more concentrated RSB for PV sample dialysis (X_1) was found to significantly decrease R_s. This can be explained from influences of the sample conductivity on the EOF magnitude, which can generate laminar flow, one of the main sources of peak dispersion [38]. Moreover, additional broadening, or even denaturation of sample components, can occur as a result of excessive heating within the sample plug when samples with higher conductivities are injected [6]. The highest dispersive effects were recorded for the experiments where PV samples with the highest conductivities were injected as the largest plug sizes (30%) (experiments 1 and 7, Fig. 2). The best results were obtained for samples dialyzed against 10 times diluted RSB (level-1 for X_1), indicating that a successful separation of large sample plugs requires a sample conductivity below this one.

The sample plug size (X_2) was found to have a significant influence on the signal intensity. Injection of larger sample plugs is one of the main dispersive factors, significantly decreasing R_s and increasing w_s (Table 2). The dispersive effects were stronger for samples injected as 30% plugs. Moreover, on the electropherograms of the experiments 1 and 7 (Fig. 2), PV was revealed as a group of peaks overlapping the EOF marker (Fig. 2). An acceptable separation and a PV peak increase of nearly 10 times were seen in the other two experiments performed with a 30% plug size (experiments 2 and 5). These experiments demonstrated that the conditions to maintain separation should be carefully chosen. Because of the negative influence on separation, it was decided to limit the maximal injected sample plug size (X_2) to the center point level (17.5%) during further experiments.

Within the tested levels, temperature had no effect on the separation. Taking into account the previous data [21], it was decided to perform further separations using the highest temperature level, 25 °C. Similarly, the water plug (X_4) had only negligible effects on the responses and therefore it was decided not to use it in further experiments. A higher BGE concentration (X_5) was found to significantly reduce w_s, and hence it was decided to increase the borate concentration to 100 mM during further experiments.

For the screening experiments, the EOF marker (dimethylformamide) and internal standard (*o*-phtalic acid) were added in

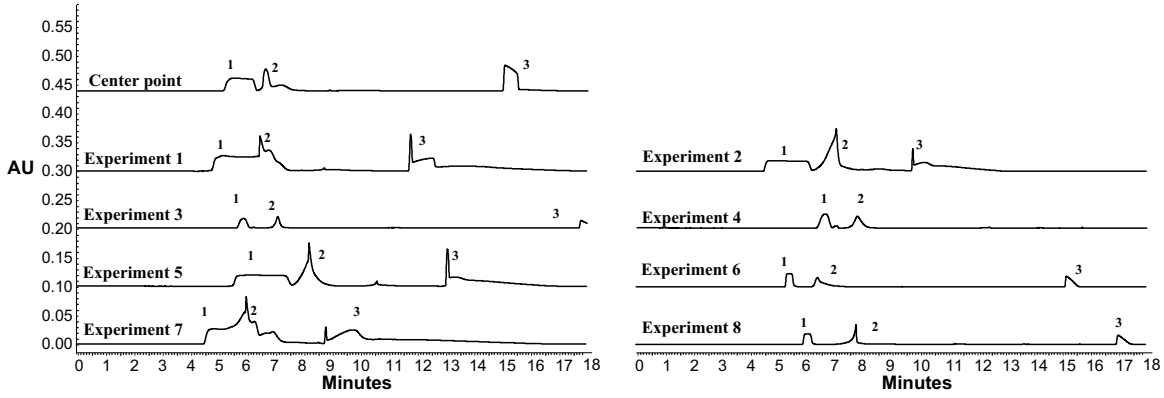

Fig. 2. Electropherograms corresponding to the screening design experiments. For experimental conditions see Tables 1 and 2. Poliovirus concentration was 490 μg/ml. Peaks: 1 – EOF marker (dimethylformamide); 2 – PV; and 3 – internal standard.

