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Research Article

Validation of capillary zone electrophoresis and capillary isoelectric focusing separations optimized for the characterization of two recombinant products of the birch pollen allergen Bet v 1a

CZE and CIEF separation systems, both developed previously for a quality control of two recombinant products of the major birch pollen allergen Bet v 1a of *Betula verrucosa*, were validated including aspects of the International Conference on Harmonization. One product contained carbamylated variants as impurities. Linearity of response was confirmed by Mandel's fitting test between 0.028 and 1.90 mg/mL for CZE and between 0.016 and 0.26 mg/mL for CIEF. Repeatability and intermediate precision were evaluated for the effective mobility (μ_{eff}) in CZE, for relative mobilization time in CIEF and the peak area ratio of Bet v 1a. LOQ for Bet v 1a was between 10 and 23 $\mu\text{g/mL}$ for both methods. Evaluation of robustness for CZE revealed susceptibility of μ_{eff} of Bet v 1a to alterations in of buffer pH and separation temperature. Selectivity was impaired by an increase in temperature, pH, and buffer concentration. In addition, pH variations influenced the separation profile of impurities. For CIEF, the ratio of narrow pH range carrier ampholytes is the critical parameter to retain robustness. Results demonstrate the suitability of both separation systems to discriminate between nonmodified Bet v 1a and carbamylated variants in the selected recombinant allergen products.

Keywords:

Capillary isoelectric focusing / Capillary zone electrophoresis / Recombinant allergen products / Validation
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1 Introduction

Allergen extracts prepared from natural source material suffer from several drawbacks, when applied in allergy diagnosis and specific immunotherapy [1]. These extracts represent complex mixtures and contain a multitude of major and minor allergens including also post-translational modifications as well as nonallergenic proteins and proteolytic enzymes [2, 3]. In the case multiple isoforms of the target allergen are present in the extract, allergic patients might react differently to individual isoforms [4, 5]. In addition,

proteolytic enzymes can degrade allergens and impair the stability of natural extracts [3, 6]. Extract stability can also be affected by extraction conditions and source material [6]. Furthermore, composition and allergic activity of extracts will also vary between different manufacturers and various batches [7–9] and thus extract standardization is an inherent problem. Consequently, immunotherapy with allergen extracts might induce systemic side effects and even new sensitizations [10].

Recombinant allergens are considered to overcome these limitations and are prerequisites in the development of standardized products of consistent quality in order to improve diagnosis and curative therapy [11–13]. Consequently, within active pharmaceutical ingredients these products have gained increased importance [14]. The major birch pollen allergen Bet v 1 is a main cause of allergic rhinitis and asthma in Europe [15], since more than 90% of birch pollen allergic patients are sensitized against this allergen [5, 16]. Structural properties of Bet v 1 have been comprehensively characterized [17] and multiple isoforms have been described [16, 18] with birch pollen allergen *Betula verrucosa* isoform 1a (Bet v 1a) most abundant [16]. Since the

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Abbreviations: **AL**, Ampholine; **Bet v 1a**, birch pollen allergen *Betula verrucosa* isoform 1a; **CA**, carrier ampholyte; **CI**, confidence interval; **ICH**, International Conference on Harmonization; **β -LgA**, β -lactoglobulin A; **PL**, Pharmalyte; **TEPA**, tetraethylenepentamine

first studies on the immunochemical properties and the potential use of three recombinant isoforms of Bet v 1 [19], a variety of host cells has been applied for the expression of recombinant allergens [20]. Selection criteria for host cells, cultivation conditions and purification strategies are outlined elsewhere [20–22]. Ideally, recombinant allergen products provide one single isoform with defined primary sequence. This way, diagnostic misinterpretations are avoided and a component-resolved diagnosis becomes accessible [7]. In addition, administration of patient tailored doses of the target allergen can be assured in immunotherapy [12, 23]. Therefore, recombinant allergens should closely resemble their natural counterparts in IgE reactivity [7] and thus wild-type conformation and epitopes have to be maintained [22]. Consequently, physicochemical and immunochemical properties of recombinant allergens have to be compared with natural counterparts. The sequence identity can be assured, *e.g.*, by PMF with Q-TOF-MS/MS [24] or LC-MS/MS [25]. Information about the allergen conformation can be gained *via* NMR [26] or circular dichroism spectroscopy [22, 24, 27, 28], whereas glycosylation pattern can be revealed by MALDI-MS [27], LC-ESI-MS [25], or by lectin binding assay [27]. For the characterization of immunochemical properties and biological activity, radio allergosorbent test (RAST) [2, 29], enzyme allergosorbent test (EAST) [7, 8] ELISA [2–4, 29], immunoblotting [10, 29] or mediator release assays [3, 29], skin prick and provocation tests [3, 30] are applied, respectively. Studies comparing recombinant products of Bet v 1a to its natural counterpart proved comparable physicochemical and immunochemical properties [5, 22].

However, up- and downstream processes in the manufacturing of recombinant products [31] can introduce impurities, which are either process- or product related [20, 32]. Consistently, the quality assurance of recombinant allergens is a relevant economic issue [31, 33]. The product quality eventually depends on the fitness of purpose of applied analytical procedures. Thus, in new drug applications a submission of validated analytical methods for regular quality control is imperative in order to achieve the product approval by regulatory authorities [34]. Relevant product specifications and validation procedures for analytical methods are exemplified by several guidelines of the International Conference on Harmonization (ICH) [32, 35–37].

Strategies for characterization of recombinant allergens will also implement analytical methods delivering information on product purity thus guaranteeing consistent composition by revealing differences in constituent profiles between different allergen batches [38–41]. The progressive implementation of CE in industrial quality control of pharmaceuticals [42–44] is related to its advanced selectivity [38, 39], especially in the separation of closely related proteins [45–47]. Further advances of CE compared to alternative separation strategies comprise minute sample consumption in the nL domain and the resolution of different conformations, even if the *pI* and the hydrodynamic radius are only slightly altered by

structural changes [47, 48]. This information is scarcely accessible from other separation strategies and might only be indirectly deduced from shifts in the charge state distribution in MS spectra. Contrary to CE, absolute quantification in MS might be troublesome due to ion suppression effects entailing an underestimation of minor constituents [49]. In addition, CE provides product information orthogonal to HPLC [40, 50]. To date, validation of CE separations of (recombinant) proteins is mainly focused on CZE and CGE-SDS [38, 51–54], whereas CIEF validation is scarcely reported [41, 45, 55, 56]. Despite the availability of several CE separations for the characterization of (recombinant) allergens [57–63], only a single validated CZE method has been published so far [57].

