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Evaluation of amino sugar, low molecular peptide and amino acid impurities of biotechnologically produced amino acids by means of CE

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Abstract

As the second part of our studies on the impurity profiles of amino acids produced by biotechnological processes, a micellar electrokinetic chromatography (MEKC) was applied to determine amino sugars, low molecular peptides and amino acids as potential expected impurities at a level 0.1% w/w. 3-(4-Carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) was found to be suitable as a labeling reagent for laser induced fluorescence (LIF) detection. The labeling reaction was optimized to achieve maximum reaction yields and reproducibility for all groups of substances. The 'level 0.1%' was chosen as quantities/concentrations in such a way that the major reagent peak becomes comparable with the impurities at this level, and the minor reagent peaks ($< 0.01\%$) are not able to cover other peaks at the level of interest. The reaction optimization and the validation studies were performed with model mixtures of representative amino sugars, low molecular peptides and amino acids. A linearity range of labeling of three orders of magnitude was achieved. The total precision of the method, including the labeling reproducibility was studied and for all test substances relative standard deviation less than 5% were obtained. The accuracy was evaluated by performing recovery experiments at three concentrations covered a 50–200% interval of the level 0.1%. Confidence intervals below $\pm 2.5\%$ ($\lambda = 0.05$, $n = 9$) of the target were found sufficient for purity tests.

Keywords: Amino acids; Impurity profile; Capillary electrophoresis; CBQCA derivatives; Amino sugars; Low molecular peptides

1. Introduction

In recent years, numerous validated capillary electrophoresis (CE) methods for assay, purity testing, stability indicating studies and dissolution tests of drug substances and products were presented in the literature [1–5]. Recently, the general monograph of CE and the first test for related substances by means of CE was included in the European pharmacopoeia, i.e. in the monograph 'Levocabastine Hydrochloride'. In our previous work, the applicability of CE to the evaluation of the impurities at level 0.1% in amino

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acids was demonstrated by the determination of other amino acids as impurities. After derivatization with 9-fluorenylmethyl chloroformate (FMOC) [6] the impurities could be quantified at a trace level of 0.1% using UV-detection at 254 nm.

In the last couple of years the production method of amino acids has changed from the chemical synthesis to fermentation processes. The question raise whether the impurity profile of biotechnologically produced amino acids consists of new uncommon impurities of biological activity which should be, therefore, limited in pharmacopoea tests for related substances. The aim of this study was to develop a CE method, which is able to determine other classes of potential impurities, especially amino sugars and low molecular peptides, as well as amino acids, all characterized by missing a chromophore or fluorophore. A suitable labeling reagent for this purpose must have a high reactivity to all these substances and has to fulfil several requirements, which make it applicable in trace impurity analysis and which were already extensively described in our previous work [6].

On the basis of the formation of isoindole derivatives a number of labeling reagents and labeling schemes for the derivatization of primary amines have been developed in the 1980s and 1990s, *ortho*-phthalaldehyde (OPA) [7,8]; naphthalene-2,3-dicarboxaldehyde (NDA) [9–13], 3-(4-carboxybenzoyl)quinoline-2-carboxaldehyde (CBQCA) [14–18]. Compared with the OPA-derivatives, 1-cyanobenz(f)-isoindoles (CBI) formed by the reaction of amino groups with NDA in presence of cyanide ions, are more stable. Another advantage of CBI-derivatives is that the excitation maximum wavelength coincides closely

