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Performance of instruments and aspects of methodology and validation in quantitative capillary electrophoresis An update[1]

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Abstract

The trial of capillary electrophoresis (CE) instruments from 1993 has been updated. The test procedure which was used there could be applied with few modifications. The data of 10 instruments are presented in a detailed table. Additional instruments are included this time. Aspects of method and instrument validation and robustness are discussed and listed in tables. Relevant instrumental features are deduced. Two-dimensional detection and a protocol of the current are very helpful for method development and validation. Some very useful methods that improve the limit of detection or resolution require pressure-driven counter-current and the possibility to control polarity and voltage during runs. Intermediate precisions corresponding to R.S.D. values of below 1% have become state of the art during the last few years. An overall day-to-day precision of 0.1% R.S.D. seems already possible if the multiple injection mode can be used. The detector technology has been improved, therefore injection became the main error source. This error source can be decreased by using internal standards or relative peak areas. In the future CE will be superior to other separation techniques not only in terms of performance, but also in terms of precision.

Keywords: Capillary electrophoresis: test of instruments; Instrumentation

1. Introduction

Commercial instruments for CE have been available since 1988. In the beginning precision was reported corresponding to about 5% R.S.D. for peak areas and heights — too poor for quantitations. However, in 1993 it was shown that R.S.D. values of 1–2% are possible with most instruments [1,2]. In the following years the interest in quantitative CE increased because of its advantages compared to LC: higher separation efficiency, different selectivity and short analysis times. The growing experience with CE, especially with aspects of validation, has been causing a flourishing use of this technique. As a positive feed-back this will lead to a further increase of the CE community.

Meanwhile new aspects of quantitation and validation came into the foreground. When the attainable precision in CE is discussed, not only the instrumental progress should be considered, but also methodological improvements, new methods of CE application and aspects of validation. In the last years more instruments were made available. Today about 20 companies are offering CE equipment (compare: Ref.

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[1]Dedicated to Professor Dr. G. Blaschke on the occasion of his 60th birthday.

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[3]). Therefore an update of the former work became necessary. To our knowledge we include in this study all complete CE systems that are commercially available.

Our concept to compare the performance of instruments was broadly accepted, so the former test procedure could be used with few modifications. In order to understand which additional instrumental aspects have become relevant during the last years, some recent developments in CE will be summarised and discussed here. A number of aspects such as temperature control, power supply and control, capillary length, and autosampler features are still important. These were already comprehensively discussed in previous articles, together with the backgrounds leading to the test procedure [1,2].

2. Experimental

2.1. Instrumentation: test design

A questionnaire (very similar to the one used in the earlier study [1]) was sent to German representatives and international head offices of all companies producing CE instrumentation. Data concerning reproducibility and the limit of detection were provided by the companies and verified in our laboratory. Information about the Hewlett-Packard and the Prince/Unicam instruments has been included for the first time, Perkin-Elmer and Beckman have been presenting new instruments, the data of BioRad, Dionex, Grom, Thermo Separations and Waters have been re-evaluated but remained essentially the same compared to the preceding study. The data obtained are shown in Table 1.

2.2. Test procedure

2.2.1. Borate buffer 100 mmol/l (pH 8.5)

6.183 g of boric acid were dissolved in 100 ml of HPLC-grade water. The pH of this solution was adjusted to 8.50 by a freshly prepared 1 *M* sodium hydroxide solution. This solution was taken into a 1-l measuring flask and filled up.

2.2.2. Sample solution

Approximately (±2 mg) 90 mg acetaminophen, 150 mg acetylsalicylic acid, 60 mg 3,5-dihydroxybenzoic acid and 120 mg nicotinic acid were weighed and filled up in a 100-ml measuring flask with the borate buffer described above.

2.2.3. Capillary electrophoresis

The experiment is carried out with a 50 cm (capillary inlet to detection window) fused-silica capillary, 50 μm I.D. It is conditioned by rinsing with 0.1 *M* sodium hydroxide for 30 min. Then it is filled with the buffer and equilibrated for at least 2 h. During this time a voltage of 25 kV is applied.

The sample is injected by pressure or vacuum. The product of pressure or vacuum and time shall be about 2.5 p.s.i. s. If the injection is hydrodynamic the product of height difference and time shall be about 172.5 cm s (1 p.s.i.=6.894.76 Pa=69 cm Δh). The same amount will then be injected with all instruments. The detection wavelength is set to 254 nm, rise time is 1 s. If the instrument provided a thermostating system, this was set to 25°C. When a stable separation is obtained (migration times of 5 subsequent runs do not differ more than 1%), the repeatability test described below can be performed.