The present paper focuses on two batches containing recombinant Bet v 1a in different purities. Whereas one batch contained nonmodified Bet v 1a, the second also included chemically modified (carbamylated) variants of the target allergen, which have been introduced during a modified manufacturing procedure by the application of urea to increase solubility and allergen yield. Our research group has set up CZE and CIEF separation systems for nonmodified and partially carbamylated products of recombinant Bet v 1a recently. The initially applied buffer system consisted of 100 mmol/L phosphate (pH 6.50) with 2.0 mmol/L tetraethylenepentamine (TEPA) [57]. Based on this buffer, a novel CZE system was developed which applied 100 mmol/L MES at pH 6.50 instead of phosphate and changed the concentration of TEPA to 0.4 mmol/L [58]. The TEPA concentration was adjusted by stepwise testing 0, 0.2, and 0.4 mmol/L and the separation voltage was raised from 15 to 25 kV in order to accelerate the separation, whereas all other separation parameters of the phosphate system were maintained [58] and thus might require further refinement for MES. In the case of CIEF, the optimization outlined in *ref.* [58] comprised the testing of different brands of wide and narrow pH range carrier ampholytes (CA) and the selection of the pH domain for narrow pH range CAs applied in the combined CA mixture. Finally, a combination of 0.2% m/v pH 3–10 Beckman with 0.5% m/v pH 5–7 Ampholine (AL) and 0.5% m/v pH 5–6 Pharmalyte (PL) was applied [58]. However, other influences such as changes in the ratio of the selected CA constituents or different gel batches have not been considered yet and both methods have not been validated. Based on the aforementioned separation conditions, the aim of this work is to validate both the CZE and CIEF system including also robustness evaluation. This will provide information about the suitability of both methods to distinguish between nonmodified Bet v 1a and carbamylated variants present in recombinant products and might reveal the refinement of separation parameters not addressed so far. Currently, both methods are intended for quality control of final recombinant Bet v 1a products with suspected carbamylation.

2 Materials and methods

2.1 Chemicals

CHES was purchased in a quality of 99% from Aldrich (Steinheim, Germany). TEPA pentahydrochloride and glycine were from Sigma (St. Louis, MO, USA), and MES was from Calbiochem (La Jolla, CA, USA). NaOH solutions of $w = 32\%$, 1.00 and 0.10 mol/L and orthophosphoric acid (H_3PO_4 , $w = 85\%$) were purchased in analytical grade quality from Merck (Darmstadt, Germany). Ultrapure water was supplied in a quality higher than $18.2 \text{ M}\Omega \cdot \text{cm}$ by a Milli-Q Plus 185 system (Millipore S.A., Molsheim, France). Wide pH range CA with pH 3–10 was purchased from Beckman (Fullerton, CA, USA) at 40% m/v. Narrow pH range CAs, all of 36–40% m/v, comprised pH 5–6 for PL and pH 5–7 for AL and were obtained from GE Healthcare. CIEF gel was from Beckman.

2.2 Allergens and proteins

Recombinant Bet v 1a was obtained as lyophilized material from Biomay AG (Vienna, Austria) and specified as batch 21. The preparation procedure for Bet v 1a, which was expressed in *E. coli*, is outlined elsewhere [64]. A modified product, i.e., batch 16, was purchased from Biomay. Bet v 1a is known to be partially carbamylated in this batch due to a modification of the manufacturing procedure by the employment of urea [57], which is a frequently applied solubilization reagent in protein extraction [65]. The lyophilized material was reconstituted in ultrapure water to give stock solutions of 1.0 and 2.0 mg/mL, respectively. Aliquots of 50 μL of these stocks were kept at -20°C until their injection into CE and could be stored at $+4^\circ\text{C}$ for at least 2 wk. β -Lactoglobulin A (β -LgA from bovine milk, pI 5.15) was included in the Proteome Lab cIEF 3–10 Kit from Beckman and used as a reference marker in CIEF in order to calculate relative mobilization times (t_m) in the determination of precision. Subsequent to its dissolution in ultrapure water β -LgA was stored at -20°C .

2.3 CE

Separations were performed on a Beckman P/ACE™ System MDQ CE equipped either with a photodiode array (PDA) detector for CZE or with an UV detector for CIEF. Data treatment and integration were carried out using the 32 Karat software version 5.0. A second P/ACE MDQ system equipped with 32 Karat version 7.0 and an UV detector was applied for the evaluation of intermediate precision in CIEF. Initial separation conditions applied in the validation of the described CZE and CIEF systems have been set up previously (see Section 1 and [58]), but will be briefly recalled in the subsequent sections for reasons of completeness.

2.3.1 CZE

The CZE buffer consisted of 0.4 mmol/L TEPA in 100 mmol/L MES adjusted to pH 6.50 with 32% NaOH. Bare fused-silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA) and capillary dimensions were 50 μm id and 375 μm od, with an effective length (L_D) of 40.0 cm and a total length (L_T) of 50.0 cm [58]. Conditioning of new capillaries prior to their first use, their storage and the prewash protocol performed between individual runs are outlined elsewhere [57]. Conditioning comprised a rinsing sequence of 1.0 mol/L NaOH, ultrapure water, 0.1 mol/L HCl, and buffer (all at 1500 mbar) followed by a final application of $+25.0 \text{ kV}$. Samples were injected at 35 mbar for 10 s with subsequent separation at $+25.0 \text{ kV}$ and 35.0°C applying a voltage ramp of 0.50 min at the start of each run. In addition, separation parameters not yet refined for MES were varied within this paper in the course of robustness evaluation to reveal critical issues. For univariate robustness testing, concentrations of MES and TEPA, the buffer pH, and the separation temperature were varied symmetrically around their preliminary optima. When changing the buffer composition the capillary was rinsed with the novel buffer for 10 min (1000 mbar) and conditioned for 15 min with $+25.0 \text{ kV}$ prior to the first run. Detection was done with a photodiode array (PDA) detector at 210 nm, simultaneously recording UV scans between 190 and 300 nm.