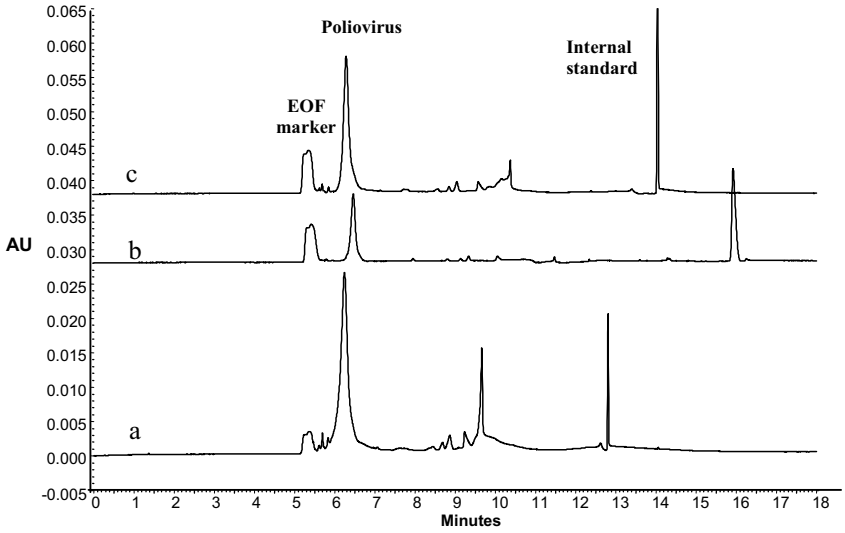

Fig. 3. The effect of sample dilution when PV samples are injected as 5% plugs. (a) AEX undiluted sample; (b) AEX sample diluted 1:4; and (c) AEX sample diluted 1:1. Separation conditions: fused silica capillary, 40 cm effective length, 10 kV, 26 °C, borate buffer 100 mM pH 8.3 containing 25 mM SDS, detection wavelength 205 nm, injection plug length 5%. Poliovirus concentration was 910 μg/ml.

the same amount as in [21], but an overload effect was noticed. To avoid it, the concentration of the markers in the sample was reduced almost seven times for DMFA and almost three times for *o*-phtalic acid and an improvement in the peak shape was noticed (Figs. 2 and 3).

The screening experiments indicated that when a large sample plug size with a higher conductivity is injected, the separation will be compromised. On the other hand, a large sample plug is necessary in order to have a signal increase. Further experiments, with 100 mM borate buffer pH 8.3 and 25 mM SDS as BGE, and a temperature of 25 °C, are needed to determine the maximal sample plug that can be injected to obtain the maximum PV height, without compromising the separation.

3.4. Identification of the maximal sample loadability

The screening experiments showed that a significant increase in PV peak height can be obtained injecting large plugs of PV samples with low conductivity. Under these conditions, the injection of a diluted PV sample was expected to still preserve the analytical information. Consequently, to circumvent the inconveniences of dialysis, the dilution of samples with water to reduce the sample conductivity was considered.

AEX samples were diluted in several ratios and injected as 5% plugs to identify the dilution level providing baseline separation. This plug size accounted for a higher R_s compared to larger plugs (Table 2) during screening. In this way, the effect of the sample matrix on the separation can be easily observed. PV is found baseline resolved from its neighboring peaks when samples are diluted at least 1:1 with water (Fig. 3). The injection of an AEX sample diluted 1:4 with water revealed extremely well separated peaks with excellent resolution, but with some loss of analytical information since several peaks seen in the undiluted sample were not detected anymore (Fig. 3). These experiments also showed that, for the internal standard, migration time and peak parameters are strongly influenced by the matrix conductivity (Fig. 3). Therefore, the use of the relative PV peak area to compare samples with different conductivity might be subjected to a bias error due to differences in IS separation. For this reason, PV corrected areas (peak areas divided by the corresponding migration times) instead of relative PV areas (peak areas divided by corresponding internal standard areas) were used further to compare the electropherograms of samples with different sample conductivities.

The samples tested during screening had different conductivities; hence the separations were performed at a constant voltage to avoid an increase in the system's complexity. When short plugs of SGPV samples were injected, an increase in the applied voltage from 10 to 30 kV had a significant negative effect on resolution, altering both selectivity and efficiency [21], whereas, when testing a stepwise increase in voltage (from 10 kV up to 25 kV) on AEX samples, only a small decrease in R_s was noticed (data not shown). For a larger plug injection of PV sample, a non-uniform electrical field will develop across the capillary and a different effect of the voltage was expected.

Several literature reports indicate voltage as a critical factor for development and optimization of CE separations [39,40]. The effect of voltage can easily be explained if we consider a fundamental CE equation to estimate the number of theoretical plates (N),

$$N = \frac{(\mu_{\text{effective}} + \mu_{\text{EOF}}) \times V \times l}{2 \times D \times L} \quad (7)$$

where $\mu_{\text{effective}}$ is the effective mobility of the analyte, μ_{EOF} the EOF mobility, V the applied voltage, L total capillary length, l the effective capillary length and D the diffusion coefficient of the target analyte [6].