2.2.4. Repeatability

When a stable separation is obtained (compare above) the sample solution is measured 10 times. If the instrument provides an autosampler, separate vials must be used for each injection. Before each run the capillary is rinsed with running buffer until the capillary content is exchanged twice. The repeatability is reported as R.S.D. for peak height, area and relative peak area respectively. Instead of area the corrected area (area/migration time) may be used.

2.2.5. Day-to-day precision

During 48 h at least 60 samples are injected out of at least 10 different sample vials. The obtained R.S.D. values correspond to the day-to-day precision.

2.2.6. Concentration corresponding to the smallest detectable signal (limit of detection)

Varying from the conditions given above the wavelength is 200 nm in the following experiment.

Table 1
Technical data for CE instruments

Parameter	Company		Applied Biosystems GmbH	Beckman Instruments GmbH	Bio-Rad Laboratories GmbH	Dionex GmbH[9]	Grom GmbH[9]
	Address		Perkin Elmer[9] Brunnenweg 13 64331 Weiterstadt Germany	Frankfurter Ring 115 80807 München Germany	Heidemannstr. 164 80939 München Germany	Richard-Klinger-Str. 15 65510 Idstein Germany	Herrenberger Str. 54 71083 Herrenberg Germany
	Telephone		06150/ 101-0	089/ 358700	089/ 31884127	06126/ 9910	07032/ 73261
	Fax		06150/ 101-101	089/ 35870-262	089/ 31884123		07032/ 76115
	Described instrument		270A-HT (ABI PRISM 310[9])	P/ACE 5000 Series	BioFocus 3000	CES	CE-System 100
High Voltage	voltage range (kV)		0 - 30	0 - 30	0 - 30	0 - 30	0 - 30
	reverse voltage		yes[11]	yes	yes[31]	yes[41]	yes[41]
	possibility of working under constant current		no	yes	yes	yes	yes
	voltage ramp possible		yes	yes	no	yes	no
	recording of current		yes	yes	yes	no	no
	recording of voltage		no	yes	yes	no	no
Capillary	minimum total length (cm)		[12]	27[13]	17	[12]	20
	minimum effective length (cm)		30	20[21]	12.4	40	13
	cartridge system		no	yes	yes[32]	no	no
Injection	pressure/ vacuum (principle)		yes	pressure injection (0.5 psi)[22]	pressure injection (5 psi)	yes[42]	yes
	electrokinetic		yes	yes	yes (only under constant current/ voltage)	yes[42]	yes
	wait for temperature		no	yes	temperature set during ringing cycles	no	no
Temperatur control	thermostat		yes	yes, Peltier	yes	no[43]	no[43]
	temperature range (°C)		5 above ambient - 60	15 - 55	15 - 40	ambient temperature	ambient temperature
	tempering medium		air	Peltier; inert cooling liquid	water	forced air	forced air
	record of temperature		no	no	yes	no	no
Detection	wavelength range (nm)		190 - 360[13]	190 - 600	190-365 or 366-800 respectively	190 - 800	190-800
	multiwavelength detection		no	yes	yes[33]	no	no[51]
	band width (nm)		5	1		6	[52]
	monochromator/ fibre optic		grating/no	no, DAD	grating	grating/ yes	grating/ no[52]
Autosampler	number of sample positions		50	23	31[34]	40	-
	number of diff. buffers available		1[36], 8	23	1[35], 31[34]	3[35], 43[34]	-
	tray thermostatable		yes	yes	yes	no	-
	can sample vials be covered against evaporation		yes	yes	yes	yes	yes
	humid athmosphere for microvials		yes	no	yes[36]	no	yes
	sample volume (µL)		5 - 4200	> 1	5 - 1500	5 - 500	1 - 500
	fraction collector		yes	yes	30	no	-
Instrument control/ Data processing	max. data rate (sec^{-1})		[14]	20	96	20	[14]
	rise time (sec)		0.02 - 5	0.1 - 1	1 - 5 programable	0.1 - 3[44]	0.1 - 3
Reproducibility	repeatability (10 subsequent injections)	height:	0.6	0.4 - 0.6[23] [24]	-	0.7 - 1.3	[53]
	area	area:	1.2 - 1.3	0.6 - 0.7[23] [24]	<3[37]	1.0 - 1.2	
		rel. area:					
	day-to-day (measurement during 2 days)	height:	1.8 - 3.1	0.5 - 1.3[23] [24]	-	-	[53]
		area:	1.9 - 2.7	2.1 - 2.7[23] [24]	<3[37]	-	
		rel. area:					
	Amount corresponding to the smallest detectable signal (mg/ L)[1]		0.12	0.2[23] [24]	-	-	[54]
	Special features		Genetic analyzer: complete automated system for DNA sizing, quantitation, and sequencing	coupling to LIF[25] or MS optional	coupling to ESI-MS-instr. from Finnigan, Fisons optional; kits for SDS-Page, DNA-Sep. and IEF deliverable; high pressure capillary flush	fluorescence detection	on-line coupling with NMR, MS; electro chromatography with gradient elution
	List Price (DM)[9]		72,000.- (270A-HT)	91,000.-[38]	98,500.-	[12]	32,950.-
	Time of Delivery (weeks)		4	8	4 - 6	6	from stock
	Dimensions (mm)[9]		508 x 597 x 584	635 x 381 x 514	490 x 580 x 560	710 x 640 x 460	three modules
	Places of Service (Ger)		Weiterstadt, Kiel, München, Basel, Wien	45 mobile service engineers	München, Krefeld, Berlin	München, Hamburg, Idstein, Herrenberg, Zürich (CH) Berlin, Mühlheim/ R	
	Other CE-instruments		no	-	-	[45]	-