2.3.2 CIEF

Separations were performed in neutral coated capillaries from Beckman. Capillary dimensions were 50 μm id, 375 μm od with L_D 20.0 cm and L_T 30.2 cm. The CAs were mixed with the CIEF gel and appropriate amounts of allergen stock solutions to achieve a final CA composition of 0.2% m/v pH 3–10 (Beckman)/0.5% m/v pH 5–7 AL/0.5% m/v pH 5–6 PL as outlined previously [58]. The protein/CA/gel mixture was centrifuged at 14 000 rpm for 5 min to remove air bubbles and any particulate matter. The anolyte consisted of 91 mmol/L glycine pH 2.12 dissolved in gel and 20 mmol/L CHES adjusted to pH 10.00 with 32% NaOH was applied as catholyte. The whole capillary was filled with the protein/CA/gel mixture by an injection for 90 s at 2068 mbar. Focusing was performed by application of $+15.0 \text{ kV}$ for 6.0 min, with anolyte and catholyte both pressurized at 48 mbar. Subsequent protein mobilization was done at $+21.0 \text{ kV}$ with application of 48 mbar at the anolyte only. The temperature was maintained at 20.0°C . Between CIEF runs capillaries were rinsed with anolyte (1 min) and ultrapure water (2 min) both with 2068 mbar. When not in use capillaries were filled with CIEF gel and stored at $+4^\circ\text{C}$, subsequent to a rinse with ultrapure water (2 min) and CIEF gel (5 min). In the course of robustness testing 20 mmol/L NaOH and 91 mmol/L H_3PO_4 in CIEF gel were alternatively applied as catholyte and anolyte, respectively. Besides, the ratio of the selected narrow range CAs was varied and different batches of CIEF gel were tested.

3 Results and discussion

For reasons of comparison, the separation pattern achieved with the conditions stated elsewhere [58] are depicted for batches 16 and 21 (Fig. 1). For batch 16 three peak clusters were distinguished which represent Bet v 1a and single and double carbamylated variants. Carbamylation has been revealed by MS [58]. Additional confirmation was performed by artificial in-lab carbamylation of unmodified Bet v 1a with potassium cyanate modifying a previous procedure [66]. Cyanate is an inevitable degradation product of urea in aqueous solutions [67]. This control resulted in MS spectra and separation profiles in CE equivalent to batch 16 (to be published). Minor peaks within peak clusters (Figs. 1A and B, trace b) are due to impurities. Validation of both separation systems was performed by implementing aspects of the ICH guideline Q2(R1) [37], considering equally issues of the US Guidance for Industry [68] and the guidance of the FDA [69].

3.1 Accuracy

Recommended procedures [37, 70] failed in the accuracy determination for the target allergen as well as for contained impurities. This is related to the situation that neither a (certified) reference material nor primary methods are currently available for Bet v 1a [34, 68]. In addition, encountered impurities cannot be purchased to blend the allergen product with defined amounts [41, 69]. As outlined elsewhere [37, 68, 70], accuracy can be evaluated by crossvalidation instead, *i.e.*, comparison of relative purity for batch 16 achieved by CZE and CIEF. Both methods provided corresponding relative purities for the target allergen (Table 1).

3.2 Specificity

The specificity of an analytical method is defined as its possibility to unequivocally discriminate the analyte from closely related compounds (*e.g.*, impurities) most likely to be present [37, 69, 71]. Due to the aforementioned limitations spiking of the Bet v 1a standard with impurities was impossible. Instead, specificity can be demonstrated by comparing results of two analytical procedures [37]. Due to its higher heterogeneity, allergen batch 16 was analyzed by CZE and CIEF. Carbamylated variants were distinctly resolved from the target allergen (Figs. 1A and B, trace b). Instead, a minor peak (Fig. 1A, peaks 1a and b) migrated closest to Bet v 1a and was present in all batches tested by CZE so far [58]. Consistently, the specificity of the CZE method was defined by the resolution (R_s) of this peak from Bet v 1a. The FDA proposes a desirable $R_s > 2.0$ between the analyte and the closest interfering peak to provide specificity [45, 69]. The R_s -values of 1.75 ± 0.12 (mean $\pm$ 95% confidence interval, CI; $n = 5$) and 2.00 ± 0.20 ($n = 6$) achieved in CZE for batches 21 and 16, respectively, are close to this limit. Contrarily, in CIEF the minor impurity closest to Bet v 1a could not be identified unambiguously (Fig. 1B), which is either related to the high absorbance of the CA/gel mixture or an inadequate resolution. Thus, in CIEF selectivity refers to the relative t_m of Bet v 1a and its single carbamylated variant (Table 1).

3.3 Linearity

Linearity of response was determined independently for CIEF and CZE. In total, 6 and 8 calibration standard solutions were prepared out of batch 21 for CIEF and CZE, respectively (Fig. 2). For Bet v 1a a relative purity of 95.2 ± 0.9 (% mean $\pm$ 95% CI, $n = 7$) was determined in

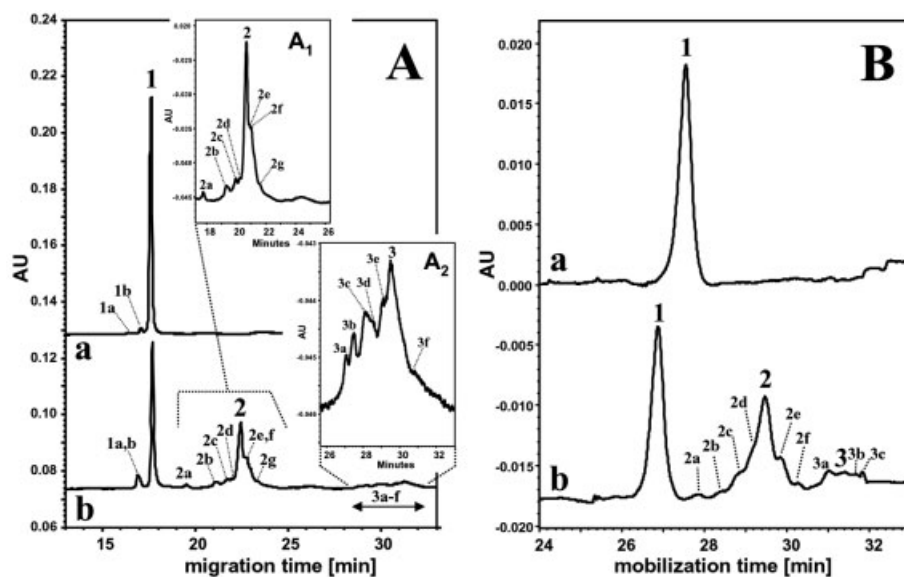


Figure 1. CZE (A) and CIEF (B) separations of recombinant Bet v 1a prepared as batch 21 (traces a) or batch 16 (traces b). Insets (A₁) and (A₂) depict details of the CZE separation of batch 16. Protein concentrations were 2.0 mg/mL (CZE) and 0.26 mg/mL (CIEF) for batch 16 and 1.0 mg/mL (CZE) and 0.13 mg/mL (CIEF) for batch 21. 1 = Bet v 1a; 2,3 = single and double carbamylated Bet v 1a, respectively. Minor peaks (annotations a–g) are impurities and progressively carbamylated in batch 16. Minor peaks are numbered chronologically, and identity might differ despite identical annotation. Separation conditions are given in Section 2 and refer to [58].