Eq. (7) stipulates that application of higher electrical fields is beneficial for large molecules, such as proteins and nucleic acids, which have low diffusion coefficients. Theoretically, the low diffusion coefficients should account for less zone broadening compared to small molecules. In the specific case of PV samples, the estimated diffusion coefficient is around 1.44×10^{-7} [41], and therefore, theoretically, extremely narrow peaks are expected. Practically, the separation of the PV generated rather broad peaks, due to sample matrix interactions and electrophoretic heterogeneity among PV particles, originating from their colloidal nature (Fig. 1) [2].

Based on the above considerations, we supposed that higher voltages to separate large plugs of PV suspensions might reduce the broadening, and indirectly increase the peak height. A central composite design (Table 3) with the factors and levels presented in Table 1 was used to determine the largest sample plug that can be injected without losing the separation efficiency. During this study, AEX samples diluted 1:4 with water were used. The BGE and the

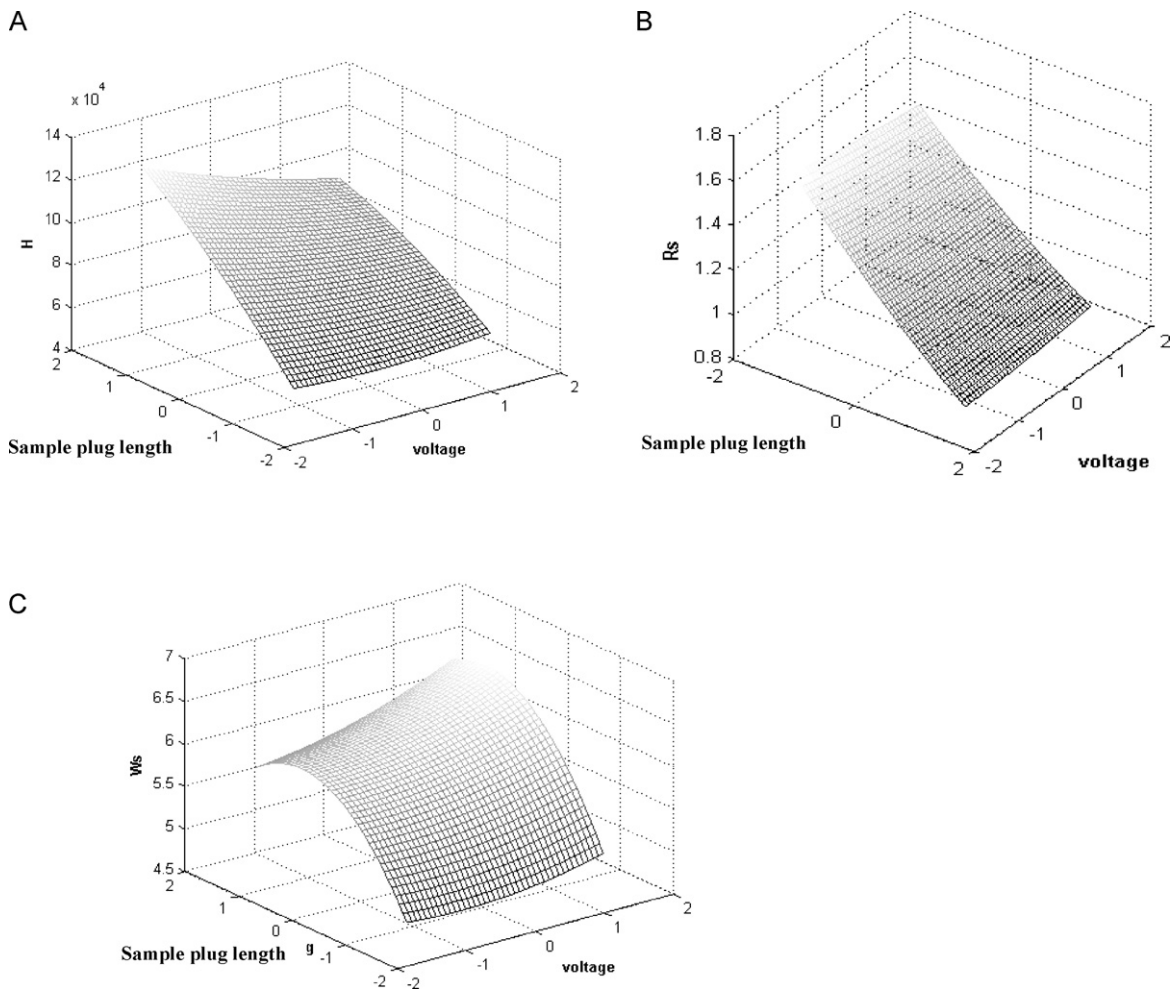

Fig. 4. Response surfaces generated for (A) peak height, (B) resolution, (C) w_s. For experimental conditions see Tables 1 and 3.

capillary temperature were selected based on the conclusions from the screening design.