Table 1. Continued

Parameter							
	Company		Hewlett-Packard	Prince Technologies B.V.	Thermo Separation Products	UNICAM Analytische Systeme GmbH	Waters GmbH[9]
	Address		Hewlett-Packard-Str. 8 76337 Waldbronn Germany	P.O. Box 2194 Handelsweg 7b 7801 CD Emmen The Netherlands	Guerickeweg 7 64291 Darmstadt Germany	Postfach 420144 Korbacher Str. 75-77 34132 Kassel Germany	Postfach 5029 Hauptstr. 87 65760 Eschborn Germany
	Telephone		07243/ 602840	31-(0)5917/ 629184	06151/ 930-0	0561/ 940870	06196/ 400-600
	Fax			31-(0)5917/ 626345	06151/ 930-222	0561/ 9409795	06196/ 462396
	Described instrument		HP[3D]CapillaryElectrophoresis system	Prince 466	Spectra PHORESIS 1000/Ultra[21]	Crystal 310	Quanta 4000
High Voltage	voltage range (kV)		0 - 30	0 - 30	0 - 30	0 - 30	0 - 30
	reverse voltage		yes[21]	yes[71]	yes	yes	yes[101]
	possibility of working under constant current		yes	yes, no constant power	yes	yes	yes
	voltage ramp possible		yes	yes	yes	yes	no
	recording of current		yes	yes	yes	yes	yes
	recording of voltage		yes	yes	yes	yes	yes
Capillary	minimum total length (cm)		33	46	44 \ 22	65	[12]
	minimum effective length (cm)		8.5[21]	-	37 \ 8[22]	46	20
	available options		yes	no	yes	no	no
Injection	pressure/ vacuum (principle)		yes, pressure (positive and negative)[22]	yes (both)	yes (just vacuum) \ yes[47]	yes	yes[102]
	electrokinetic		yes	yes	yes	yes	yes[103]
	wait for temperature		yes	yes	yes[80]	yes	no
Temperatur control	thermostat		yes	yes	yes, Peltier	yes	yes
	temperature range (°C)		10 - 60	5 below ambient temperature - 60	15 - 60 \ 10 below amb. temp. - 60	5 below ambient temperature - 80	10 below ambient temperature - 45[104]
	tempering medium		air	air	air	Peltier element	Peltier element
	record of temperature		yes	yes	yes	yes	yes[105]
Detection	wavelength range (nm)		190-600	190 - 800	190 - 365 or 366 - 800 respectively	190 - 800	185 - 546[106]
	multiwavelength detection		yes	no	yes[20]	yes	no
	band width (nm)		2-400	6	6	1.3	[107]
	monochromator/ filter optic		no, DAD[22]	grating	grating (LIS) / yes	yes	line spectrum filter/ no
Autosampler	number of sample positions		48	48	80 + 2 \ 108[80]	48	20 hydrostatic/ 6 electrokinetic injection
	number of diff. buffers available		20[24]	30	1[35], 80 + 2[22] \ 108[80]	30	4 or 6
	tray thermostatable		yes, 10 - 40°C	yes, 4 - 40°C	no[80] \ yes[87]	yes, 4 - 40°C	yes
	can sample vials be covered against evaporation		yes	yes	yes	yes	yes
	humid atmosphere for microvials		no	yes	no \ yes	no	no
	sample volume (µL)		> 3	5 - 4000	5 - 1500 \ 20 or 50[80]	1 - 4000	25 - 500
	fraction collector		yes[44]	yes	no \ yes	possible	yes[108]
Instrument control/ Data processing	max. data rate (sec^{-1})		-	75	98	50	300
	rise time (sec)		[12]	0.02 - 2	0.1 - 9.9[44]	0.1	0.1 - 1
Reproducibility	repeatability (10 subsequent injections)	height:	~1.5[80]	0.4[72] / 0.15 - 0.32	0.78 - 1.03 \ [80]	0.15 - 0.32	0.3 - 0.7
	area	area:	~1.5[80]	0.4[72] / 0.24 - 0.35	1.03 - 1.23 \ [80]	0.24 - 0.35	0.7 - 0.8
		rel. area:	0.27 - 0.90[80]	/ 0.13 - 0.26	0.22 - 0.27 \ [80]	0.13 - 0.26	
	day-to-day (measurement during 2 days)	height:	~1.5 - 2[80]	0.5 - 1[72] / 1.65	1.19 - 2.05 \ [80]	1.65	1.8 - 2.5
		area:	~2[80]	0.5 - 1[72] / 1.86	1.08 - 1.48 \ [80]	1.86	1.5 - 1.9
		rel. area:	~0.5 - 2[80]	/ 0.31	0.24 - 0.85 \ [80]	0.31	
	Amount corresponding to the smallest detectable signal (ng/ L)[1]		0.12[20]	0.3[72]	0.12 \ [80]	0.48	[109]
	Special features		various coupling possibilities e.g. MS[47]; full validation program available; high pressure flush; can operate under const. pressure/ vacuum; vial access during analysis; capillary electro chromatography standard operation mode	coupling to conductivity[78], DAD[74], FPD (Flame Photometric Detector)[76], LIF[79], thermo optical absorption[77], MS[75] detectors possible	online sample derivation by diluting, mixing, heating, cooling, extracting; automat. mixing and replenishment of buffers; coupling to MS; electrochromatography	coupling to[21] UV/ VIS, DAD, fluorescence, conductivity, MS detectors possible; polarity reversal during run possible; programable pressure profile during run; [22]	original Eppendorf-vials fit to autosampler tray; isomigration modus; hardware controlled by chromatography software Millennium 2010
	List Price (DM)[2]		104,304.-	46,500.-	59,000.- \ 84,500.-	65,000.-	[12]
	Time of Delivery (weeks)		3-6	4 - 6	4 \ [80]	4 - 6	4 - 6
	Dimensions (mm)[2]		800 x 700 x 520	350 x 420 x 500	470 x 650 x 470 \ 820 x 510 x height	480 x 500 x 480	560 x 550 x 880
	Places of Service (tier)		Frankfurt, Hamburg, Berlin, Nürnberg, Ratingen, München, Leipzig, Bad Homburg, Waldbronn	no	Darmstadt, Hamburg, Solingen, München	München, Stuttgart, Frankfurt, Kassel, Recklinghausen, Berlin, Hamburg	Berlin, Frankfurt/M., München, Düsseldorf, Pforzheim, Hamburg
	Other CE-Instruments			Prince 200, 250, 400, 410, 450, 550, 560[70]	Spectra PHORESIS 100 & 500	[99]	no