Table 1. Repeatability and intermediate precision of CZE and CIEF separations

Allergen batch ^{a)}			t_{EOF} (min)	μ_{eff} (m ² ·V ⁻¹ ·s ⁻¹)	Relative t_{m} 1 × carb. ^{b)}	Peak area ratio % ^{c)}
CZE	21 (<i>n</i> = 6; A1)	Mean ± CI	7.71 ± 0.22	$-8.63 \times 10^{-9} \pm 3.65 \times 10^{-11}$	/	95.2 ± 0.9%
		CV%	2.81	0.40		0.97
	16 (<i>n</i> = 6; A1)	Mean ± CI	7.28 ± 0.24	$-8.54 \times 10^{-9} \pm 5.09 \times 10^{-11}$	1.21 ± 0.013	40.8 ± 1.1%
		CV%	3.12	0.57	1.00	2.48
	16 (<i>n</i> = 6; A2)	Mean ± CI	7.59 ± 0.21	$-8.36 \times 10^{-9} \pm 1.69 \times 10^{-11}$	1.19 ± 0.013	39.4 ± 0.8%
		CV%	2.58	0.19	1.00	1.94
	Intermediate precision ^{d)}	Mean ± CI	7.43 ± 0.17	$-8.45 \times 10^{-9} \pm 6.49 \times 10^{-11}$	1.20 ± 0.010	40.1 ± 0.9%
		CV%	3.47	1.20	1.23	3.46
Allergen batch			Relative t_{m} ^{e)} p/ marker		Relative t_{m} 1 × carb. ^{b)}	Peak area ratio ^{f)}
CIEF	16 (<i>n</i> = 4; A2)	Mean ± CI	1.102 ± 0.001		1.118 ± 0.002	42.4 ± 1.9%
		CV%	0.05		0.12	2.86
	16 (<i>n</i> = 4; A3)	Mean ± CI	1.122 ± 0.003		1.130 ± 0.001	45.2 ± 2.2%
		CV%	0.14		0.08	3.03
	16 (<i>n</i> = 6; A2)	Mean ± CI	/		1.102 ± 0.002	44.8 ± 1.3%
		CV%	/		0.16	2.63
	16 (<i>n</i> = 6; A3)	Mean ± CI	/		1.120 ± 0.001	40.8 ± 0.4%
		CV%	/		0.12	0.96
	Intermediate precision ^{d)}	Mean ± CI	/		1.111 ± 0.006	42.8 ± 1.4%
		CV%	/		0.86	5.22

a) A1–3 denote different analysts performing the measurement series used to calculate intermediate precision. In CZE, intermediate precision considers different capillaries and buffer batches. For CIEF, intermediate precision implements different capillaries, batches of gel, of anolyte and catholyte and different CE equipment.

b) Ratio of migration times (CZE) and mobilization times (CIEF) of single carbamylated Bet v 1a and Bet v 1a.

c) The t_{m} -corrected peak area of Bet v 1a was related to the total peak area.

d) Intermediate precision of CZE and CIEF refers to two independent measurement series, respectively, with $n = 6$ in either case and is expressed as cumulative mean and CV [45]. Further details are given in the text.

e) Ratio of mobilization times of Bet v 1a and the flanking p/ marker β -LgA.

f) For CIEF, ratio was calculated from uncorrected peak area of Bet v 1a related to the total peak area.

batch 21 by CZE on basis of peak area ratio. The calculation of concentrations is based on this purity. Peak areas and heights were plotted against allergen mass concentration, respectively. In CZE, peak areas were corrected by t_{m} to compensate for slight variability in EOF and migration velocity [72]. Equations for linear least square fitting with their corresponding coefficients of determination, r^2 , are given in Fig. 2. However, an r^2 close to one is not a proof of linear relationship between two parameters [73–75]. Thus, the goodness of fit of the linear calibration was evaluated by Mandel's fitting test, which compares residual errors of a linear first order regression with those of an alternative quadratic regression model by means of an F -test on a significance level of 0.01 [76, 77]. In CZE, a quadratic equation did not create an improved regression model for peak areas within the tested concentration range since the F -value calculated by Mandel's fitting test (F_{calc} 0.74) was lower than the tabulated limit (F_{tab} 16.26). Thus, linearity was confirmed between 0.028 and 1.90 mg/mL (Fig. 2A). In contrast, Mandel's test clearly indicated a quadratic relationship between peak heights and concentration within this

range (F_{calc} 189.65 $>$ F_{tab} 16.26). Linear relationship for peak height and mass concentration was confirmed up to 0.67 mg/mL (F_{calc} 42.76 $<$ F_{tab} 98.50) (Fig. 2B). In CIEF, linearity between peak area and mass concentration was proven between 0.016 and 0.26 mg/mL with Mandel's test (F_{calc} 9.84 $<$ F_{tab} 34.12) (Fig. 2C). For peak heights Mandel's test proved linearity up to 0.12 mg/mL (F_{calc} 10.21 $<$ F_{tab} 98.50). At higher concentrations the response flattened, apparently due to the high allergen concentrations achieved during CIEF focusing (Fig. 2D).

