Journal of Pharmaceutical and Biomedical Analysis 50 (2009) 320–327

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Improving precision in gel electrophoresis by stepwisely decreasing variance components

Simone Schröder[a], Asita Brandmüller[a], Xi Deng[a], Aftab Ahmed[b], Hermann Wätzig[a,*]

[a] Institute of Pharmaceutical Chemistry, TU Braunschweig, Beethovenstraße 55, 38106 Braunschweig, Germany
[b] RI-INBRE Research Core Facility, College of Pharmacy, University of Rhode Island, Kingston, USA

ARTICLE INFO

Article history:
Received 26 January 2009
Received in revised form 27 April 2009
Accepted 29 April 2009
Available online 7 May 2009

Keywords:
Gel electrophoresis
Quantitative analysis
Precision
Near-infrared detection
Plackett–Burman design

ABSTRACT

Many methods have been developed in order to increase selectivity and sensitivity in proteome research. However, gel electrophoresis (GE) which is one of the major techniques in this area, is still known for its often unsatisfactory precision. Percental relative standard deviations (RSD%) up to 60% have been reported. In this case the improvement of precision and sensitivity is absolutely essential, particularly for the quality control of biopharmaceuticals. Our work reflects the remarkable and completely irregular changes of the background signal from gel to gel. This irregularity was identified as one of the governing error sources. These background changes can be strongly reduced by using a signal detection in the near-infrared (NIR) range. This particular detection method provides the most sensitive approach for conventional CCB (Colloidal Coomassie Blue) stained gels, which is reflected in a total error of just 5% (RSD%). In order to further investigate variance components in GE, an experimental Plackett–Burman screening design was performed. The influence of seven potential factors on the precision was investigated using 10 proteins with different properties analyzed by NIR detection. The results emphasized the individuality of the proteins. Completely different factors were identified to be significant for each protein. However, out of seven investigated parameters, just four showed a significant effect on some proteins, namely the parameters of: destaining time, staining temperature, changes of detergent additives (SDS and LDS) in the sample buffer, and the age of the gels. As a result, precision can only be improved individually for each protein or protein classes. Further understanding of the unique properties of proteins should enable us to improve the precision in gel electrophoresis.

1. Introduction

Gel electrophoresis, especially two-dimensional gel electrophoresis (2-DE), is a favoured separation tool in protein analysis. 2-DE is a powerful technique to simultaneously separate hundreds to thousands of protein species in complex protein mixtures within a single gel. Therefore, it has already been applied in many fields of research ranging from clinical diagnostics over various basic research projects to the development of new drugs. Although achieving high resolution, 2-DE shows limited precision as expressed by high percental relative standard deviation (RSD%) values ranging from 10% to 60% (RSD%) of observed spot areas [1–5]. However, in quantitative analysis reproducibility and precision are important parameters [3]. Determining these statistical values provides a way to evaluate the quality of analytes, e.g. pharmaceuticals. Without analytical precision a reliable estimation of changes in an investigated system is impossible.

Direct and indirect detection and visualization strategies with different sensitivity are available [6–9]. Traditional protein detection methods include staining with organic dyes such as Coomassie Brilliant Blue (CBB) [7,9–12], silver staining [7,13], fluorescent dyes [7,14,15], or radioactive labelling (with ^{14}C, ^{3}H, ^{35}S, etc.) [8,9,16]. A common major drawback in all of these staining methods is the high background staining. A limitation in sensitivity is a direct consequence of this high background signal. Many detection methods have been developed with the goal of increased stain selectivity and sensitivity. An advancement in the Coomassie Brilliant Blue dye is the Colloidal Coomassie Blue (CCB) dye [7,11]. This colloidal formulation was developed to reduce the high background staining which improves the sensitivity of this dye. Nevertheless, the detection limit is still worse than that of silver or fluorescence staining. However, due to its low cost, ease of use and compatibility with downstream protein characterization and identification methods such as mass spectrometry, CCB is the most widely used stain for protein detection.

Recently published works report an enhanced sensitivity of CCB stained gels by using a near-infrared (NIR) imaging system

* Corresponding author. Tel.: +49 531 391 2764; fax: +49 531 391 2799.
E-mail addresses: si.schroeder@tu-bs.de (S. Schröder), asita_b1@freenet.de (A. Brandmüller), x.deng@tu-bs.de (X. Deng), aftab@mail.uri.edu (A. Ahmed), h.waetzig@tu-bs.de (H. Wätzig).

0731-7085/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2009.04.034

for detection instead of a conventional densitometric detection method [6,17,18]. In the present study, we have shown the improved protein detection by using CCB stained one-dimensional (1D) gels in combination with a NIR imaging system. This combination provides the most sensitive protein detection method. Thus, a total error of just 5% (RSD%) for main assays should be in close reach in GE.

In a previous work, we analyzed the entire 2-DE workflow in detail and identified the following three points as the main error sources of variability in quantitative 2-DE [2]: first, the staining or rather detection of separated proteins; second, the transfer between the first and the second dimension; and third, the researcher's skills on 2-DE technique. Many other minor sources of error are conceivable, such as sample preparation, staining time, or temperature changes. According to the law of error propagation, the major sources of error dominate the total error. By reducing the major ones, it becomes possible to investigate the next larger predominant sources of error. Spot detection was already identified as the dominant source of error. Consequently, this main source of error can be reduced by using an adequate detection method, such as the combination of CCB stained gels with the NIR imaging system.