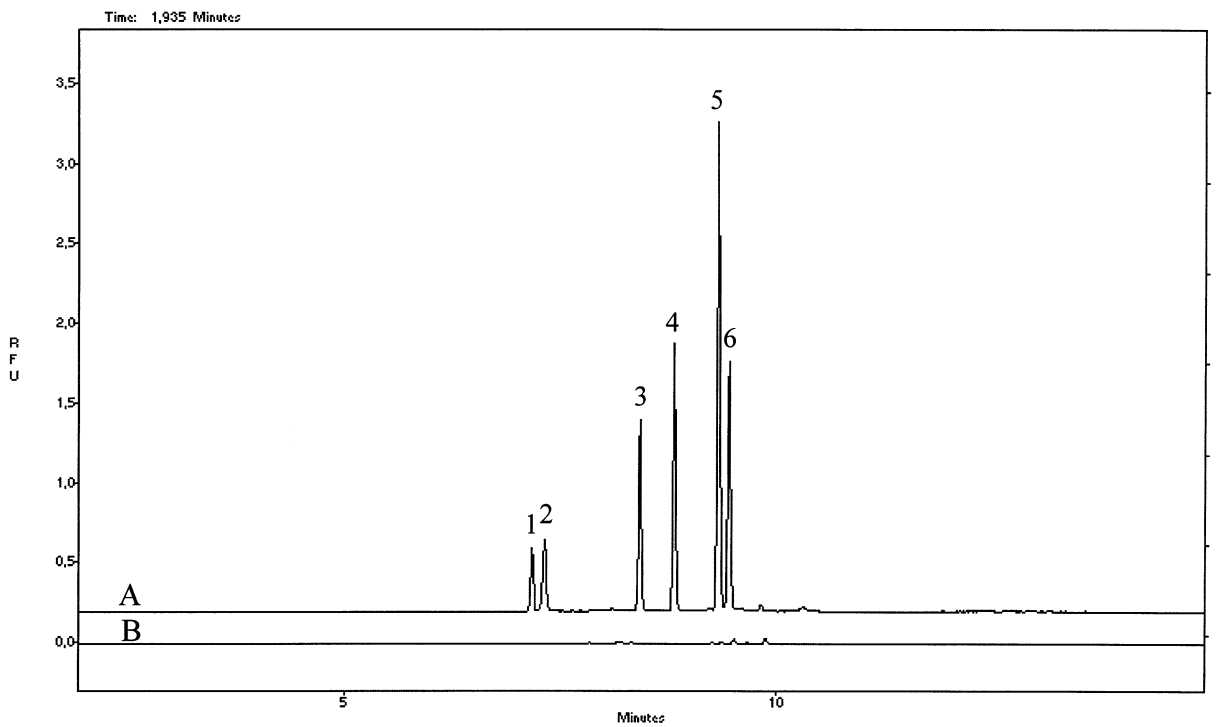

Fig. 1. Electropherograms of derivatized with CBQCA (A), complex sample, consisting of D(+)-glucosamine (peak 1), D(+)-galactosamine (peak 2), Gly–Pro–Gly–Gly (peak 3), Gly–Gln (peak 4), Phe (peak 5), Ser (peak 6) each 0.1 mM; (B), blank. (CE), Running buffer: borate (pH 9.3, 20 mM), containing 25 mM SDS; Capillary: fused-silica 70/60 cm, 75 μm i.d.; Voltage: 20 kV; Temperature: 25 °C; Injection: 3448 Pa, 5 s; Detection: LIF 488/520 nm.