For the CCD experiments, PV peaks significantly higher than those obtained using the conditions from [21], were obtained. The peaks had a nice, symmetrical shape with a mean asymmetry factor of 1.07. Moreover, they were resolved from the EOF marker peak in all experiments. This indicates that the PV peak will be resolved from the EOF marker when samples diluted 1:4 are injected as plugs with lengths between 5% and 15% and when a voltage between 10 and 20 kV is applied.

A quadratic model was fitted to each response, i.e. PV peak height, resolution and w_s, were modeled as a function of the sample plug length and the voltage, and the corresponding response surfaces were drawn (Fig. 4). The estimated coefficients of the quadratic model indicated a strong significant effect of the sample plug length on the responses while the effect of voltage usually accounted much less (Table 3, Fig. 4). The voltage was found to have an insignificant effect on all responses.

The model for height (H) predicts that maximal signals are obtained when sample plugs larger than 10% (level 0) are injected and the voltage is maintained at 10 kV. On the contrary, the R_s model predicts that the resolution will decrease drastically when large plugs (Fig. 4B) are injected. The R_s model also indicated that voltage hardly had an influence (Fig. 4). The w_s model predicts that minimal broadening will be obtained for sample plugs smaller than 10% (Fig. 4).

Based on these considerations, a set of experimental conditions consisting of a voltage of 10 kV and a sample plug of 12% were selected, resulting in a PV peak almost 14 times higher than at the conditions of [21]. When the sample plug was stepwise increased from 1% (initial conditions from [21]) to 12% (Table 4), resolution decreased gradually down to 42%, i.e. from 2.12 to 1.22. Simulta-

Table 4
The effect of various sample plugs and voltages on H, w_s and R_s.

Voltage (kV)	Size of the sample injection plug (% column effective length)	× Increase in H compared to initial conditions	Responses		
			H	w_s	R_s
10	1	–	11259	3.85	2.12
10	5	7.3	82562	5.00	1.51
10	8	7.7	86787	4.54	1.62
10	12	13.7	154781	4.93	1.22
19	8	6.4	71558	5.84	1.28
25	8	5.6	63571	5.61	1.23

neously, broadening increased with 30%, from 3.85 to 5.00, when a 5% sample plug was injected, and remained more or less constant, regardless of a further increase of the plug. These results indicate that 12% is about the maximal size of sample plug to inject, at the tested conditions, without compromising the separation.

Although no significant effect was found for voltage, for the same injected plug length a lower voltage usually generated higher PV peaks, larger R_s and smaller $w_{s,}$ as indicated in Fig. 4. These results indicate that, for diluted viral suspensions injected as larger plugs, higher voltages will not increase the separation efficiency, but rather have an unfavorable effect on the separation by increasing the heat generation. To verify this hypothesis, two higher voltages were tested. The sample was injected as an 8% plug because minimal broadening was recorded for this plug (Table 4). H decreased down to 26% when voltage increased from 10 kV to 25 kV. A similar decrease was noticed for R_s, while peak broadening increased with 24%, i.e. from 4.54 to 5.61. The results confirmed that to separate viral suspensions injected as larger plugs, lower voltages are needed to avoid broadening associated with extreme Joule heating.

To study the effect of sample conductivity on the separation quality, the optimal conditions, i.e. 12% sample plug and 10 kV, were also applied for AEX samples diluted only 1:1 (in design 1:4). The peaks were still baseline separated and a slight signal increase was noticed while similar peaks were revealed as on the electropherogram of the undiluted sample (Fig. 5 trace (b) vs Fig. 3 trace (a)). However, the group of peaks corresponding to contaminants or subviral particles was better revealed for the 1:1 dilution (Fig. 5). Although the PV concentration was 2.5 times lower, injection of a sample diluted 1:4 resulted in a PV peak of comparable height to the PV peak of the 1:1 diluted sample. Still, for the 1:1 diluted sample injected as a 12% plug, the resolution was less than half, while the broadening was 25% larger than for the 1:4 sample. It was concluded that acceptable separation can be obtained when the sample plug size us within the 5–12% range, even for 1:1 diluted samples.

The results also indicated that the type of target analyte, i.e. PV, or sub-viral particles/contaminants, will determine the level of dilution and the sample plug size to achieve maximal signals for the target analyte. For instance, a sample dilution 1:1 and a plug size of 5% could be considered when the target analytes are sub-viral particles or contaminants, while a dilution 1:4 and a plug size of 12% could be selected when the target analyte is intact PV. Based on the above considerations the minimal amount of PV suspension necessary for a CE run was reduced from 20 to 5 μl.