After isolating and reducing these major sources of error, the minor sources of error can be analyzed and optimized, because they are no longer covered by the major error sources. For this purpose, different methods are available. The classical method for optimization involves changing just one independent variable at a time, such as temperature, staining time, sample preparation, etc. and fixing the others at a certain level. This classical approach is very laborious and time-consuming, especially for a large number of variables. Consequently, the combination of variables unfortunately does not ensure the optimum conditions. An alternative approach is a full factorial experimental design, which examines every possible combination of independent variables at appropriate levels. However, full factorial designs are impractical for a large number of variables because of the huge number of experiments that are required to complete the investigation. For example, the evaluation of seven possible influencing factors at two different levels would require 128 (2^7) experimental trials. Therefore, a more practical method is required, a two-level screening design proposed by Plackett and Burman [19–23]. In some cases also a three-level Plackett–Burman design was executed for factor optimization. Screening designs are an efficient way to identify the main factors from a list of many potential factors, which have a large influence on the response of a particular method. These screening designs allow for the evaluation of a relatively high number of factors in a relatively small and feasible number of experiments. The investigation of up to $N-1$ variables at two different levels in N experiments is possible. Initially, screening designs give a general overview. After screening the important factors and the significant main effects, these important factors have to be investigated in detail through further experiments. In many cases such Plackett–Burman designs were already applied successfully to screen for important factors in robustness tests [21,24,25] or during method optimization. For instance, in the field of microbiology a lot of working groups used screening designs to optimize nutrient levels of bacterial cultures [26–30]. Furthermore, in separation science experimental designs are useful tools. Analysis time, separation and resolution as well as the peak symmetry of different capillary electrophoresis (CE) methods were optimized by using the Plackett–Burman design [31–33]. Furthermore, many other techniques were optimized using these screening designs such as RT-PCR (reversed transcription-polymerase chain reaction) [34], ELISA (enzyme-linked immunosorbant assay) [35], or HG-GPMAS (hydride generation gas phase molecular absorption spectrometry) [36,37]. Investigating the lipase-catalyzed synthesis of anthranilic acidic esters, the Plackett–Burman design provided useful information on the choices of reactants for a better yield [38].

In the present paper, two different aspects were mentioned. In the first part, different detection approaches were compared in order to evaluate the sensitivity of these methods by using the signal-to-noise ratio with the objective of reduction the dominant source of error, the detection of separated proteins. In the second part a Plackett–Burman sample design was employed to investigate the influence of further, potential sources of error on the total variability, and to achieve a further improvement in quantitative gel electrophoresis.

2. Materials and methods

2.1. Chemicals

Polyacrylamide, Tris–HCl buffer (pH 6.8; 0.5 M) and Tris–HCl buffer (pH 8.8; 1.5 M), ammonium persulfate (APS), sodium dodecyl sulfate (SDS), *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and ready gel 12% Tris–HCl were purchased from BioRad (Hercules, CA, USA). All water used was obtained from a Millipore Milli-Q Synthesis purification system (Billerica, MA, USA).

2.2. Sample

Mark 12™ unstained standard solution was purchased from Invitrogen (Carlsbad, CA, USA), and diluted with XT sample buffer, containing lithium dodecyl sulfate (LDS), or rather Laemmli sample buffer, containing sodium dodecyl sulfate (SDS). Both 1D sample buffers were acquired from BioRad (Hercules, CA, USA). The standard solution consisted of 12 different proteins, which were analyzed by one-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE). A detailed composition of the Mark 12™ unstained 1D standard is shown in Table 1.

2.3. SDS-PAGE

The SDS-PAGE was performed using a constant voltage of 200 V for 45 min in a Mini-PROTEAN 3 electrophoresis cell system (Bio-Rad, Hercules, CA, USA) by using Tris–glycine SDS running buffer (Invitrogen, Carlsbad, CA, USA).

2.4. Visualization

2.4.1. CCB staining

After electrophoresis, gels were rinsed twice with water for 5 min, stained with BioSafe™ Coomassie (BioRad, Hercules, CA, USA), destained with water, and imaged by Odyssey near-infrared

Table 1
Composition, molecular weight and amount of the different proteins contained in Mark 12™ unstained 1D standard solution.

Protein	Mr (kDa)	Concentration (μg/μl)
Myosin (1)	200.0	0.076
β-Galactosidase (2)	116.3	0.040
Phosphorylase B (3)	97.4	0.070
BSA (4)	66.3	0.040
Glutamic dehydrogenase (5)	55.4	0.120
Lactate dehydrogenase (6)	36.5	0.080
Carbonic anhydrase (7)	31.0	0.044
Trypsin inhibitor (8)	21.5	0.064
Lysozyme (9)	14.4	0.050
Aprotinin (10)	6.0	0.076
Insulin B chain (64%) (11)	3.5	0.143
Insulin A chain (35%) (12)	2.5	0.085
Total		0.888

imaging system (LiCor Biosciences, Lincoln, NE, USA) using the fluorescence of CCB stain after excitation in the near-infrared (700 nm channel) and Typhoon Multimode Fluorescence detector (GE Healthcare, Piscataway, NJ, USA) using an excitation source red laser (633 nm).

2.4.2. Fluorescence staining

Two fluorescence stains were used: SYPRO Ruby protein stain (BioRad, Hercules, CA, USA) and Deep Purple protein stain (GE Healthcare, Piscataway, NJ, USA). Both stainings were performed as described in detail in the instruction manual of the manufacturer. These gels were imaged using Typhoon Multimode Fluorescence scanner (GE Healthcare, Piscataway, NJ, USA) using excitation source green laser (532 nm).

2.5. Analysis

The digitalized gel images were converted into dimension reduced electropherograms by using MATLAB software as described in detail previously [2]. Quantitative data analysis of these electropherograms was performed by Correct Integration Software System (CISS) [39]. The peak areas of each protein were normalized relative to the total peak area corresponding to the investigated lane.

3. Results and discussion

3.1. Detection of CCB stained gels

The CCB stained gels were imaged by two different high-quality scanners, NIR imaging system and fluorescence detector, using the fluorescence of CCB stain. For further analysis of sensitivity, the digitalized gel images were converted into dimension reduced electropherograms. These electropherograms were analyzed by Correct Integration Software System (CISS) [39] in order to determine sensitivity by using the detection limit (DL), precision and linearity. Fig. 1 shows a direct comparison of the same gel imaged by the NIR scanner (A) and the fluorescence scanner (B). Fig. 1C and D illustrates the converted electropherograms of the same lane loaded with the Mark 12™ standard solution in a total protein concentration of 0.4 μg/μl. The electropherograms point out different strong fluctuating baselines between the two scanners. The background signal is markedly reduced by using the NIR detector. Particularly, a closer look at the first five proteins emphasizes the differences between the two scanners. The standard solution contains these first five proteins in a comparatively low concentration. In the case of the fluorescence scanner an assignment of the first five proteins to the peaks is impossible. These peaks disappear in the baseline noise. By contrast, the electropherogram obtained by using the NIR detector exhibits well-defined peaks and an excellent signal-to-noise ratio, which enables an exact identification of all proteins.