3.4 LOQ

The LOQ is defined as the lowest analyte amount, which can be quantified with acceptable precision and accuracy. The LOQ for Bet v 1a was calculated in a triplicate approach according to the ICH [37]. In the first approach (i) LOQ determination was based on a 10:1 ratio of S/N. Further calculations related the SD of response to the slope of the calibration curve for peak heights. Responses, i.e., peak heights,

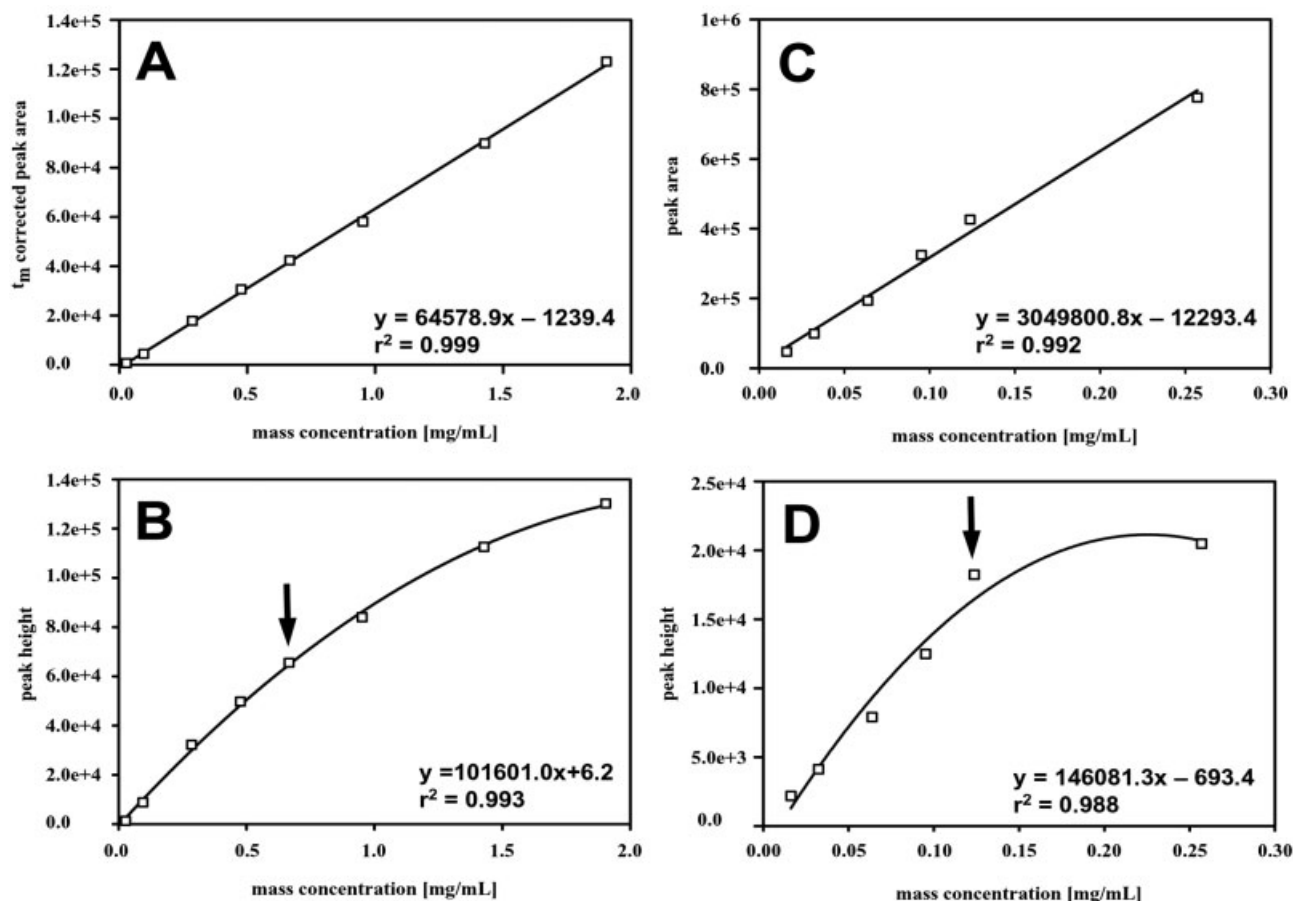


Figure 2. Linearity evaluation of detector response for Bet v 1a (batch 21). Measurements of (A) t_m -corrected peak areas and (B) peak heights in CZE at 210 nm as well as (C) peak areas and (D) peak heights in CIEF at 280 nm. The entire concentration range tested is given. Upper concentration limits for the linear range of peak heights were confirmed by Mandel's test and indicated with arrows. Regression equations and r^2 refer to ranges with confirmed linearity. Allergen mass concentrations in CIEF refer to the injected CA/gel/allergen mixture.

were calculated either from (ii) blank samples or from (iii) allergen solutions prepared at concentrations approximate to the estimated LOQ. Slopes of calibration curves refer to concentration ranges with confirmed linearity of peak heights (see Section 3.3; Figs. 2B and D). Strictly interpreted, for (i) and (ii) the noise has to be determined from a placebo formulation, *i.e.*, a blank matrix derived from a nontransfected *E. coli* culture which has been submitted to the whole extraction and purification procedure. As those blanks were not available the noise was determined instead by injecting either ultrapure water in CZE or in the case of CIEF by applying a mixture of CAs and CIEF gel containing ultrapure water at a volume equivalent to the allergen standard solution added otherwise. For both approaches baseline noise of the model blanks was measured over 2 min centered at the averaged t_m of Bet v 1a. Depending on the chosen calculation procedure (i)–(iii), the LOQ for Bet v 1a was between 10–20 and 12–23 $\mu\text{g/mL}$ for CZE and CIEF, respectively. Calculated limits were re-examined by analyzing allergen solutions with concentrations approximate to the LOQ (Figs. 3A and B) [37].