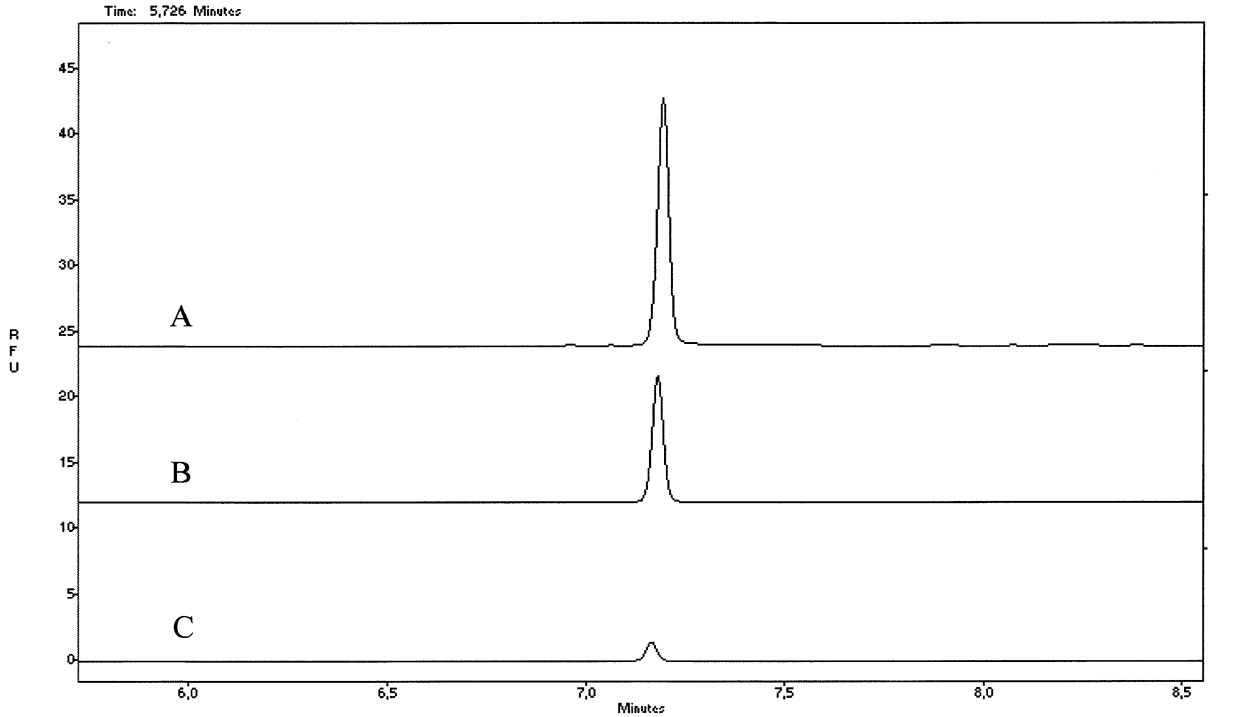

Fig. 2. Electropherograms of 5 mM D(+)-glucosamine sample derivatized with CBQCA in (A) phosphate buffer (pH 7.0, 50 mM); (B) borate buffer (pH 7.5, 100 mM); (C) water. CE conditions as described in Fig. 1.

with the blue line of the more popular He–Cd lasers. Different examples of successful application of NDA as labeling reagent of amino acids and peptides were reported in the literature [9–13]. However, also several side reaction products were reported, even when no amine-containing analyte is present [19]. Therefore, a evaluation of impurity profile at trace level after labeling with NDA is difficult. CBQCA seem to be a more 'promising candidate'. CBQCA was introduced as a fluorogenic reagent for labeling of primary amines by Novotny et al. [14,15]. One important advantage of CBQCA as a labeling reagent is that it does not fluoresce unless it reacts with an amino group. Thus, the excess used for labeling purposes has not to be removed from the sample. The secondary fluorescence products are at a very low level and the reaction quantities/concentrations could be selected in such a way that they were found to be at a lower level than the peaks of interest. Another advantage of this reagent is stability of its derivatives. Novotny et al. reported that the formed isoindoles for both amino acids and peptides are stable for more than 24 h and minimum 10 h for amino sugars [14,15]. Two disadvantages of this reagent have to be mentioned, firstly due to the formation of an isoindole ring it is suitable for labeling of primary amines only and, secondly, relative long reaction times are required. Since a number of successful other applications of CBQCA as reagent for labeling of primary amines were reported in the literature [16–18], the reagent was applied to study the impurities of the amino acids.

2. Experimental

2.1. Instrumentation

All CE separations were performed on a Beckman Coulter P/ACE System MDQ (Fullerton,

CA, USA), equipped with LIF-detector: excitation with coupled argon ion laser at 488 nm; emission at 520 nm. The fused-silica capillaries purchased from Beckman Coulter (Fullerton) were of 75 μm internal diameter and effective length of 60 cm (total length of 70 cm). The samples were loaded by pressured injection by 3448 Pa for 5 s on the anode side and the detection was performed on the cathode side. The electrophoreses were carried out at 25 °C and a voltage of 20 kV. The capillary was conditioned for 20 min with 0.1 M NaOH, and 10 min with water. Additionally, the capillary was washed for 5 min with 0.1 M NaOH, 5 min with water, and 5 min with the running buffer before each run.

2.2. Chemicals

All chemicals used were of analytical grade. The labeling reagent CBQCA was purchased from Molecular Probes (Eugene, OR, USA). Sodium dodecyl sulfate (SDS), amino acids and amino sugars from Fluka (Buchs, Switzerland), 9-FMOC, low molecular peptides Gly–Gln and Gly–Pro–Gly–Gly from Sigma (St. Louis, MO, USA) were used for the method development. A number of amino acids produced by fermentation from several manufacturers were subjected to the optimized method.