The detection limit (DL) of an individual analytical procedure is defined as the lowest amount of an analyte in a sample, which can be detected but not necessarily quantitated as an exact value [40]. Usually, the detection limit is defined using a signal-to-noise ratio of three, which is calculated by using Eq. (1) [41,42], where H is the height of the measured signal, and h_n is the maximum spread of the baseline signal within 20 signal widths at half height.

$$\frac{S}{N} = \frac{2H}{h_n} \quad (1)$$

Compared to the DL, the quantitation limit (QL) of an individual analytical procedure is defined as the lowest amount of an analyte in sample, which can be quantitatively determined with suitable precision and accuracy [40]. The considered pharmacopoeias [41,42] suggest a necessary signal-to-noise ratio of 10 for quantitative determinations.

Here, the DL is used to evaluate the sensitivity of the scanners. The calculation of DL confirms that the NIR scanner is the most sensitive scanner as seen by a visible comparison of the gels as well as by a comparison of the electropherograms in Fig. 1. The calculated signal-to-noise ratios and the corresponding concentrations are shown in Table 2, exemplarily for four proteins of the standard solution.

Both the NIR scanner and fluorescence scanner show a good quantitative precision of the peak areas as expressed in the RSD% values in a range of 3–10% (RSD%) with an appropriate signal-to-noise ratio. Each standard solution was analyzed six times on the same gel. The RSD% values and the corresponding signal-to-noise ratios are summarized in Table 3. However, this good precision as well as quite good signal-to-noise ratio can be achieved at considerably lower concentrations by the NIR scanner. In pre-tests different concentrations of samples were chosen to estimate the QL. In order to compare quantitative precision of the peak areas of both scanners, different sample concentrations were used which resulted in a similar signal-to-noise ratio.

In comparison to the other proteins Myosin shows a very high RSD% value by using the NIR imaging system. After electrophoresis Myosin appears very close to the border of the gels. Hence, the peak shape is not clearly defined in some cases, which makes the correct integration very difficult.

A lot of fluorescence stains are available for in-gel protein detection, which are more sensitive and selective than CCB stain using a standard densitometry. Harris et al. [6] investigated fluorescent signal intensity and the number of proteins detected in 2-DE gels by using different commercially available fluorescence stains. All these stains detected a comparable number of total protein spots and showed no significant difference in their signal-to-noise ratios. Two of these fluorescent stains, SYPRO (BioRad) and Deep Purple (GE Healthcare), were selected for protein detection, and the corresponding electropherograms were investigated with respect to their DL and precision. These stains were also compared with CCB using a NIR imaging system. The results of precision are summarized in Table 3. We found that sensitivity and precision of CCB detection was comparable to SYPRO fluorescence stain when using NIR detection. In contrast to previous reports [14,15], Deep Purple showed less sensitivity, than SYPRO stained gels. For a good quantitative precision, considerably higher sample concentrations are necessary.

The NIR imaging system uses two infrared fluorescent channels for detection of separated proteins. Two separate lasers (diode lasers) and detectors (solid-state detectors) simultaneously enable the detection of two fluorescence signals after excitation wavelengths of 685 nm (700 nm-channel) and 785 nm (800 nm-channel). The chemical structure CCB dye shows a triphenylmethane structure with an excitation maximum at 550 nm and an emission maximum at 670 nm [6,43]. Therefore, the 700 nm-channel of the NIR imaging system can be used for the detection of CCB stained gels. However, the detection of proteins stained with Deep Purple (excitation maximum: 540 nm, emission maximum: 610 nm [6]) or SYPRO (excitation maximum: 450 nm, emission maximum: 610 nm [6]) is not possible. The fluorescence detector also detects the emitted fluorescence of the CCB stained proteins, but with an excitation wavelength of 633 nm (compare 2.4.1). For the detection of proteins stained with other dyes (e.g. Deep Purple, SYPRO) special excitation wavelength for the corresponding dyes can be selected.

Detection by using fluorescence is a more sensitive technique, than using absorption. Furthermore, the fluorescence in the near-infrared region offers many advantages compared to the visible range. For instance, membranes and bio-molecules show a much

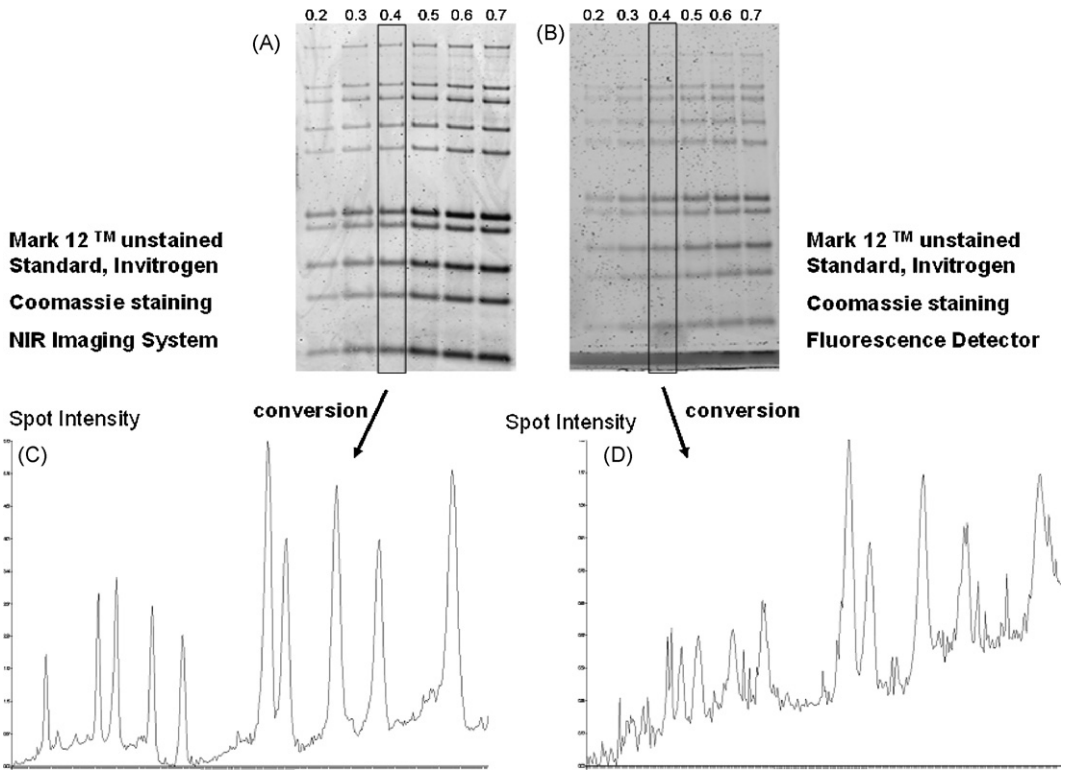

Fig. 1. Comparison of the same gel imaged by NIR scanner (A) and fluorescence scanner (B); the lane which was loaded with the standard solution in a total concentration of 0.4 μg/μl was converted in dimension reduced electropherograms; a comparison of these resulting electropherograms is shown in (C) and (D).