[1]According to the standard test procedure described in Section 2. [2]List prices in Deutschmarks according to German companies without taxes and without computer ware, unless otherwise stated (09/96: 1 US$≈1.50 DM) [3]Width×depth×height. [4]Data from [1], company confirmed that there are no significant changes up to now. [5]Genetic analyzer: complete system for DNA sizing, quantitation and sequencing based on the CE principle. [11]By input at the instrument. [12]No data available. [13]Optional: measurement in the visible range after assembling special lamp. [14]Analogue output, external data processing. [21]Only 7 cm, if injection close to detector, but in this case full field strength is not available. [22]Pressure during injection is controlled by 500 data points/s and corrected over the time. [23]Previous data, obtained by P/ACE 2100. [24]Improved detection system compared to P/ACE 2100. [25]Optional lasers: Argon ion (488 nm), Helium Cadmium (325 nm), Helium Neon (633 nm). [26]List price given for P/ACE 5510 (DAD and temperated sample tray). [31]Programmable by software. [32]Complete cartridges available, capillaries can be exchanged by users. [33]Max. 32 simultaneously; recording of spectra possible. [34]Buffers and samples can be placed in the same autosampler tray positions; overall number of positions is given. [35]At capillary outlet. [36]Sample tray can be filled with water. [37]Guaranteed by company. [41]By changing of connections or modules. [42]Injection at both sides of the capillary possible. [43]No thermostatting range. [44]Electronic damp of the analogue output may also be switched off. [45]New prototype in development. [51]Optional with additional equipment. [52]Normally a UV–Vis detector Spectra 100 with flow cell (Thermo Separations) is installed. [53]Not tested. [54]Not tested, see also [53]. [61]Only 8.5 cm, if injection close to detector, but in this case full field strength is not available; supported by software. [62]2–12 bar. [63]Optic filters available. [64]Pressure fraction collection, electr.-elution fraction collection, fraction collection from C-IEF fully automatable from software using built-in peak detector. [65]Values obtained in our lab; has to be confirmed by company. [66]Data obtained with bubble cell capillary did not always lead to significant improvement. [67]Side panel opening. [71]By input at the instrument. [72]Different test method: 50 mbar, 6 s. injection, cytosine 100 mg/l, buffer CAPS 50 m*M* (pH 10.0), $\lambda = 254$ nm. [73]Equipment: Crystal 1000-ATI, Boston, MA, USA; [47]. [74]Equip.: Crystal 240 DAD-ATI, Boston, MA, USA, [54]. [75]See [48], equipment home built by cited authors. [76]See [55,56], equipment home built by cited authors. [77]See [49], equipment home built by cited authors. [78]See [39–43]. [79]Instruments differ in certain features, list prices between DM 26 800.- and 50 000.-; for detailed information contact company. [81]New model Ultra introduced on Analytica '96; only changes are pointed out. [82]Only 6 cm, if injection close to detector, but in this case full field strength not available. [83]Programmable. [84]93 vials if sample tray is temperated. [85]94 vials if sample tray is temperated; theoretically indefinite number of buffer vials available because of automatic refilling and buffer replenishment. [86]Tray filled with cooling material optional (no active tempering). [87]Optional, by Peltier element. [88]20 μl hydrodynamic injection and 50 μl electrokinetic injection. [89]Data not yet available. [91]Simultaneous use possible. [92]Buffer replenishment during run. [93]Another instrument available (only 4 sample positions), list price 55 000.-. [101]By exchanging module. [102]Hydrostatic loading system. [103]Possible in the range from 0.1–30 kV. [104]In steps of 0.5°C. [105]External analogue recording optional. [106]Discrete line spectra: 185, 214, 229, 254, 280, 313, 365, 405, 436 and 546 nm available. [107]Discrete lines, very high wavelength accuracy. [108]Manual operation. [109]Not available with described method, anions and cations in the ppb range.