2.3. Buffers and reagents

Buffers were daily prepared in high purity water produced by a Milli-Q Water System (Millipore, Bedford, MA, USA) and filtered through 0.22 μm membrane filter (Roth, Karlsruhe, Germany). The separation buffer was a borate buffer (pH 9.3, 20 mM), containing 25 mM SDS. CBQCA was dissolved in dimethyl sulphoxide to give a 10 mM solution. A 10 mM solution of potassium cyanide (KCN) was prepared in water.

2.4. Derivatization procedure

The samples were derivatized with CBQCA by a procedure previously described by Novotny et

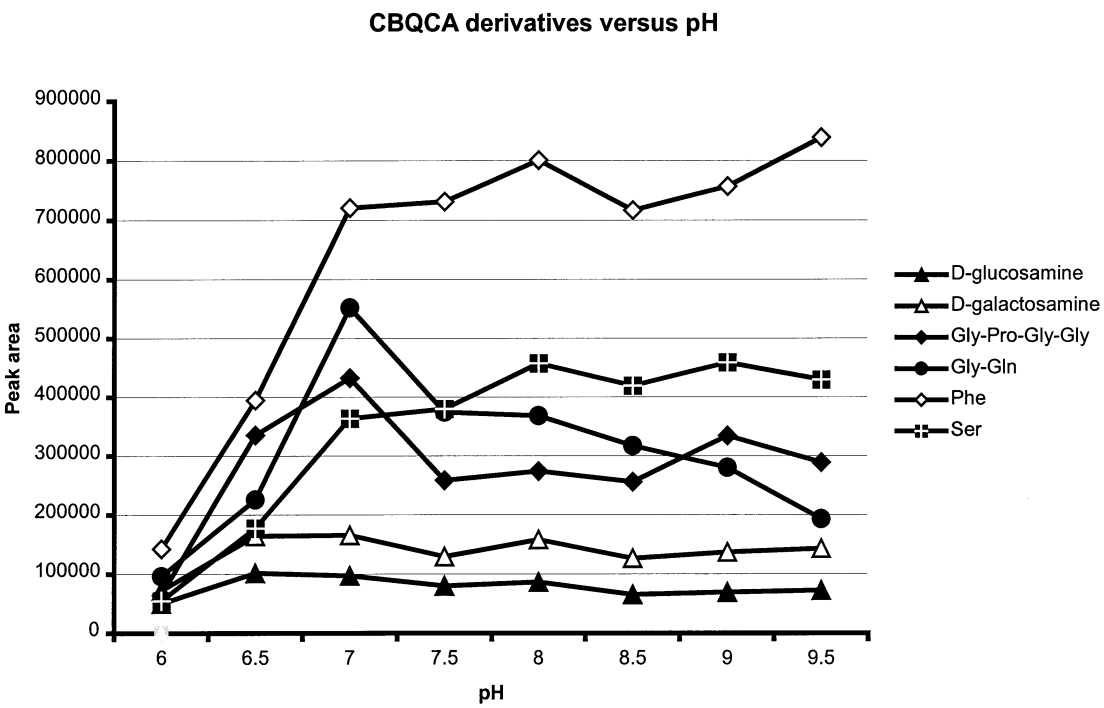

Fig. 3. Effect of pH on the labeling reaction. Sample and CE conditions as described in Fig. 1.