Table 2
Results of DL determination for NIR imaging system and fluorescence scanner.

Protein	Mr (kDa)	NIR imaging system		Fluorescence detector	
		Concentation (μg/μl)	S/N	Concentation (μg/μl)	S/N
β-Galactosidase	116.3	0.003	3.3	0.005	6.7
Phosphorylase B	97.4	0.005	3.3	0.008	6.7
BSA	66.3	0.003	5.0	0.005	7.7
Glutamic dehydrogenase	55.4	0.008	5.7	0.014	7.0

lower autofluorescence in the near-infrared, than in the visible wavelength region [44], which results in a low background signal and consequently in an improved sensitive detection. Recently published works compared the sensitivity of fluorescent stains with traditional silver and CCB staining. By imaging a CCB stained gel with NIR detection, Harris and co-workers were able to detect more than twice the number of proteins compared to traditional densitometry [6]. Thus, CCB was found to be comparable to sensitive fluorescence stains when detected using a near-infrared fluorescence imaging system. Smejkal reported that CCB fluorescence was at least twice as sensitive as SYPRO Ruby for the detection of proteins [45].

It is frequently circulated that difference gel electrophoresis (DIGE) [46] already solves the problem of insufficient precision. This technique utilizes various internal standardizations with different fluorescence markers in one gel [47–49]. It is generally accepted

Table 3
RSD% values and the corresponding signal-to-noise ratios of the Mark 12™ protein standard solution. Each standard solution was analyzed six times on the same gel.

Protein	Mr (kDa)	CCB NIR imaging system		CCB fluorescence detector		SYPRO fluorescence detector		Deep Purple fluorescence detector	
		0.309 μg/μl[a]		0.707 μg/μl[a]		0.309 μg/μl[a]		0.619 μg/μl[a]	
		RSD%	S/N	RSD%	S/N	RSD%	S/N	RSD%	S/N
Myosin	200.0	30.0	3.7	3.5	6.5	16.2	13.3	14.1	8.0
β-Galactosidase	116.3	6.2	10.3	5.8	12.3	6.0	19.0	7.0	22.1
Phosphorylase B	97.4	6.9	16.2	5.2	17.8	6.9	28.6	7.9	25.1
BSA	66.3	6.8	21.6	3.0	19.7	2.3	33.6	8.7	30.6
Glutamic dehydrogenase	55.4	9.2	20.3	5.0	20.8	7.0	38.4	7.1	41.7
Lactate dehydrogenase	36.5	4.8	43.9	1.7	31.8	2.9	66.1	3.6	39.3
Carbonic anhydrase	31.0	2.8	28.3	2.9	22.5	4.8	53.9	14.8	29.1
Trypsin inhibitor	21.5	5.6	32.9	5.5	28.5	3.3	70.9	3.1	34.9
Lysozyme	14.4	6.7	19.3	2.6	19.5	8.4	45.2	3.7	9.0
Aprotinin	6.0	3.8	33.1	5.7	22.6	3.2	64.8	5.3	43.1

[a] Total concentration.

that DIGE improves the precision in gel electrophoresis to some extent [48,50,51] [Winckler . . . Oehler, and further literature cited herein]. However, even the manufacturers just report, that RSD% values of 10% can be obtained with DIGE. This improvement is moderate related to the effort and investments which are necessary to establish DIGE. It requires very expensive equipment as well as reagent kits [52] which makes it difficult for routine use. Further, even this moderate amelioration cannot be clearly derived from the so-far published data [53].

Due to the low DL and good quantitative precision as a result of the good signal-to-noise ratios, the combination of CCB stained gels and the NIR imaging system provides the most sensitive detection method, which is currently available for in-gel protein detection. Hence, the main source of error was reduced with this detection method. Consequently, this combination was used to perform the Plackett–Burman sample design in order to further analyze potential sources of error, and to further improve precision in quantitative gel electrophoresis.

3.2. Plackett–Burman design

Using the Plackett–Burman design, seven different potential influencing factors were investigated by conducting 12 experiments. This particular design is shown in Table 4. Other Plackett–Burman designs for 8, 16, 20 up to 100 experiments (but always a multiple of four) are available, depending on the number of factors to be analyzed. Each row represents an experiment, and each column represents an independent parameter or a dummy factor. In this case (Table 4), the potential influencing factors are assigned to columns 1–7. The last four columns represent the so-called dummy variables, imaginary factors, for which the change from one level to the other has no meaning. These dummy variables are just used to estimate the experimental error. As factors, staining and destaining time, staining temperature, temperature of the electrophoretic process, composition of sample buffer (SDS and LDS containing sample buffer), age of the gels (six-week-old gels and freshly casted gels), and self- and pre-casted gels were investigated. Each of the parameter must be investigated at two different levels, which are represented by the plus and minus signal in the Plackett–Burman table. The investigated variables here and their corresponding levels are shown in Table 5. The Plackett–Burman design is usually performed using levels, which represent the upper and lower limit of the range of expected values. These limits were determined in pretests. The Plackett–Burman design assumes independence between the investigated parameters as well as linearity between the two different levels of the investigated parameters. This means a linear connection between the investigated factor and the response factor within the extreme levels ("+" and "−") is expected. The design experiments are performed in a random sequence to eliminate any biased errors. The response of interest is measured for each experiment.

In order to evaluate the precision in quantitative gel electrophoresis, we chose the percental relative standard deviation (RSD%) of the peak areas as the target parameter. The test results (calculated RSD% values) were entered in the last column of the Plackett–Burman schedule (Table 4). All following calculations are provided at http://www.pharmchem.tu-bs.de/forschung/waetzig/support/in order to facilitate the traceability of this data evaluation.

The most important variables affecting this target parameter are estimated by their effects E_x. This effect of a particular factor x was calculated from the difference between the sum of the + and − responses Y for each independent and dummy factor (Eq. (2)), where N is the number of experiments of the design. In this case the response variable Y represents the percental relative standard deviation (RSD%) of the peak areas calculated from the electropherograms per experiment.

$$E_x = \frac{\sum Y_{x(+)}}{N/2} - \frac{\sum Y_{x(-)}}{N/2} \tag{2}$$

In order to evaluate the calculated effects, both graphical and statistical interpretation methods are considered feasible. The graphical identification of important effects can be applied with a normal probability plot [54,55] or with a bar plot [23], where the estimated factor effects are represented. Statistical interpretation provides the calculation of a limit value, which can also be plotted in the bar plot diagram and which allows a less subjective evaluation than just the graphical representation.