The sample solution described above is diluted several times. The dilutions are injected. The concentration corresponding to the smallest detectable signal is calculated from the lowest concentrated dilution, that still shows all 4 main peaks distinguishable from the baseline noise in 5 subsequent runs. The limit of detection (LOD) is given as concentration of 3,5-dihydroxybenzoic acid (α=30 100 l/mol cm at 200 nm, determined in the CE buffer, pH 8.5) in mg l^{-1}.

3. Results and discussion

3.1. Methodological aspects

3.1.1. The use of internal standards

Generally there are three major instrumental error sources in CE: detection, integration and injection. New developments in detection techniques have improved the overall precision. If a good detector is used and the concentrations can be chosen freely, i.e. high enough, the injection error becomes dominant. In this case the use of internal standards is beneficial, because it is able to compensate for injection errors [4–12].

However, in some cases no improvement but even a change for the worse has been reported, when internal standards were used [8,13,14]. Probably the detector noise and the integration error were the main error sources in these cases. Thus the integration error of the internal standard significantly increased the overall error according to the law of error propagation.

Suitable internal standards should have a similar mobility as the analytes to avoid effects of EOF changes during the run. Internal standards should be used in high concentrations in order to maximize the signal-to-noise ratio and to minimize integration errors.

Using pressure-driven injection systems, internal standards or relative peak areas lead to a repeatability corresponding to about 0.5% R.S.D. and to a day-to-day precision corresponding to 1% R.S.D. or less. This value can be obtained with a number of commercially available instruments. Internal standards also improve the precision of electrokinetic injection [down to 1–2% (repeatability)] [15].

These R.S.D. values also characterise the quality of CE methods. The value of 0.5% for R.S.D. is not too far away from the theoretical limit of about 0.2%. This number corresponds to the mere integration error [6] (here [16] cited). One can remain under this threshold, if a multiple injection mode is used.

If the precision of a CE method is corresponding to an R.S.D. value of above 1%, there are still some aspects to optimize. For instance, if there is a trend in both peak areas and migration times, the stability of the EOF should be controlled. Rinsing procedures should be considered in order to control and minimize matrix effects.