3.5. Validation at optimal conditions

Six consecutive replicates were performed to assess the repeatability of each procedure proposed above. For the 12% plug injection a relative standard deviation of the PV areas of 4.1% was found, while for the 5% plug injection, it was 3.8%.

Several dilutions of an AEX sample were tested to determine the detection limit. To mimic the samples from the PV replication and purification steps, dilutions were prepared with 0.02 M phosphate buffer pH 7.3. The detection limit of the PV peak was ~19.8 μg/ml when a 12% plug, diluted 1:4, was injected, and ~31.3 μg/ml when a 5% plug of a sample diluted 1:1 was injected. Compared with the method from [21], the detection limits of the new procedures correspond to a three to four times decrease when a 12% plug was injected, while almost a 2.5 times decrease was obtained with 5% plugs.

Further, a mixture made of the AEX fractions containing PV particles and subviral particles/contaminants was used to assess the separation efficiency when more complex samples are injected (Fig. 6). Regardless the used procedure, PV was excellently resolved from the subviral particles/contaminants. The nature of the subviral particles/contaminants is not yet known since the AEX samples were not thoroughly investigated up till now.

A series of seven PV concentrations, i.e. 100%, 75%, 62%, 50%, 25%, 12% and 10%, was created by diluting the AEX mixture with 0.02 M phosphate buffer pH 7.3, to simulate samples with different PV concentrations. To investigate the linearity of the procedures, each member of the series was further analyzed by both procedures, i.e. diluted 1:1 and injected as 5% plugs or diluted 1:4 and injected as 12% plugs. In general, higher peaks were obtained for PV when samples were diluted 1:4 and injected as 12% plug. Both procedures were linear over the range 91–910 μg PV/ml, corresponding to 10–100% of the PV concentration. The equation was $y = 223.8x + 968.18$ ($R^2 = 0.9983$) for the "5%" procedure, and $y = 417.64x + 657.02$ ($R^2 = 0.9952$) for the "12%" procedure, where y is the estimated corrected area of PV peak and x is the PV concentration. For the lowest concentration, the signal-to-noise ratio was 11 for the 5% plug procedure and 25 for the 12% plug, thus above 10,

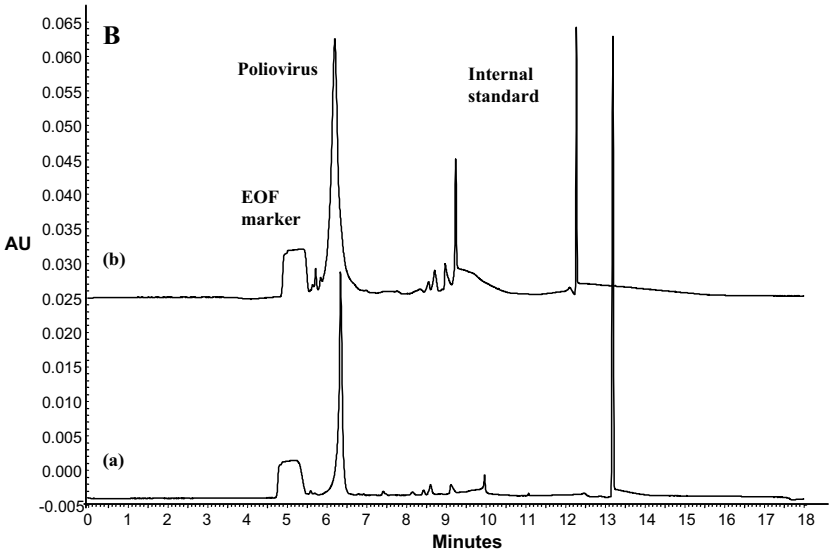

Fig. 5. The use of optimal conditions to separate PV samples with different conductivities: (a) AEX sample diluted 1:4; and (b) AEX sample diluted 1:1. Separation conditions: fused silica capillary, 40 cm effective length, 10 kV, 26 °C, borate buffer 100 mM pH 8.3 containing 25 mM SDS, detection wavelength 205 nm, injection plug length 12%. Poliovirus concentration was 910 μg/ml.