3.5 Repeatability and intermediate precision

Repeatability is defined as the closeness of agreement between results of successive measurements performed under identical conditions [71] and was determined for migration time of EOF marker (t_{EOF}), effective electrophoretic mobility (μ_{eff}), relative t_m , and peak area ratios (Table 1). For each calculation, four or six replicate injections of the same allergen standard solution were performed. Repeatability of t_{EOF} in CZE was $<3.2\%$ (CV) and variability is partially due to the dynamic wall coating with TEPA. However, for μ_{eff} , which corrects for EOF variability [72], repeatability was improved to $<0.6\%$. This corroborates results from the initial phosphate buffer [57]. Repeatability for peak area ratio of Bet v 1a was $<2.5\%$ in CZE for both allergen batches. The t_m in CIEF were prone to variations with CV $<3.7\%$ (data not shown) due to the complex composition of the injected solution. When relative t_m for Bet v 1a were calculated on basis of an adjacent marker compound (*i.e.*, $\beta\text{-LgA}$) or

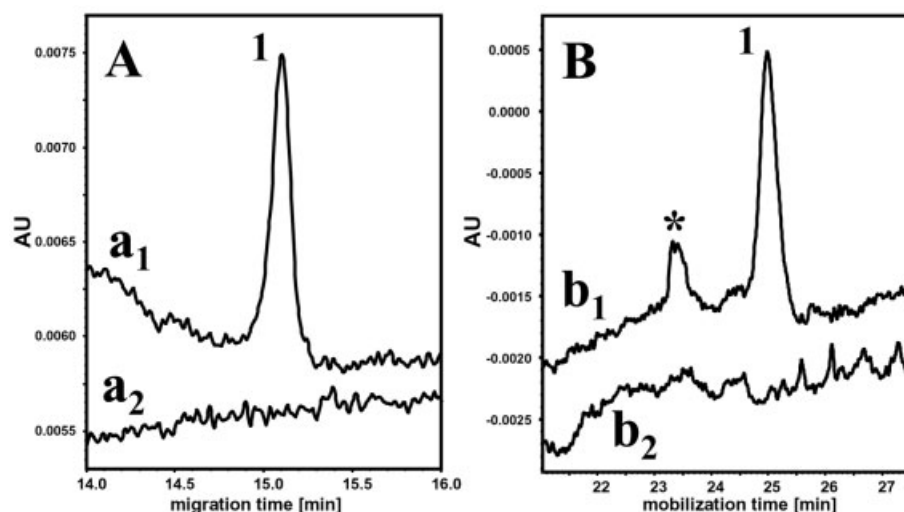


Figure 3. Electropherograms of Bet v 1a (batch 21) standards approximate to the LOQ. Concentrations were 28 $\mu\text{g/mL}$ in CZE (A) and 16 $\mu\text{g/mL}$ in CIEF (B). Corresponding blanks are depicted as traces a_2 and b_2 . Further details are given in Section 3.4. * Assigns a baseline irregularity.

single carbamylated Bet v 1a, repeatability improved to $<0.2\%$ (Table 1).

Intermediate precision expresses within-laboratory variation, when changing selected factors, *e.g.*, analysts and equipment [37]. As a measure for intermediate precision, cumulative mean and CV [45] calculated from individual data of two measurement series are reported (Table 1). In CZE, μ_{eff} differed on the 0.05 level between different measurement series (Table 1). This is most likely related to the use of different capillaries and their diverging state of the inner surface, as discussed previously for phosphate [57]. In CIEF, the covalent coating and the preparation of the complex CA/gel/allergen mixture (see Section 2.3.2) are additional factors of influence. Thus, in CIEF peak area ratio for Bet v 1a between different measurement series shows a higher variability (5.2%) than in CZE (3.5%) and relative t_m differ on the 0.05 level. In addition, the application of different CE-equipment has to be considered in CIEF (Table 1).

3.6 Robustness

Evaluation of method robustness [37, 71] allows for the elucidation of sensitive separation parameters. Consequently, these parameters should be given particular attention, especially in method transfer between different equipment and laboratories. Robustness was tested for batch 16 by fractional factorial design. Thereby, single parameters were varied symmetrically around their preliminary optima [78].

3.6.1 CZE

Parameters to be tested in CZE encompassed concentrations of MES and of TEPA, the pH of the buffer and the separation temperature (Table 2). MES concentrations of 90 and 100 mmol/L both resulted in equivalent t_{EOF} , t_m for Bet v 1a and R_s for the critical peak pair. An increase to 110 mmol/L reduced the R_s , increased t_{EOF} and t_m (data not

shown), and impaired the repeatability of these parameters. The μ_{eff} remained unaffected since 95% CI overlap for the tested MES concentrations. A variation of the TEPA concentration between 0.3 and 0.5 mmol/L had no effect on the μ_{eff} at the 0.05 level. However, at 0.3 mmol/L TEPA R_s was decreased, whereas at 0.5 mmol/L the repeatability of both the EOF and μ_{eff} became impaired. Deviations of ± 0.5 U from the selected pH of 6.50 changed the μ_{eff} in either case and also influenced the R_s of the carbamylated peak clusters (Figs. 4A and B). This is related to a change of the net charge of the allergen, but probably also to alterations in the hydrodynamic radius [47, 79]. The R_s of the critical peak pair was only reduced at pH 7.00. A temperature variation to 30 and 40°C exerted no significant effect on the R_s of the critical component pair, but changed μ_{eff} of Bet v 1a considerably. Peak area ratios for Bet v 1a were only affected by changes in pH (Table 2).

3.6.2 CIEF

Unlike CZE the critical impurity adjacent to Bet v 1a (see Section 3.2) could not be identified unambiguously in CIEF. The t_m of Bet v 1a relative to the single carbamylated variant and the area percentage of Bet v 1a and of the single carbamylated variant were selected for the evaluation of robustness (Table 3). Different gel batches as well as anolyte/catholyte combinations did not affect robustness. In contrast, the CA composition represents a key factor affecting relative t_m and area percentage. A small baseline step subsequent to the double carbamylated variants was most likely due to the CA composition in combination with the electrolytes. The position of the allergens relative to this irregularity is related to the PL content in the CA mixture: increasing the portion of PL pH 5–6 moves the double carbamylated variants towards this baseline step (Fig. 5) by spreading the pH gradient at the position of allergens. This becomes evident from the improved R_s of Bet v 1a and its carbamylated var-