The experimental error is estimated by the effects of the dummy factors E_D. Hence, the standard deviation ($\hat{\sigma}$) of the effects was calculated by using Eq. (3), where $E_{(Di)}$ is the effect of a particular dummy factor and n the number of dummy factors and degrees of freedom.

$$\hat{\sigma} = \sqrt{\frac{\sum E_{(Di)}^2}{n}} \tag{3}$$

Each effect (E_x) of a particular factor x was compared with the minimum significant factor effect (MIN), a limit value which represents the experimental variability within the design and was calculated by multiplying the standard deviation with a particular t-value (Eq. (4)).

$$\mathrm{MIN} = \hat{\sigma} \cdot t \tag{4}$$

An effect is considered as significant at a given probability value α if $|E_x| > \mathrm{MIN}$.

For each experiment, one gel was prepared with six lanes containing ten proteins in a total concentration of 0.309 μg/μl per lane. Each experiment was done twice and each gel was scanned five times. All lanes were converted into dimension reduced electropherograms, which resulted in 60 electropherograms per experiment, from which the peak areas rather the RSD% values were determined. For each lane, the sum of all peak areas was calculated as total peak area. Each individual peak area was then related to this total peak area. The calculation of RSD% is described in Eq. (5), where $\hat{\sigma}$ is the estimated standard deviation (Eq. (6)), $\hat{\sigma}^2$ the estimated variance (Eq. (7)) and $\bar{x}$ the estimated arithmetic mean (Eq. (8)) calculated from the six electropherograms of one gel image.

$$\mathrm{RSD\%} = \frac{\hat{\sigma}}{\bar{x}} \times 100\% \tag{5}$$

$$\hat{\sigma} = \sqrt{\hat{\sigma}^2} \tag{6}$$

$$\hat{\sigma}^2 = \frac{\sum_{i-1}^{n}(x_i - \bar{x})^2}{n-1} \tag{7}$$

$$\bar{x} = \frac{\sum_{i=1}^{n} x_i}{n} \tag{8}$$

The total RSD% values for each protein were calculated by using the pooled variance. Therefore, the RSD% values obtained from one gel image were squared to yield the variances. All 10 variances of each protein (each experiment was done twice and each gel was scanned five times) were summarized by Eq. (9), where $\hat{\sigma}^2_{\mathrm{pooled}}$ is the pooled variance, $\hat{\sigma}^2_{\mathrm{m}}$ the calculated variance of each protein, n the number of lanes and m the number of considered gel images. By extracting a root of this pooled variance the total RSD% values for each SDS concentration were obtained.

$$\hat{\sigma}^2_{\mathrm{pooled}} = \frac{(n_1-1)\hat{\sigma}_1^2 + (n_2-1)\hat{\sigma}_2^2 + \cdots + (n_m-1)\hat{\sigma}_m^2}{n_1 + n_2 + \cdots + n_m - m} \tag{9}$$

For our investigations, we considered only seven of the 10 proteins (q.v. Table 1, protein numbers 2–8), which were well separated

Table 4
Plackett–Burman design for 12 trials.

Number	Mean	Influencing factors							Dummy factors				Response
		1	2	3	4	5	6	7	8	9	10	11	RSD%
1	+	+	+	–	+	+	+	–	–	–	+	–	
2	+	+	–	+	+	+	–	–	–	+	–	+	
3	+	–	+	+	+	–	–	–	+	–	+	+	
4	+	+	+	+	–	–	–	+	–	+	+	–	
5	+	+	+	–	–	–	+	–	+	+	–	+	
6	+	+	–	–	–	+	–	+	+	–	+	+	
7	+	–	–	–	+	–	+	+	–	+	+	+	
8	+	–	–	+	–	+	+	–	+	+	+	–	
9	+	–	+	–	+	+	–	+	+	+	–	–	
10	+	+	–	+	+	–	+	+	+	–	–	–	
11	+	–	+	+	–	+	+	+	–	–	–	+	
12	+	–	–	–	–	–	–	–	–	–	–	–	
Effect													

Table 5
Investigated influencing factors and the corresponding levels.

Influencing factors		Levels	
		–	+
1	Staining time	1 h	5 h
2	Destaining time	2 h	20 h
3	Temperature of staining/staining solutions	Cooled in the refrigerator	Room temperature
4	Temperature of electrophoretic process	Cooled in the refrigerator	Room temperature
5	Sample buffer	SDS	LDS
6	Age of gels	Old (six-week-old gel)	New (casted freshly)
7	Casting type	Pre-casted gel	Self-casted gel

Table 6
Results of the Plackett–Burman design; calculated RSD% values of each protein.

Experiment number	β-Galactosidase	Phosphorylase B	BSA	Glutamic dehydrogenase	Lactate dehydrogenase	Carbonic anhydrase	Trypsin inhibitor
1	19.5	7.8	9.0	4.3	3.7	6.1	5.5
2	6.6	5.0	5.8	5.4	4.6	4.0	5.6
3	9.0	5.0	7.7	7.4	6.3	5.9	5.2
4	11.5	4.2	4.0	6.1	3.8	3.3	3.2
5	17.7	12.0	18.5	10.2	8.0	8.1	9.9
6	6.9	26.2	8.1	9.4	5.4	5.8	6.5
7	5.3	6.4	5.3	7.9	5.3	3.8	5.5
8	7.0	7.9	10.9	12.8	5.4	3.9	8.9
9	23.3	20.0	12.6	8.2	3.9	7.1	5.7
10	6.8	6.6	8.8	7.0	5.7	6.8	9.0
11	8.9	7.7	6.4	7.0	3.8	3.9	10.1
12	7.4	7.2	11.2	5.3	3.5	3.7	6.0

in the centre of the gel to avoid effects resulted of the gel borders. Also in some cases, depending on the electrophoretic conditions or rather defined parameters, some of the separated proteins were run out the gel. Thus, the proteins, which were located on all gels, were chosen for the evaluation of the Plackett–Burman design.

For each protein, a Plackett–Burman schedule was averaged from all the data of the twelve trials by using the pooled variance as described above. The obtained RSD% values and the calculated effects are summarized in Tables 6 and 7, respectively.