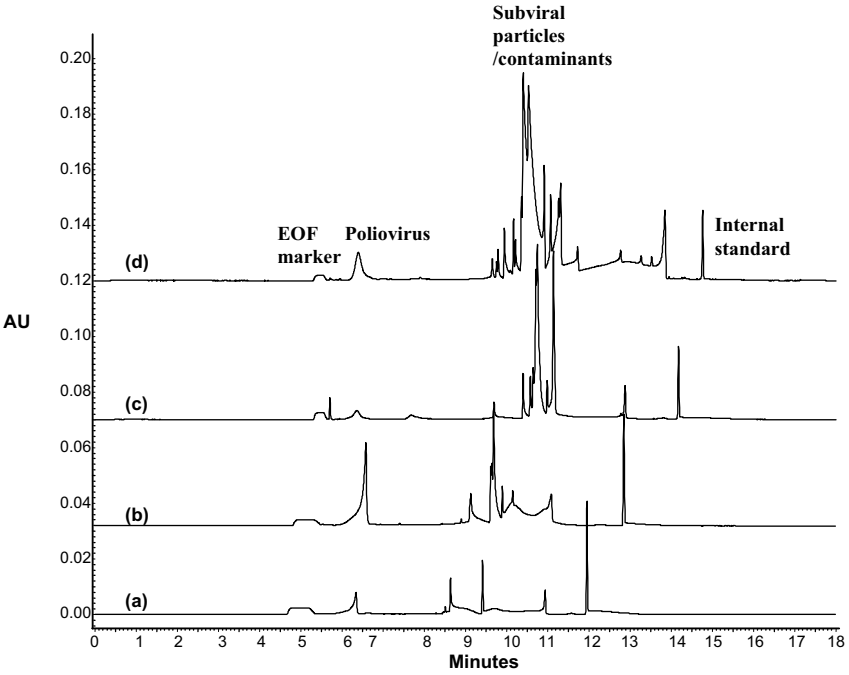

Fig. 6. Application of the proposed procedures on AEX mixture samples. Separation conditions: fused silica capillary, 40 cm effective length, 10 kV, 26 °C, borate buffer 100 mM pH 8.3 containing 25 mM SDS, detection wavelength 205 nm. Poliovirus concentration was 910 μg/ml. The sample was: (a) diluted 1:4 with water and injected as a 5% plug; (b) diluted 1:4 with water and injected as a 12% plug; (c) diluted 1:1 with water and injected as a 5% plug; and (d) diluted 1:1 with water and injected as a 12% plug. For (a) and (c), the sample was initially diluted up to 25% PV using phosphate buffer 0.02 M pH 7.4.

the ICH-defined signal-to-noise ratio for the limit of quantification [42].

Several PV samples from different origin and with different degrees of purity were further investigated using the proposed strategies and earlier method. PV was always detected using the proposed strategies. The earlier method failed to detect PV from the samples below 90 μg/ml PV (data not shown). The proposed strategy also should allow parallel investigation of PV, subviral particles and different AEX fractions [33].

4. Conclusions

An intra-capillary signal enhancing approach was successfully applied to a PV suspension, once the factors with most influence were identified. Differences in sample conductivity had strong effects on the CE analysis of the PV suspensions. Samples with higher conductivities largely reduced the resolution, although a promising enhancement of the analytical signal was also observed.

Reduction of sample conductivity in a reproducible and reliable manner was essential for designing an appropriate signal enhancement strategy for PV samples. Such step is less important in the signal enhancement procedure for small molecules. Dialysis was found not reproducible enough. An effective way to reduce the conductivity of the PV samples was a limited dilution of the sample, with water. This allows the injection of larger sample plugs without much sample treatment.

Surprisingly, voltage was not found to be a critical separation factor for PV samples, unlike the separation of some small molecules or some peptides and their immune complexes. However, the higher voltages anyway promote excessive Joule heating and thus are detrimental for the PV analysis.

Two improved procedures were proposed for qualitative and quantitative analysis, i.e. (i) a "5%" procedure, when samples were diluted 1:1 with water and injected as 5% plugs and (ii) a "12%" procedure, where samples diluted 1:4 with water were injected as 12% plugs. The choice of the applied procedure could be based on the targets, i.e. the study of PV or subviral particles/contaminants on one hand, or a PV concentration assay, on the other. Both procedures showed to be able to separate PV in more complex samples, such as mixtures from the AEX fractions, containing PV particles and subviral particles/contaminants. The procedures showed a good repeatability, were linear for PV particles and detected PV in much lower concentrations than the earlier method. The procedures also allow a significant reduction in the necessary volume of PV sample, from 20 to 5 μl, while the analytical signal is enhanced up to 14 times. The strategies were successfully applied to test several PV samples from different origin and with different degrees of purity.

The results of this study can provide a basis for the development of routine CE methods for viral particles analysis, especially when rational and reproducible signal enhancement is required.