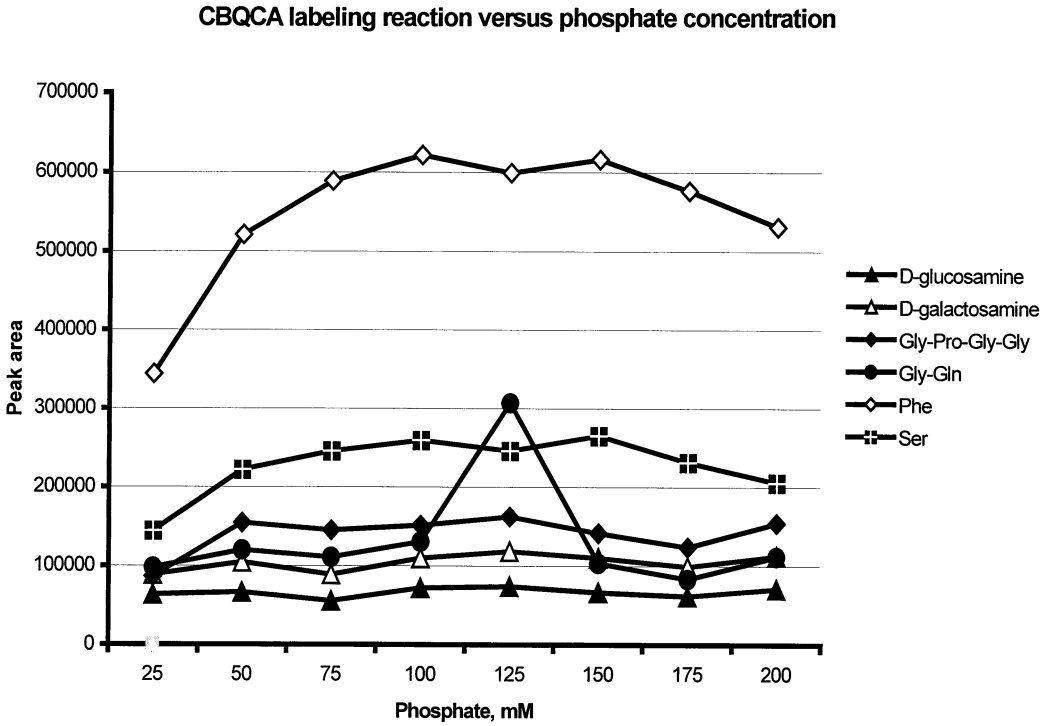

Fig. 4. Effect of phosphate concentration in the solvent buffer on the labeling reaction. Sample and CE conditions as described in Fig. 1.

al. [14,15]: to 10 μl of a sample solution in phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.0, 125 mM) 10 μl CBQCA solution and 10 μl KCN solution were added. After vortexing, the reaction was allowed to stand for 10 h at room temperature (RT). Derivatized samples were 100-fold diluted with water before loading.

3. Results and discussion

3.1. Optimization of derivatization procedure

The aim of this study was to develop a suitable method for purity evaluation of amino acids at level 0.1%, considering amino sugars, low molecular peptides and amino acids. Since most of these impurities do not have a chromophore or fluorophore, CBQCA was chosen as a labeling reagent. Several parameters of the labeling reaction have to be optimized in order to achieve a high yield of labeling and reproducibility for each individual class. The parameters, optimized are, type of solvent buffer; its pH and ion strength; reaction temperature and reaction time. The optimization of the labeling reaction was carried out with a model mixture, consisting of two representative substances for each group of interest, D(+)-glucosamine; D(+)-galactosamine, Gly–Gln, Gly–Pro–Gly–Gly, Phe and Ser.

The MEKC method was first proposed by Terabe et al. [20] and successfully applied utilising borate/SDS running buffer in our previous studies on amino acids [6]. The borate buffers were recommended for separation of sugars and aminosugars such as structurally related glucosamine and galactosamine [15]. Since borate buffer (pH 9.3, 20 mM), containing 25 mM SDS was successfully applied to the FMOC derivatives of amino acids, the same conditions were used with the CBQCA derivatives. Fig. 1 shows a typical electropherogram of a sample derivatized with CBQCA.

As the first step of the optimization of the labeling procedure, a series of samples dissolved in water, borate buffers or phosphate buffers was derivatized for 1 h at RT in order to study the influence of solvent type. With respect to the yield of the labeling reaction the phosphate buffer was found to be suitable. For example (see Fig. 2), in the case of D(+)-glucosamine the peak area obtained from the reaction in phosphate buffer (Na_2HPO_4/NaH_2PO_4, pH 7.0, 50 mM) is 2-fold higher compared to the sample dissolved in borate buffer (pH 7.5, 100 mM) and 14-fold higher compared with the sample dissolved in water.

Next, the effect of pH was studied. Previously, Novotny et al. reported an optimum pH of 7.0 for amino sugars and 9.0 for amino acids [14,15]. Thus, the pH was varied in a range of 6.0–9.5. No significant difference in the reaction yields for D(+)-glucosamine and D(+)-galactosamine was observed in the pH interval from 6.5 to 8.0, and in the same interval both peptides Gly–Gln and Gly–Pro–Gly–Gly showed maxima at pH 7.0 (see Fig. 3). Although the reaction yields obtained for Phe and Ser at this pH value were approximately 80 and 70% of the maximal values, respectively, pH 7.0 was chosen for this complex mixture. However, using a sufficient long time of reaction at RT (see below), a quantitative conversion of Phe and Ser can be achieved even at the low pH of 7.0.