The calculation of the limit value to identify statistical significant effects is usually derived from the *t*-distribution. The *t*-test statistics provide the evaluation of location parameters such as the arithmetic mean or the median. However, in the present case, the percental relative standard deviation, a scattering parameter, was

Table 7
Results of the Plackett–Burman design; the calculated effects which are considered as significant are represented in bold values; the MIN value was calculated at a significance level of $\alpha = 0.2$.

Estimated effects of investigated factors[a]								
Protein	1	2	3	4	5	6	7	MIN
β-Galactosidase	1.37	**8.30**	**−5.07**	1.85	2.42	0.08	−0.75	3.80
Phosphorylase B	1.27	−0.45	**−7.22**	−2.40	**5.55**	−3.18	4.37	5.19
BSA	0.017	1.35	−3.53	−1.65	−0.45	1.58	−3.00	4.08
Glutamic dehydrogenase	−1.04	−0.76	0.03	−1.76	0.53	1.23	0.03	2.86
Lactate dehydrogenase	0.48	−0.05	−0.05	−0.07	−0.97	0.72	−0.58	1.61
Carbonic anhydrase	0.96	1.08	−1.16	0.84	−0.13	0.46	−0.18	1.77
Trypsin inhibitor	−0.28	−0.34	0.50	−1.36	0.59	**2.79**	−0.18	2.01

[a] Assignment of the investigated factors according to Table 5.

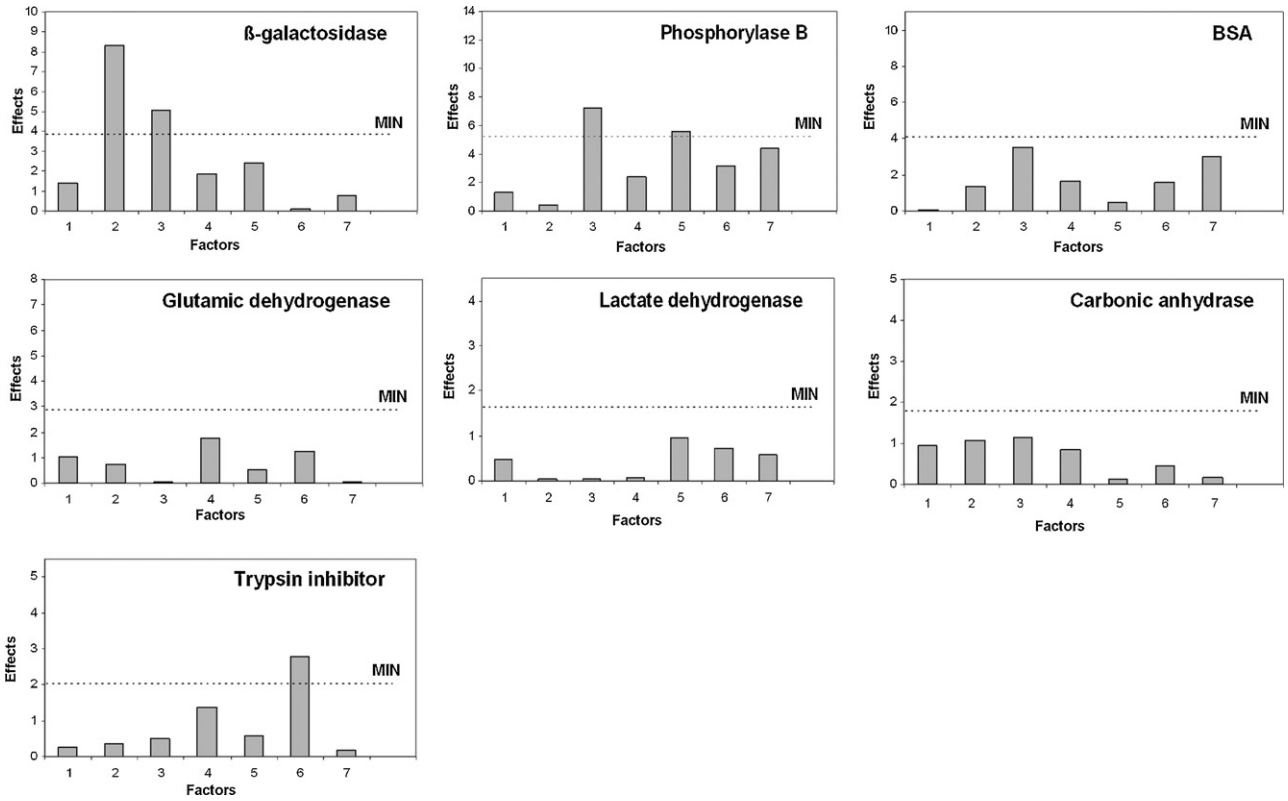

Fig. 2. Bar plots for the effects of each single protein. The bars represent the absolute values of the calculated effects, the horizontal broken line the calculated limit value, i.d. minimum significant factor effect (MIN); for denomination of factors see Table 5.

investigated. Thus, the evaluation of this target parameters by a *t*-distribution has to be considered with care. Therefore, we initially preferred a graphical analysis using a bar plot. Fig. 2 shows the bar plots for each single investigated protein. The bars represent the absolute values of the calculated effects. The influence of the factors on the precision was evaluated by the height of the bars. Additionally, the limit values were calculated by using the *t*-distribution and were plotted as a horizontal broken line in each diagram. For the calculation of the MIN value, a relatively high probability value α of 20% was used. In this way, a low β-error was obtained, which expresses the probability with which an investigated factor is not identified as significant, although, in fact, this factor influences the precision significantly. Thus, we effectively reduce a false-negative decision. The comparison of the graphical and statistical interpretation provides essentially the same results.

Fig. 2 and Table 7 show that completely different factors are considered to be significant for each protein. For β-galactosidase, the destaining time and the temperature during staining had a major influence on spot area precision. Whereas, the temperature during staining and possibly the nature of the surfactant (SDS or LDS) were most influential for phosphorylase B. The age of the gels plays an important part for the quantification of trypsin inhibitor.

The detection of glutamic dehydrogenase, lactate dehydrogenase, BSA, and carbonic anhydrase seems to be quite resistant against external effects and changes. The staining time, the temperature of the electrophoretic process, and the sort of gels (pre- or self-casted) do not have any impact on the precision. These parameters need not be closely investigated in further experimental designs.