Acknowledgements

Bieke Dejaegher and Sigrid Pieters thank the Research Foundation (FWO) – Flanders, Belgium for the financial support. This work was supported by a Horizontale Onderzoeksactie (HOA) of the Vrije Universiteit Brussel and a research grant (G.0051.08) of the FWO. We thank Monique De Pelsmacker for the preparation of the viruses, and Katrien Decq and Frank Van der Kelen for the technical and logistic assistance.

References

[1] T. Douglas, M. Young, Viruses: making friends with old foes, Science 312 (2006) 873–875.
[2] M.A. Rodriguez, D.W. Armstrong, Separation and analysis of colloidal/nanoparticles including microorganisms by capillary electrophoresis: a fundamental review, J. Chromatogr. B 800 (2004) 7–25.
[3] L. Kremser, D. Blaas, E. Kenndler, Virus analysis using electromigration techniques, Electrophoresis 30 (2009) 133–140.
[4] M.J. Desai, D.W. Armstrong, Separation, identification, and characterization of microorganisms by capillary electrophoresis, Microbiol. Mol. Biol. Rev. 67 (2003) 38–51.

[5] L. Krivankova, P. Pantuckova, P. Gebauer, P. Bocek, J. Caslavska, W. Thormann, Chloride present in biological samples as a tool for enhancement of sensitivity in capillary zone electrophoretic analysis of anionic trace analytes, Electrophoresis 24 (2003) 505–517.
[6] R.P. Oda, J.P. Landers, in: J.P. Landers (Ed.), Handbook of Capillary Electrophoresis, 1st ed., CRC Press, Boca Raton, 1994, pp. 9–42.
[7] M.C. Breadmore, J.R.E. Thabano, M. Dawod, A.A. Kazarian, J.P. Quirino, R.M. Guijt, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips (2006–2008), Electrophoresis 30 (2009) 230–248.
[8] S.L. Simpson Jr., J.P. Quirino, S. Terabe, On-line sample preconcentration in capillary electrophoresis. Fundamentals and applications, J. Chromatogr. A 1184 (2008) 504–541.
[9] M.C. Breadmore, Recent advances in enhancing the sensitivity of electrophoresis and electrochromatography in capillaries and microchips, Electrophoresis 28 (2007) 254–281.
[10] B.M. Simonet, A. Rios, M. Valcarcel, Enhancing sensitivity in capillary electrophoresis, Trends Anal. Chem. 22 (2003) 605–614.
[11] M. Urbanek, L. Krivankova, P. Bocek, Stacking phenomena in electromigration: from basic principles to practical procedures, Electrophoresis 24 (2003) 466–485.
[12] J.L. Beckers, P. Bocek, Sample stacking in capillary zone electrophoresis: principles, advantages and limitations, Electrophoresis 21 (2000) 2747–2767.
[13] R.L. Chien, D.S. Burgi, Sample stacking of an extremely large injection volume in high-performance capillary electrophoresis, Anal. Chem. 64 (1992) 1046–1050.
[14] Z.K. Shihabi, Organic solvent high-field amplified stacking for basic compounds in capillary electrophoresis, J. Chromatogr. A 1066 (2005) 205–210.
[15] Y. Xiong, S. Park, H. Swerdlow, Base stacking: pH-mediated on-column sample concentration for capillary DNA sequencing, Anal. Chem. 70 (1998) 3605–3611.
[16] M.E. Hadwiger, S.R. Torchia, S. Park, M.E. Biggin, C.E. Lunte, Optimization of the separation and detection of the enantiomers of isoproterenol in microdialysis samples by cyclodextrin-modified capillary electrophoresis using electrochemical detection, J. Chromatogr. B 681 (1996) 241–249.
[17] D.M. Osbourn, D.J. Weiss, C.E. Lunte, On-line preconcentration methods for capillary electrophoresis, Electrophoresis 21 (2000) 2768–2779.
[18] Z. Mala, L. Krivankova, P. Gebauer, P. Bocek, Contemporary sample stacking in CE: a sophisticated tool based on simple principles, Electrophoresis 28 (2007) 243–253.
[19] J. Palmer, N.J. Munro, J.P. Landers, A universal concept for stacking neutral analytes in micellar capillary electrophoresis, Anal. Chem. 71 (1999) 1679–1687.
[20] P. Britz-McKibbin, D.D.Y. Chen, Selective focusing of catecholamines and weakly acidic compounds by capillary electrophoresis using a dynamic pH junction, Anal. Chem. 72 (2000) 1242–1252.