3.1.2. Sample concentration

Meanwhile the dependence of S.D. and sample concentration has been established as a fundamental law for quantitative CE [8,13,17]. High sample concentrations are favourable; often they can easily be obtained. If the sample concentration is low, precision can be improved by back-pressure assisted stacking [18]. By this method a highly concentrated sample zone is obtained by a focusing step.

For the same reason the quantitation of side compounds often becomes more precise, if the column is overloaded with the main compound. If the main peak becomes off-scale, it is possible to compare the area of minor peaks in this electropherogram directly to the area of the main peak from another electropherogram where a smaller known amount was injected, because the UV detection is linear over a wide range [19,20].

3.1.3. Appropriate calibration functions

Calibration curves are linear over a wide range, if peak areas are used. CE peak heights usually lead to non-linear calibration functions and low sensitivity for higher concentrations and thus, errors of the analytical result [21]. Even though peak heights are sometimes reported to be more reproducible [10], their use can only be advantageous close to the LOD. Quantitative assays are always performed far above the LOD; thus heights are rarely suitable for reliable quantitations, whereas peak areas are much more relevant when instrument performances are compared.

It has been shown that heteroscedasticity can be a relevant source of error in CE, if the concentration

range exceeds one order of magnitude and ordinary least squares regression methods are used. This error can be completely avoided using weighted least squares [22]. Heteroscedasticity is mainly caused by the injection error; therefore internal standards can partly compensate for it.

3.1.4. Miscellaneous important aspects

Thermally induced fluctuations of the baseline are stronger at low wavelengths, because here the refraction index is higher [23]. Therefore the use of a higher wavelength is often favourable.

A drift in migration times causes the reproducibility of peak areas to deteriorate. Often corrected areas are not helpful, because of the dynamic changes of surface equilibria [24]. The stability of the electroosmotic flow (EOF) is an important criterion for CE validation. The EOF can be influenced by small amounts of impurities. Thus the purity of the reagents and the cleaning of the glassware used for sample pretreatment can become very important for reproducibility.

More pitfalls are summarized that should be avoided to spoil the obtainable repeatability of about 1% R.S.D. with most commercial instruments. Not only preconditioning should be carefully described [8,25], but also two runs should be done with buffer injections only before a sample series is analysed in order to let the system stabilise. This helps to equilibrate the capillary surface and allows the solutions in the autosampler to reach a constant temperature. It is important to check that the filling height of all vials is approximately the same; otherwise siphoning effects are observed. It was also observed that the distance of sample and buffer vials should be small. Special attention should be paid to the vial caps: wrong positioning can lead to evaporation, but also to pressure differences from injection to injection [8].

3.2. Validation

Validation is substantial for the quality of analytical results, thus it is a main issue for CE as well. General guidelines to validate CE methods were already proposed [26,27]. Method and instrument validation will be separately discussed in the following paragraphs.

Method validation does not mean method development but is defined as a systematic inspection of relevant aspects. A validated instrument is required for method validation. Aspects of importance are listed in Table 2: peak homogeneities should be checked for all relevant peak groups. The sensitivity (slope of calibration function, response factor) is a good indicator to assure wavelength accuracy and reproducibility. Linearity can be checked in CE as well, although there are almost no non-linear curves reported, when peak areas are used for calibrations. Heteroscedasticity is relevant if the working range exceeds one order of magnitude (compare above). The test of the EOF reproducibility is a CE-specific aspect. The EOF depends on the inner surface structure of the fused-silica capillaries; thus the EOF varies from capillary batch to batch, but also from different pieces from a capillary spool [28]. Even more important, the EOF can be strongly influenced by adsorbed analytes or matrix constituents [28]. Rinsing procedures and their effectiveness are therefore another important aspect of validation.

As outlined above, impure reagents and glassware for sample pretreatment can strongly influence the EOF (compare [29]). Thus the suitability of different

Table 2
Important aspects of method validation (compare [26,27])

Aspects that are generally relevant to validate analytical methods
Peak homogeneities of all relevant peaks, freedom from matrix interferences
Wavelength accuracy and reproducibility; reproducibility of sensitivity/response factors
Linearity, rectilinearity
Heteroscedasticity
Peak shapes and efficiencies
Short and long term precision of migration time and peak area (=repeatability, intermediate/day-to-day precision...)
Limit of detection (LOD), limit of quantitation (LOQ)
Rules to exclude outliers
Reproducibility between analysts and instruments
Robustness
Accuracy
Crossvalidation
Recovery
Additional aspects that are relevant to validate CE methods
EOF stability
Rinsing procedures
Buffer stability and shelf-life
Reproducibility between capillaries

reagent batches should be tested. The shelf-life of the buffers can differ over a wide range and should be validated as well. For instance, citrate buffers are nutritious for many microorganisms, thus these buffers have a short shelf-lifetime. A shelf-life of three months was reported for a borate–phosphate buffer [30]