The influence of phosphate concentration on the reaction is shown in Fig. 4. The variation of the phosphate concentrations between 25 and 200 mM revealed no significant difference for both D(+)-glucosamine and D(+)-galactosamine, for Gly–Pro–Gly–Gly above 50 mM and for Phe

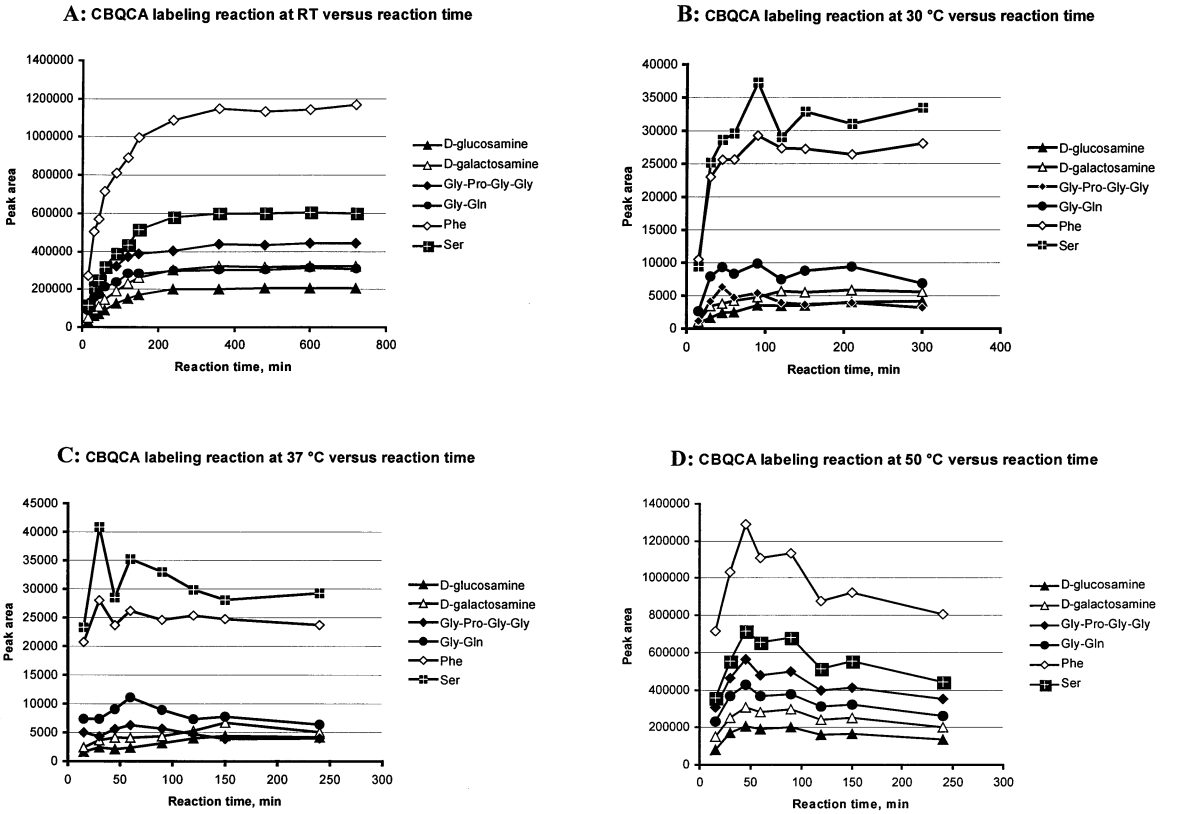

Fig. 5. Effect of the temperature on the labeling reaction. Samples A and D, and CE conditions as described in Fig. 1; sample B and C consists of the same substances, but their concentrations are 0.00043 mg/ml each.