[21] I. Oita, H. Halewyck, S. Pieters, B. Dejaegher, B. Thys, B. Rombaut, Y. Vander Heyden, Improving the capillary electrophoretic analysis of poliovirus using a Plackett–Burman design, J. Pharm. Biomed. Anal. 50 (2009) 655–663.
[22] L. Kremser, G. Bilek, D. Blaas, E. Kenndler, Capillary electrophoresis of viruses, subviral particles and virus complexes, J. Sep. Sci. 30 (2007) 1704–1713.
[23] C.P. Gerba, Applied and theoretical aspects of virus adsorption to surfaces, Adv. Appl. Microbiol. 30 (1984) 133–168.
[24] B. Rombaut, R. Vrijsen, P. Brioen, A. Boeyé, A pH-dependent antigenic conversion of empty capsids of poliovirus studied with the aid of monoclonal antibodies to N and H antigen, Virology 122 (1982) 215–218.
[25] V. Okun, D. Blaas, E. Kenndler, Separation and biospecific identification of subviral particles of human rhinovirus serotype 2 by capillary zone electrophoresis, Anal. Chem. 71 (1999) 4480–4485.
[26] B. Rombaut, R. Vrijsen, A. Boeyé, Stabilization by host cell components and Mg^{2+} of the neutralization epitopes of poliovirus, J. Gen. Virol. 66 (1985) 303–307.
[27] L. Levintow, J.E. Darnell, A simplified procedure for purification of large amounts of poliovirus: characterization and amino acid analysis of Type 1 poliovirus, J. Biol. Chem. 235 (1960) 70–73.
[28] J. Charney, R. Machlowitz, A.A. Tytell, J.F. Sagin, D.S. Spicer, The concentration and purification of poliomyelitis virus by the use of nucleic acid precipitation, Virology 15 (1961) 269–280.
[29] Y. Vander Heyden, C. Perrin, D.L. Massart, Optimization strategies for HPLC and CZE, in: K. Valko (Ed.), Separation Methods in Drug Synthesis and Purification, Handbook of Analytical Separations, vol. 1, Elsevier, Amsterdam, 2000, pp. 163–212.
[30] Y. Vander Heyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, Guidance for robustness/ruggedness tests in method validation, J. Pharm. Biomed. Anal. 24 (2001) 723–753.
[31] D.L. Massart, B.G.M. Vandeginste, L.M.C. Buydens, S. De Jong, P.J. Lewi, J. Smeyers-Verbeke, Handbook of Chemometrics and Qualimetrics, Part A, Elsevier, Amsterdam, 1997.
[32] X. Huang, W.F. Coleman, R.N. Zare, Analysis of factors causing peak broadening in capillary zone electrophoresis, J. Chromatogr. 480 (1989) 95–110.
[33] H.M. Halewyck, I. Oita, B. Thys, B. Dejaegher, Y. Vander Heyden, B. Rombaut, Identification of poliovirions and subviral particles by capillary electrophoresis, Electrophoresis 31 (2010) 3281–3287.
[34] A. Boeye, B. Rombaut, The proteins of poliovirus, in: J.L. Melnick (Ed.), Progress in Medical Virology, vol. 39, Karger, Basel, 1992, pp. 139–166.
[35] C.U.T. Hellen, E. Wimmer, Maturation of poliovirus capsid proteins, Virology 187 (1992) 391–397.
[36] J.R. Putnak, B.A. Phillips, Picornaviral structure and assembly, Microbiol. Rev. 45 (1981) 287–315.
[37] R.L. Chien, D.S. Burgi, Amplified sample injection in high-performance capillary electrophoresis, J. Chromatogr. 559 (1991) 141–152.
[38] B. Găs, E. Kenndler, Dispersive phenomena in electromigration separation methods, Electrophoresis 21 (2000) 3888–3897.
[39] S. Furlanetto, S. Orlandini, I. Giannini, G. Beretta, S. Pinzauti, Pitfalls and success of experimental design in the development of a mixed MEKC method for the analysis of budesonide and its impurities, Electrophoresis 30 (2009) 633–643.
[40] S.M. Babar, E.J. Song, M.N. Hasan, Y.S. Yoo, Experimental design optimization of the capillary electrophoresis separation of leucine enkephalin and its immune complex, J. Sep. Sci. 30 (2007) 2311–2319.
[41] F. Koch, G. Koch, The Molecular Biology of Poliovirus, 1st ed., Springer, Wien, 1985, p. 31–32.
[42] International Conference on Harmonisation of Technical Requirements for the Registration of Pharmaceuticals for Human Use (ICH), Validation of Analytical Procedures: Text and Methodology, Q2(R1), 2005, pp. 1–13, http://www.ich.org/ (accessed August 2010).