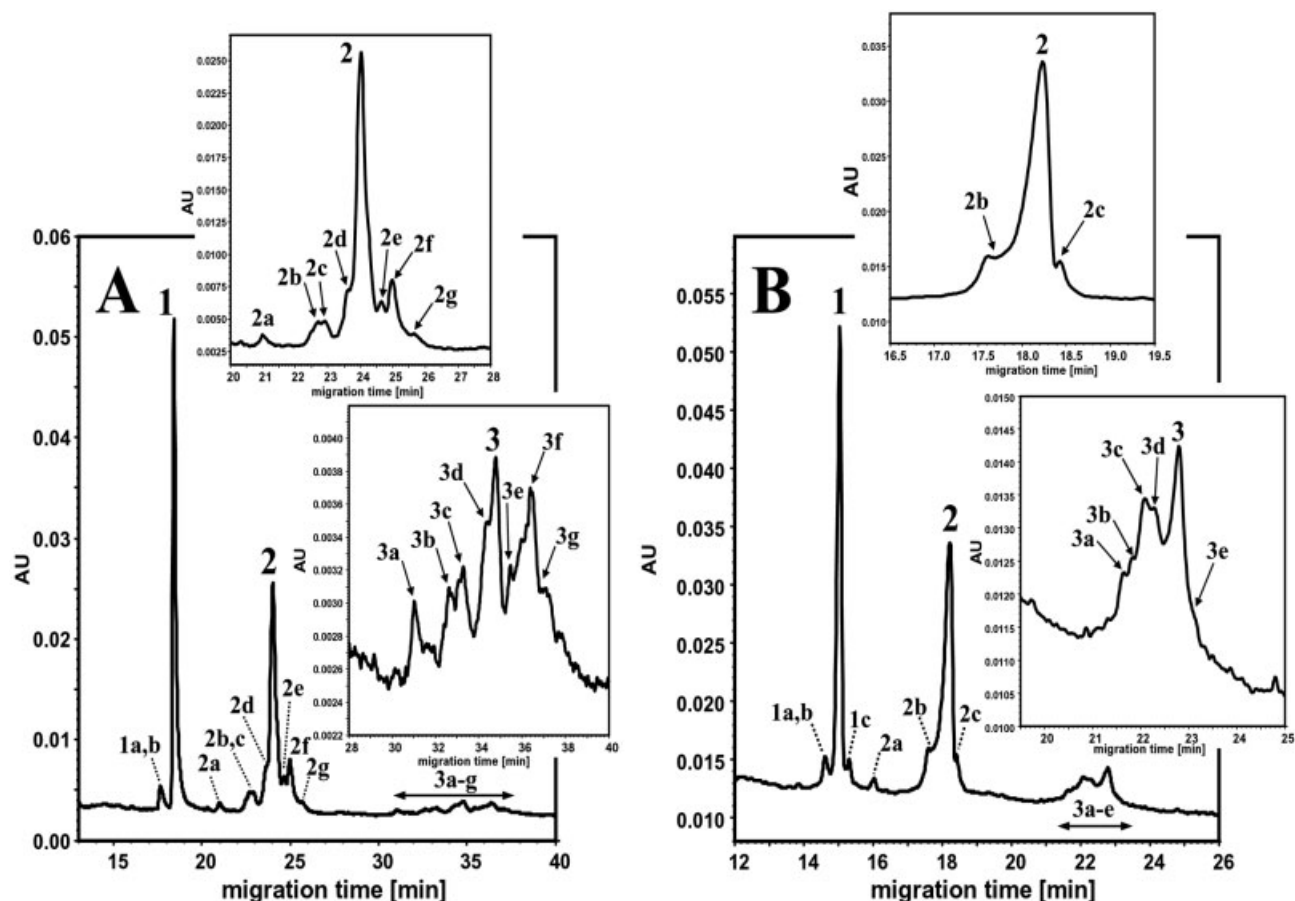


Figure 4. Robustness evaluation in CZE by variation of the pH to (A) 6.00 and (B) 7.00. Selectivity and resolution of impurities was improved at pH 6.00 compared to pH 7.00. However, at pH 7.00 a minor peak (peak 1c) occurred adjacent to Bet v 1a which was absent in all other separation systems. For peak annotations see Fig. 1.

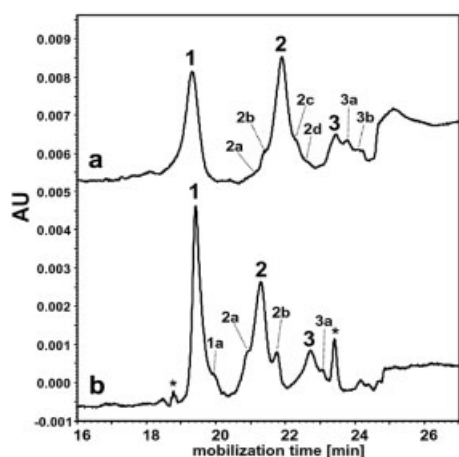


Figure 5. Robustness evaluation in CIEF by variation of the CA composition. (a) 0.75% m/v PL and 0.25% m/v AL; (b) 0.25% m/v PL and 0.75% m/v AL. The 0.2% m/v wide pH range (3–10) CA was additionally contained in both mixtures. Protein concentration was 0.26 mg/mL. For peak annotations and separation conditions see Fig. 1. * Assigns a baseline irregularity.

iants, the increased t_m -ratios and a broadening of the Bet v 1a peak (Fig. 5, Table 3). However, a combination of 0.75% m/v PL/0.25% m/v AL reduced the R_s of minor peaks within the carbamylated peak cluster compared to the optimized CA combination, whereas a higher ratio of AL gave rise to a minor shoulder of the Bet v 1a peak (Fig. 5).

3.7 System suitability test

Prior to the initiation of the actual analysis sequence the appropriate performance of the analytical systems should be assessed by a suitability test, which has to meet critical limits for selected key parameters. The CZE separation was considered properly equilibrated when the R_s of the critical peak pair was >1.8 , t_{EOF} was below 8.0 min and μ_{eff} of Bet v 1a was $-8.5 \times 10^{-9} \pm 0.2 \times 10^{-10} \text{ m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$ (mean $\pm$ 95% CI). In combination with the separation profile of the single carbamylated peak group, these parameters can reveal deviations from the optimized conditions. In CIEF, t_m of Bet v 1a should be >20.0 min. The partial separation of the sin-