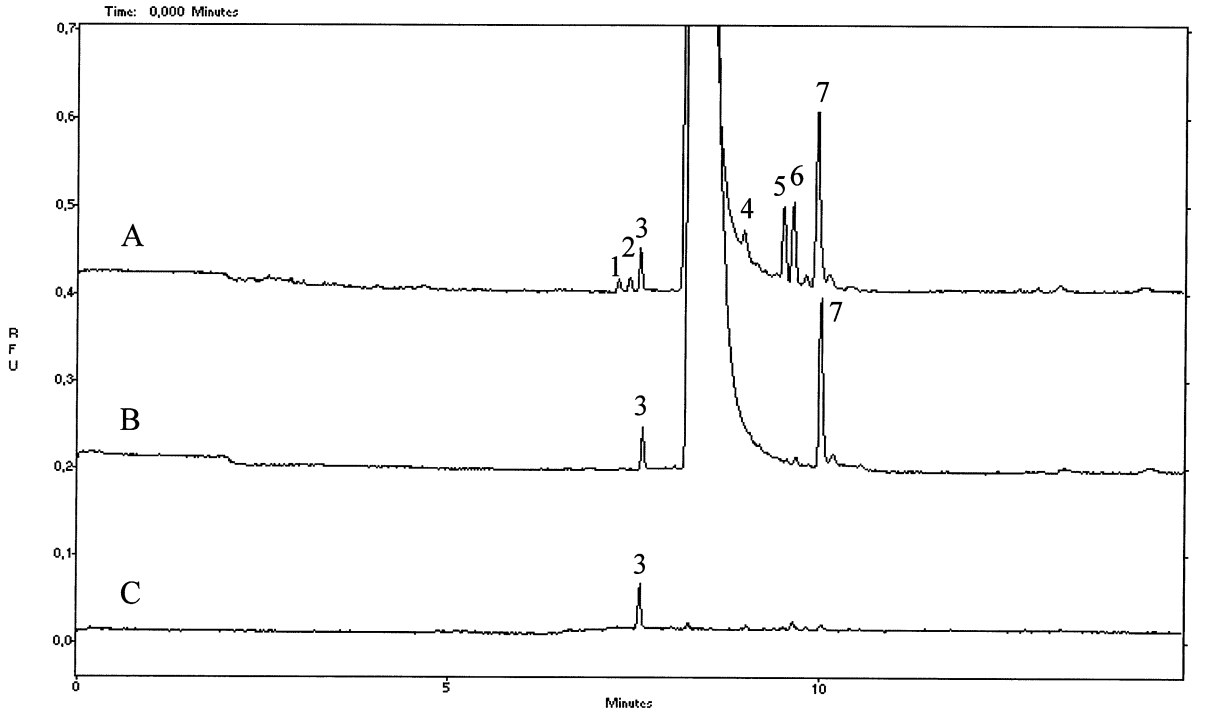

Fig. 6. Electropherograms of CBQCA derivatized samples: (A) Arg 0.45 mg/ml, spiked with 'impurities' each of 0.1%; (B) Arg 0.45 mg/ml; (C) blank. Peak identity: (1) D(+)-glucosamine; (2) D(+)-galactosamine; (3) major reagent peak; (4) Gly–Gln; (5) Phe; (6) Ser; (7) unknown. CE conditions as described in Fig. 1.

Table 1
Linearity of the method, tested ranges and correlation coefficients

Substance	Tested range			Correlation coefficient
	(mM)	(mg/ml)	(% of target)	
D(+)-glucosamine	0.001–0.01	0.00018–0.0018	40–400	0.9999
D(+)-galactosamine	0.001–0.01	0.00018–0.0018	40–400	0.9768
Gly–Pro–Gly–Gly	0.001–0.01	0.00029–0.0029	44–444	0.9999
Gly–Gln	0.001–0.01	0.00025–0.0025	64–644	0.9958
Phe	0.001–0.01	0.00017–0.0017	37–367	0.9999
Ser	0.001–0.01	0.00011–0.0011	23–233	0.9947

and Ser within an interval of 75–175 mM phosphate buffer. For Gly–Gln, a maximum of reaction yield was achieved at a phosphate buffer concentration of 125 mM. Thus, all experiments were carried out at this concentration.