Method robustness is an important aspect of validation. In order to test robustness, parameters that can influence the analytical result in CE, must be identified (Table 3). These parameters can subsequently be varied and, depending on the analytical problem, their influence on parameters such as peak resolutions, efficiencies and reproducibility of migration times and peak areas is noted. Here a fractional factorial design is helpful to reduce the number of experiments [31,32].

Similar to method validation, instrument validation means the systematic inspection of all relevant instrumental parameters. Most parameters can only be tested by a reference method. This method must be validated, and parameters obtained with a validated instrument using this method must be known. When this reference method is used with another instrument, the obtained parameters can be compared to the ones obtained with validated instrument. Aspects of importance are listed in Table 4.

The test method described in Section 2 proved to be very useful for instrument validation as well. Typical values for many parameters of CE instruments are already listed in Table 1.

Table 3
Relevant parameters for robustness

Parameters to vary in order to test robustness
Temperature
pH
Ionic strength, buffer concentrations
Rinse times
Additive concentrations
Detector wavelength
Sample loading conditions (t_{inj}, Δp)
Capillaries
Integration parameters
Parameters to measure in order to test robustness
Resolutions
Efficiencies
Migration times
Peak areas

Table 4
Important aspects of instrument validation and system suitability (compare [26,27])

Relevant parameters for full instrument validation
Reproducible slope (=sensitivity, response factor); linearity
Efficiencies, peak shapes
Short and long term precision of migration time, peak area... (=repeatability, intermediate/day-to-day precision...)
Limit of detection (LOD), limit of quantitation (LOQ)
Relevant parameters for system suitability
Repeatability of migration time and peak area
Resolution

Full instrument validation should be repeated from time to time: at least half a year routinely, even if routine measurement was successfully performed every day. Unscheduled validation is recommended, if valuable samples will be analysed next, if the lamp exceeds its specified lifetime, or if unusual performance data are obtained although validated methods were used.

In addition to that, a quick system suitability check should be done every day and when a system is restarted. The system suitability test should restrict to repeatability of migration time and peak area and to resolution in order to waste not too much analysis time.

A validation test certificate, at least according to the system suitability protocol, should be supplied when an instrument is sold.

3.3. Instrumental aspects

3.3.1. Test procedure: benefits and limitations

The test procedure given in [1,2] has been widely accepted. However, some details may be critically discussed:

In order to have the same amount injected for all instruments, the sample concentrations and the product of pressure difference and injection time is specified. However, the test procedure must rely on the nominal value of pressure difference specified by the companies. If any instrument used a higher pressure difference than displayed, better values for the concentration LOD and also for the reproducibility would be obtained. The improvement of reproducibility with increasing sample concentration is at least in part due to improvements in the signal-to-

noise ratio and hence in peak integration: different loading volumes using the same concentration influence reproducibility as well.

Perrett and Hows [33] investigated the real injected volume (by microscopy and multiple injection) and compared this to the nominal one. Deviations of about 100% were indeed found in some cases. However, this fact does not lead to unfair results. Nominal and real injected volume was always matching for instruments with good reproducibility or low LOD.

There is another problem concerning the fairness of a comparison of instrument reproducibility. Sometimes very significant differences in the performance on instruments of the same type are noted. Instruments are not identical, and the same instrument can have a different status of maintenance. The check of the reproducibility data in Table 1 was usually performed on one instrument only. Thus every company participating in this study could maintain that they obtained better data on other instruments.

Meanwhile it is obvious, that the composition of the test substances is not optimal. Acetylsalicylic acid is unstable in the buffer solution, thus a decrease in peak area is observed in some of the long-term reproducibility tests. Furthermore the concentration of nicotinic acid is rather low causing relatively high standard deviations; these shortcomings were intentionally accepted when the method was released for the first time.

When these problems were reported by the companies, the honesty of the submitted data were proved. In future it may be sufficient to use acetaminophen and 3,5-dihydroxybenzoic acid only; of course other stable, well detectable, non-toxic and easily available test compounds are suitable as well.

3.3.2. Relevant parameters for instrument performance

As outlined above, the three major error sources in CE are injection, detection and integration. Improvements in detection techniques have made the injection error dominant for most instruments. This is indirectly demonstrated: otherwise internal standards would not be beneficial. Technical efforts have been made to decrease this injection error, and certainly further developments can be expected. Day-to-day precisions of about 0.5% R.S.D. can already be reported (Table 1).