Table 2. Robustness evaluation of the CZE method

Separation conditions		t_{EOF} (min)	μ_{eff} ($\text{m}^2 \cdot \text{V}^{-1} \cdot \text{s}^{-1}$)	R_s	Bet v 1a Area ratio (%)	1 × carb. Area ratio (%)
100 mmol/L MES–0.4 mmol/L TEPA, pH 6.50, 35.0°C (ref)	Mean ± CI ^{a)}	7.39 ± 0.31	$-8.55 \times 10^{-9} \pm 4.08 \times 10^{-11}$	2.00 ± 0.24	41.2 ± 1.25	44.3 ± 1.95
	CV%	2.63	0.30	7.53	1.91	1.38
MES						
90 mmol/L	Mean ± CI	7.50 ± 0.38	$-8.43 \times 10^{-9} \pm 4.37 \times 10^{-11}$	1.98 ± 0.25	40.4 ± 1.1	44.1 ± 1.6
	CV%	3.15	0.33	8.07	1.70	2.21
110 mmol/L	Mean ± CI	8.07 ± 0.52	$-8.45 \times 10^{-9} \pm 3.09 \times 10^{-11}$	1.61 ± 0.33	40.0 ± 1.0	44.4 ± 1.6
	CV%	4.04	0.23	12.74	1.61	2.27
TEPA						
0.3 mmol/L	Mean ± CI	7.66 ± 0.23	$-8.46 \times 10^{-9} \pm 7.52 \times 10^{-11}$	1.64 ± 0.18	40.9 ± 0.4	43.1 ± 0.2
	CV%	1.91	0.56	6.99	0.63	0.30
0.5 mmol/L	Mean ± CI	8.14 ± 0.55	$-8.40 \times 10^{-9} \pm 1.31 \times 10^{-10}$	2.17 ± 0.05	41.4 ± 0.8	43.9 ± 1.9
	CV%	4.26	0.98	1.47	1.21	2.64
pH						
pH 6.00	Mean ± CI	9.70 ± 0.58	$-7.00 \times 10^{-9} \pm 1.05 \times 10^{-10}$	1.92 ± 0.16	43.9 ± 2.1	46.1 ± 1.6
	CV%	5.30	1.21	5.91	3.05	2.18
pH 7.00	Mean ± CI	7.39 ± 0.63	$-9.26 \times 10^{-9} \pm 2.80 \times 10^{-10}$	1.53 ± 0.23	38.1 ± 1.4	41.9 ± 0.7
	CV%	6.88	2.44	12.15	3.01	1.34
Temperature						
30.0°C	Mean ± CI	9.06 ± 0.64	$-7.64 \times 10^{-9} \pm 1.39 \times 10^{-11}$	2.01 ± 0.26	40.0 ± 2.0	45.5 ± 3.6
	CV%	4.47	0.11	8.15	3.08	4.90
40.0°C	Mean ± CI	7.37 ± 0.28	$-9.33 \times 10^{-9} \pm 1.19 \times 10^{-10}$	1.89 ± 0.18	41.4 ± 1.1	47.0 ± 4.0
	CV%	2.42	0.80	6.05	1.70	5.38

The optimized CZE system is given as a reference (ref). Individual parameters were varied stepwise as given in the table and all other conditions of the reference system were maintained. The influence of MES and TEPA concentration, pH, and temperature on t_{EOF} , μ_{eff} , R_s of the critical peak pair (Bet v 1a and the most adjacent impurity), peak area ratios for Bet v 1a and the single carbamylated variant were tested ($n = 4$ in either case). Peak areas were t_m -corrected. All runs were performed with the same capillary.

a) 95% CI.

gle carbamylated peak cluster into one major peak and two partial resolved minor peaks on either flank provides an additional decision parameter.

4 Concluding remarks

Two complementary CZE and CIEF separation systems for nonmodified and carbamylated products of recombinant Bet v 1a were validated considering aspects of the ICH. Data allow for a crossvalidation and can provide information about the general method applicability in monitoring conformity of recombinant allergen products with respective specifications of the manufacturer. Relative purities derived from both methods for the selected products corresponded. Regression calculation by peak areas offered an improved linearity compared to heights. The LOQ of 10–23 µg/mL for Bet v 1a in both systems corresponds with allergen concentrations frequently applied in diagnostic skin-prick tests [30]. Repeatability of

migration behavior was <0.2% for relative t_m and <0.6% for μ_{eff} in CIEF and CZE, respectively. Repeatability for peak area ratio was better than 3.1%. The specificity of CZE met the proposed FDA criteria in the investigated samples. In CIEF the critical impurity adjacent to Bet v 1a was not detected either due to increased background or impaired R_s . However, impurity profiles of both methods coincide, although CZE achieved a slightly improved separation. Robustness evaluation in CZE revealed susceptibility of μ_{eff} to deviations of pH (± 0.5 U) and temperature ($\pm 5.0^\circ\text{C}$), whereas the selectivity was affected by an increase in temperature, pH, and buffer concentration and a reduction in the TEPA concentration. For CIEF, the composition of the CA mixture is the key parameter to retain. Results suggest both methods applicable for quality control of recombinant Bet v 1a products with suspected carbamylation.

The authors have declared no conflict of interest.

Table 3. Robustness evaluation of the CIEF method

Separation conditions		Relative t_m	Bet v 1a area ratio	1 × carb. area ratio
0.50 PL/0.50 AL glycine/CHES ^{a)}	Mean ± CI CV%	1.112 ± 0.001 0.06	43.2 ± 2.2 3.26	42.9 ± 4.9 7.11
Anolyte/catholyte				
H ₃ PO ₄ /NaOH	Mean ± CI CV%	1.116 ± 0.007 0.40	41.5 ± 2.7 4.12	43.8 ± 3.8 5.39
H ₃ PO ₄ /CHES	Mean ± CI CV%	1.109 ± 0.004 0.24	41.8 ± 1.0 3.74	44.7 ± 0.9 1.54
CIEF gel				
Batch 1	Mean ± CI CV%	1.112 ± 0.001 0.04	42.6 ± 4.0 3.26	42.9 ± 1.5 7.11
Batch 2	Mean ± CI CV%	1.125 ± 0.010 0.34	41.2 ± 3.3 3.20	44.4 ± 4.9 4.42
CA composition				
0.25 PL/0.75 AL	Mean ± CI CV%	1.101 ± 0.002 0.13	48.6 ± 2.0 3.26	41.8 ± 2.0 3.0
0.75 PL/0.25 AL	Mean ± CI CV%	1.132 ± 0.002 0.13	42.8 ± 0.8 1.11	44.6 ± 1.5 2.10

Individual parameters were varied as given in the table and all other conditions of the reference system were maintained. The influence of the anolyte and catholyte combination, of different batches of CIEF gels and of the ratio of narrow pH range CAs were tested applying the same capillary in either case ($n = 3$).

a) The optimized CIEF system with a CA composition of 0.2% m/v pH 3–10 Beckman/0.5% m/v pH 5–6 PL/0.5% m/v pH 5–7 AL, employing glycine and CHES as anolyte and catholyte, respectively, is given as the reference system.

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