Seven proteins with different biophysical properties were systematically investigated applying the Plackett–Burman design. However, the experiment does not result in a general conclusion for all proteins. Any factor, which influences the precision for one protein, may be without meaning in another case. A general improvement of precision in quantitative gel electrophoresis cannot, therefore, be reached by changes in the electrophoretic conditions under investigation. Instead, a stepwise process for different proteins or maybe classes of proteins seems necessary.

4. Conclusion and outlook

The background signal, as a consequence of the detection method, was already identified as a predominant source of error in quantitative gel electrophoresis [2]. This background signal is markedly reduced by using the NIR imaging system, which results in well-defined peaks and an excellent signal-to-noise ratio.

The sensitivity and precision of CCB staining using NIR imaging systems is comparable to or even better than the sensitive fluorescence stains. The high cost of such fluorescence stains presents a disadvantage in the routine use of these products. Furthermore, using fluorescence in the near-infrared region results in a low background signal and consequently in an improved sensitivity. Due to the low DL and the good quantitative precision as a result of the good signal-to-noise ratio, CCB stained gels imaged by NIR detector provide the most sensitive and cost effective detection method, which is currently available for in-gel protein detection.

The influence of seven potential factors (namely, the staining and destaining time, the staining temperature, the temperature of the electrophoretic process, the composition of sample buffer, the age of the gels, and casting type) on the precision was investigated using a Plackett–Burman design with seven proteins with different representative properties. Individually, different influence factors were identified to be significant for each protein. Thus, the Plackett–Burman design emphasized the individuality of the proteins. A general direct optimization of protein quantification by changing the investigated parameters of the electrophoretic process was not possible. However, out of seven investigated

parameters, just four showed a significant effect on some proteins, namely the destaining time, the staining temperature, the use of either SDS or LDS, and the age of the gels

Instead, precision can only be improved individually for each protein, and only if its biophysical properties are well characterized. Protein properties such as constitution and structure, hydrophilicity or flexibility should be carefully considered, keeping in mind that the proteins are analyzed in at least a partly denatured form. Further classification of proteins into special groups, according to their structural and analytical properties, should be performed. Possibly, further information about the proteins, e.g. about their function, will be tried and incorporated into this consideration.

In practice, protein subgroups will be tentatively defined. Therefore, we shall look for "relatives" of these proteins that were investigated in this study. As a result, we shall be able to carefully optimize precision for this subgroup; but by using only the factors which already have proved to be significant for the parent compound. By investigating a group of proteins responding to the factor(s), recognizing significant effects is facilitated. In contrast, the effort to find general optimal parameters for quantitation in gel electrophoresis failed in earlier works. Possibly, because positive effects on some proteins were masked by the data scattering caused by the majority of non-responders.

If the optimization result is still unsatisfactory, one has to take one step back and improve the classification system, in order to find the right subgroup of proteins for which optimization becomes effective. When the precision in one subgroup is optimized without influencing others, the overall precision for protein quantitation is improved to some extent as well. Step-by-step optimization for various subgroups will have a major effect over time.

More understanding of the uniqueness of the proteins should enable us to further improve precision in gel electrophoresis. Thus, the RSD% values of 2% for the main assays should be achievable within the next years.

Acknowledgement

We are grateful to LiCor Biosciences, especially, to Dr. Ulrike Englisch and Dr. Harry Osterman for their technical support as well as for their fruitful contributions to discussions. In addition, we are appreciative to Nathan Nous, Dr. Isabel Astner, Dr. Sami El Deeb, Dr. Markus Blümel, Dr. Marc Hassel, Dr. Luma Baydoun, Dr. Peter Fleischmann, and Phillip Hasemann for critically reading the manuscript. We also thank Prof. Dr. John Grandin, IEP program University of Rhode Island and the Transatlantic Program of the Federal Republic of Germany with funds of the European Recovery Program of the Federal Ministry of Economics and Technology (BMWi), for providing financial support to Asita Brandmüller and Simone Schröder. This research was made possible in part by use of the RI-INBRE Research Core Facility supported by Grant # P20RR16457 from NCRR/NIH.