High voltage, capillary length, temperature control and autosampler are important instrumental features. These were comprehensively discussed in previous articles [1,2]. There have not been much changes in these aspects. A reproducible temperature at the moment of injection is essential for reproducible injection volume; however, differences in injection volume from run to run can be partly compensated by using internal standards.

Analysis times of only a few seconds were already achieved using prototype instruments. The capillary length was reduced to less than 10 cm [34] and even to less than 1 cm [35]. This development is very promising. Short analysis times can be used to further improve the reproducibility. If the analysis time was 5 s, 25 subsequent injections of the same sample would need only 2 min. This would still be a very acceptable analysis time. The R.S.D.(x_a) of the single analytical results is reduced by the factor of 5, when 25 values are averaged and R.S.D.($\bar{x}$) is calculated (Eq. (1) [36]). Here n_a is the number of repeated measurements.

An overall day-to-day precision of 0.1% R.S.D. is already in sight, if a multiple injection mode is used. In CE it is mostly possible to use multiple injections from the same sample, because the injected amount is only a few nanoliters. Hopefully these possibilities will be soon provided for commercial instruments as well.

$$\text{R.S.D.}(\bar{x}_a) = \frac{\text{R.S.D.}(x_a)}{\sqrt{n_a}} \quad (1)$$

This possibility to reduce data scattering by multiple injection does not exist in LC. Here many attempts to reduce analysis time were made, but still it remains in the minute range. Thus the reduction of analysis time will decide the competition between CE and LC. Micellar electrokinetic chromatography methods may substitute LC methods one by one.

3.3.3. Additional instrumental options

Although the UV detector is still standard in CE, there are several sensible options for other detectors, which are applied to improve selectivity or sensitivity. Two-dimensional detectors make the test of peak

homogeneity much easier and facilitate method validation. Here the UV–visible photodiode array detector is most common; the possibilities of peak purity testing and of identification via spectra data bases are very similar to LC [37]. CE–MS coupling is used more and more [38–43]. It is desirable to avoid surfactants when MS interfaces are used. Thus electrochromatography with the option to use CE–MS is becoming more relevant [44]. Laser-induced fluorescence detectors can enormously enhance sensitivity (e.g. [45,46]). Moreover conductivity detectors [47], flame photometric detectors [48] and thermooptical absorption detectors [49] can be coupled with commercial instruments.

The possibility to report the current during sample tables is extremely useful, especially when working with complex matrices. It helps to sort out failed runs, which are characterised by current irregularities or breakdowns.

The LOD in CE was significantly improved by various sample zone focusing procedures. [18,50]. The resolution can be improved almost to infinity, if the mobility is balanced by a hydrodynamic back-pressure flow (e.g. [51]). The use of these techniques imply the possibility to control polarity and voltage during runs or during a sample table, as well as the possibility to use a back-pressure counter current.

A possibility to improve sensitivity in capillary electrophoresis is due to Lambert–Beer's law. A higher absorption can be obtained by increasing the pathlength. This can be done by enlarging the inner diameter of a limited region ('bubble cell') of the capillary three to five times compared to a standard capillary. These capillaries are made commercially available by Hewlett-Packard, but can also be used with some other instruments. The theoretical increase in sensitivity of the factor three to five is difficult to obtain [37,52]. The limiting factors are slit dimension and positioning as well as reproducibility of the bubble cell capillary production. However, an increase of about the factor 2 is often reported.

4. Conclusions and outlook

Precision in CE has been improved a lot during the last 5 years. The use of high sample concentrations and of internal standards or relative peak areas makes a R.S.D. of about 0.5% state of the art. Meanwhile the precision of quantitative results is superior to most analytical techniques. However, the limit of detection has to be further improved.

In addition to that, instruments have been improved as well. They are all very well suited for a wide range of applications.

For a long time it seemed, that LC would still win against CE, and CE would only be used for special problems, when LC failed. However, in future CE will be superior not only in terms of separation performance, but also in precision, because CE analysis can be performed extremely fast. Furthermore know-how for CE validation is readily available. CE methods may substitute LC applications one by one during the next years.

5. Closing remarks

We are hoping very much that we did not forget any instrument manufacturer. If this should have happened, be sure that we feel very sorry! Please do not hesitate to get in touch with us: we promise to include your instrument(s) in the next update!

We would also like to encourage manufactures to keep us regularly up-to-date with instrumental improvements in future.

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