References

[1] K.K. Challapalli, C. Zabel, J. Schuchhardt, A.M. Kaindl, J. Klose, H. Herzel, Electrophoresis 25 (2004) 3040–3047.
[2] S. Schröder, H. Zhang, E.S. Yeung, L. Jänsch, C. Zabel, H. Wätzig, J. Proteome Res. 7 (2008) 1226–1234.
[3] A. Koller, H. Wätzig, Electrophoresis 26 (2005) 2470–2475.
[4] A. Blomberg, L. Blomberg, J. Norbeck, S.J. Fey, P.M. Larsen, M. Larsen, P. Roepstorff, H. Degand, M. Boutry, A. Posch, A. Görg, Electrophoresis 16 (1995) 1935–1945.
[5] M.P. Molloy, E.E. Brzezinski, J. Hang, M.T. McDowell, R.A. VanBogelen, Proteomics 3 (2003) 1912–1919.
[6] L.R. Harris, M.A. Churchward, R.H. Butt, J.R. Coorssen, J. Proteome Res. 6 (2007) 1418–1425.
[7] I. Miller, J. Crawford, E. Gianazza, Proteomics 6 (2006) 5385–5408.
[8] B. Moritz, H.E. Meyer, Proteomics 3 (2003) 2208–2220.
[9] W.F. Patton, J. Chromatogr. B 771 (2002) 3–31.
[10] G. Candiano, M. Bruschi, L. Musante, L. Santucci, G.M. Ghiggeri, B. Carnemolla, P. Orecchia, L. Zardi, P.G. Righetti, Electrophoresis 25 (2004) 1327–1333.
[11] V. Neuhoff, N. Arold, D. Taube, W. Erhardt, Electrophoresis 9 (1988) 255–262.
[12] V. Neuhoff, R. Stamm, H. Eibl, Electrophoresis 6 (1985) 427–448.
[13] J. Poland, T. Rabilloud, P. Sinha, in: J.M. Walker (Ed.), The Proteomics Protocols Handbook, Humana Press, Totowa, 2005, pp. 177–184.
[14] J.A. Mackintosh, H.-Y. Choi, S.-H. Bae, D.A. Veal, P.J. Bell, B.C. Ferrari, D.D.V. Dyk, N.M. Verrills, Y.-K. Paik, P. Karuso, Proteomics 3 (2003) 2273–2288.
[15] N.S. Tannu, G. Sanchez-Brambila, P. Kirby, T.M. Andacht, Electrophoresis 27 (2006) 3136–3143.
[16] B. Warscheid, in: M. Hamacher, K. Marcus, K. Stühler, A. vanHall, B. Warscheid, H.E. Meyer (Eds.), Proteomics in Drug Research, Wiley-VCH, Weinheim, 2006, pp. 57–88.
[17] S. Luo, N.B. Wehr, R.L. Levine, Anal. Biochem. 350 (2006) 233–238.
[18] C. Loebke, H. Sueltmann, C. Schmidt, F. Henjes, S. Wiemann, A. Poustka, U. Korf, Proteomics 7 (2007) 558–564.
[19] R.L. Plackett, J.P. Burman, Biometrika 33 (1946) 305–325.
[20] Petersen, H., Grundlagen der Statistik und der statistischen Versuchsplanung, Band 2, ecomed Verlagsgesellschaft mbH, 1991.
[21] Y. VanderHeyden, A. Nijhuis, J. Smeyers-Verbeke, B.G.M. Vandeginste, D.L. Massart, J. Pharm. Biomed. Anal. 24 (2001) 723–753.
[22] B. Dejaegher, Y. Vander Heyden, LC-GC Eur. (2007) 526–532.
[23] B. Dejaegher, Y. Vander Heyden, LC-GC Eur. (2008) 96–102.
[24] Y. Vander Heyden, C. Hartmann, D.L. Massart, L. Michel, P. Kiechle, F. Erni, Anal. Chim. Acta 316 (1995) 15–26.
[25] Y. Vander Heyden, K. Luypaert, C. Hartmann, D.L. Massart, J. Hoogmartens, J.D. Beer, Anal. Chim. Acta 312 (1995) 245–262.
[26] G. Volpato, R.C. Rodrigues, J.X. Heck, M.A.Z. Ayub, J. Chem. Technol. Biotechnol. 83 (2008) 821–828.
[27] F.-X. Ma, J.H. Kim, S.B. Kim, Y.-G. Seo, Y.K. Chang, S.-K. Hong, C.-J. Kim, Process Biochem. 43 (2008) 954–960.
[28] B. Jia, Z.H. Jin, L.H. Mei, Appl. Biochem. Biotechnol. 144 (2008) 133–143.
[29] K. Chauhan, U. Trivedi, K.C. Patel, Bioresour. Technol. 98 (2007) 98–103.
[30] T.G. Schimmel, S.J. Parsons, Biotechnol. Tech. 13 (1999) 379–384.
[31] S. Boonkerd, M.R. Detaevernier, Y. Vander Heyden, J. Vindevogel, Y. Michotte, J. Chromatogr. A 736 (1996) 281–289.
[32] S.A.A. Rizvi, S.A. Shamsi, Electrophoresis 28 (2007) 1762–1778.
[33] J. Vindevogel, P. Sandra, Anal. Chem. 63 (1991) 1530–1536.
[34] S. Lauwers, Y. Vander Heyden, B. Rombaut, J. Pharm. Biomed. Anal. 25 (2001) 131–142.
[35] S. Lauwers, Y. Vander Heyden, B. Rombaut, J. Pharm. Biomed. Anal. 29 (2002) 659–668.
[36] J. Sanz, M. Pérez, M.T. Martinez, M. Plaza, Talanta 50 (1999) 149–164.
[37] J. Sanz, M. Pérez, M.T. Martinez, M. Plaza, Talanta 51 (2000) 849–862.
[38] C.V.S. Babu, S. Divakar, J. Am. Oil Chem. Soc. 78 (2001) 49–52.
[39] B. Schirm, H. Wätzig, Chromatographia 48 (1998) 331–346.
[40] ICH Guidline Q2 (R1): Validation of Analytical Procedures: Text and Methodology (2005) www.ich.org.
[41] European Pharmacopoeia (Ph. Eur.) Official Edition, 5th edition, Deutscher Apothekerverlag, Stuttgart, 2005.
[42] United States Pharmacopeia (USP), Chapter: Interpretation of chromatograms, 29th edition, The United States Pharmacopeial Convention, Rockville, MD, 2006.
[43] S.B. Bukallah, M.A. Rauf, S. Ashraf, Dyes Pigments 72 (2007) 353–356.
[44] G. Patonay, M.D. Antoine, Anal. Chem. 63 (1991) 321–327.
[45] G.B. Smejka, Expert Rev. Proteomics 1 (2004) 381–387.
[46] M. Ünlü, M.E. Morgan, J.S. Minden, Electrophoresis 18 (1997) 2071–2077.
[47] A. Alban, S.O. David, L. Bjorkesten, C. Andersson, E. Sloge, S. Lewis, I. Currie, Proteomics 3 (2003) 36–44.
[48] J.F. Timms, R. Cramer, Proteomics 8 (2008) 4886–4897.
[49] R. Tonge, J. Shaw, B. Middleton, R. Rowlinson, S. Rayner, J. Young, F. Pognan, E. Hawkins, I. Currie, M. Davison, Proteomics 1 (2001) 377–396.
[50] N.A. Karp, M. Spencer, H. Lindsay, K. O'Dell, K.S. Lilley, J. Proteome Res. 4 (2005) 1867–1871.
[51] W. Winkler, M. Zellner, M. Diestinger, R. Babeluk, M. Marchetti, A. Goll, S. Zehetmayer, P. Bauer, E. Rappold, I. Miller, E. Roth, G. Allmaier, R. Oehler, Mol. Cell. Proteomics 7 (2008) 193–203.
[52] P.G. Righetti, A. Castagna, F. Antonucci, C. Piubelli, D. Cecconi, N. Campostrini, P. Antonioli, H. Astner, M. Hamdan, J. Chromatogr. A 1051 (2004) 3–17.
[53] M. Eravci, S. Fuxius, O. Broedel, S. Weist, S. Eravci, U. Mansmann, H. Schluter, J. Tiemann, A. Baumgartner, Proteomics 7 (2007) 513–523.
[54] G.E.P. Box, W.G. Hunter, J.S. Hunter, Statistic for Experimenters, An Introduction to Design, Data Analysis, and Model Building, John Wiley & Sons, New York, 1978, pp. 306–418.
[55] Y. Vander Heyden, D.L. Massart, in: M.M.W.B. Hendriks, J.H. de Boer, A.K. Smilde (Eds.), Robustness of Analytical Chemical Methods and Pharmaceutical Technological Products, Elsevier Science B.V, Amsterdam, 1996, pp. 79–147.