Four temperatures, 25, 30, 37 and 50 °C, were examined in order to find the optimal reaction temperature. As can be seen from Fig. 5 temperatures higher than 30 °C result in a short reaction time, but simultaneously a degradation of the labeling products was observed. Hence, it is difficult to achieve reproducible, quantitative derivatizations. Performing the labeling reaction at RT, 10 h of reaction time is necessary to achieve a quantitative labeling for all components of the model mixture. In addition, the uniformity

of the curve indicates that no degradation reaction takes place during this time course. Thus, all labeling reactions were performed at RT for at least 10 h.

The next goal was to chose quantities/concentrations for the representative sample substances at a fixed labeling reagent concentration of 10 mM in such a way that the major reagent peak is comparable to the level of the impurity peaks and the minor reagent peaks remains negligible and do

Table 2
Precision of the method

Component	Run-to-run precision		Total precision	
	R.S.D. of peak areas (%)	R.S.D. of migration times (%)	R.S.D. of peak areas (%)	R.S.D. of migration times (%)
D(+)-glucosamine	2.8	0.12	3.3	0.13
D(+)-galactosamine	2.6	0.14	3.2	0.14
Gly–Gln	4.2	0.16	4.9	0.18
Phe	3.4	0.16	3.6	0.17
Ser	3.2	0.17	4.2	0.18
Unknown peak	2.5	0.17	4.1	0.19

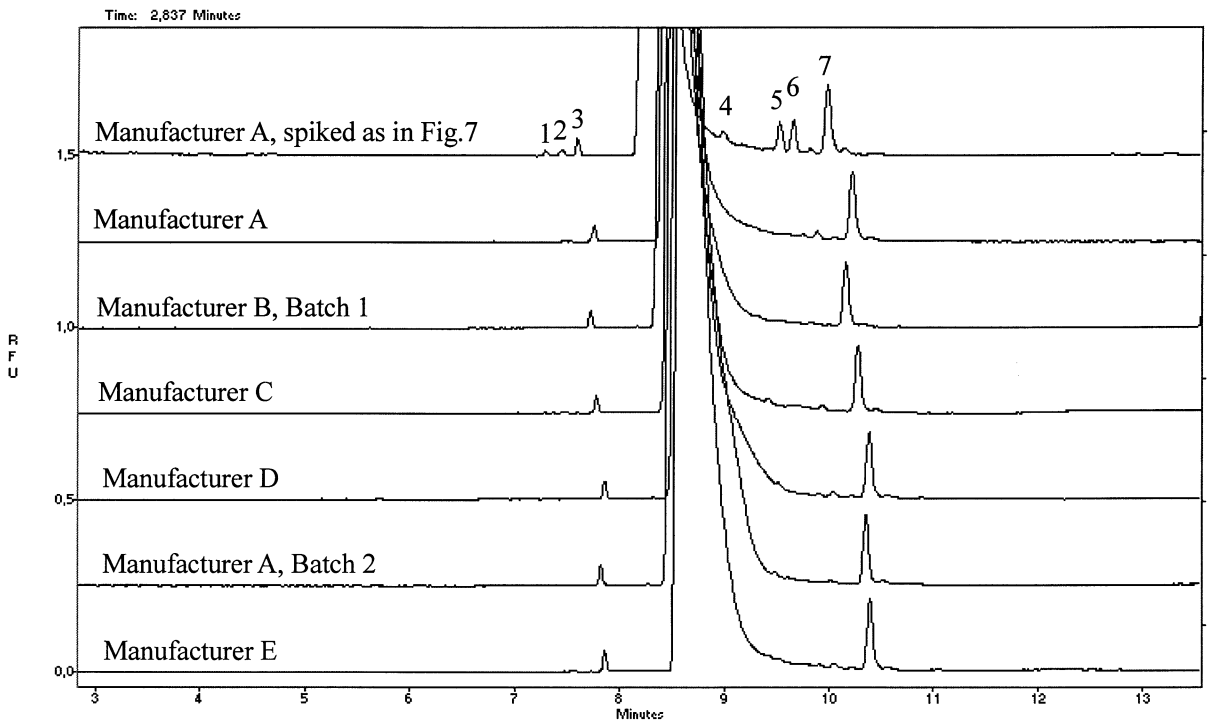

Fig. 7. Electropherograms of CBQCA derivatized samples Arg 0.45 mg/ml from different manufacturers and batches. Peak identity as in this figure. CE conditions as described in Fig. 1.

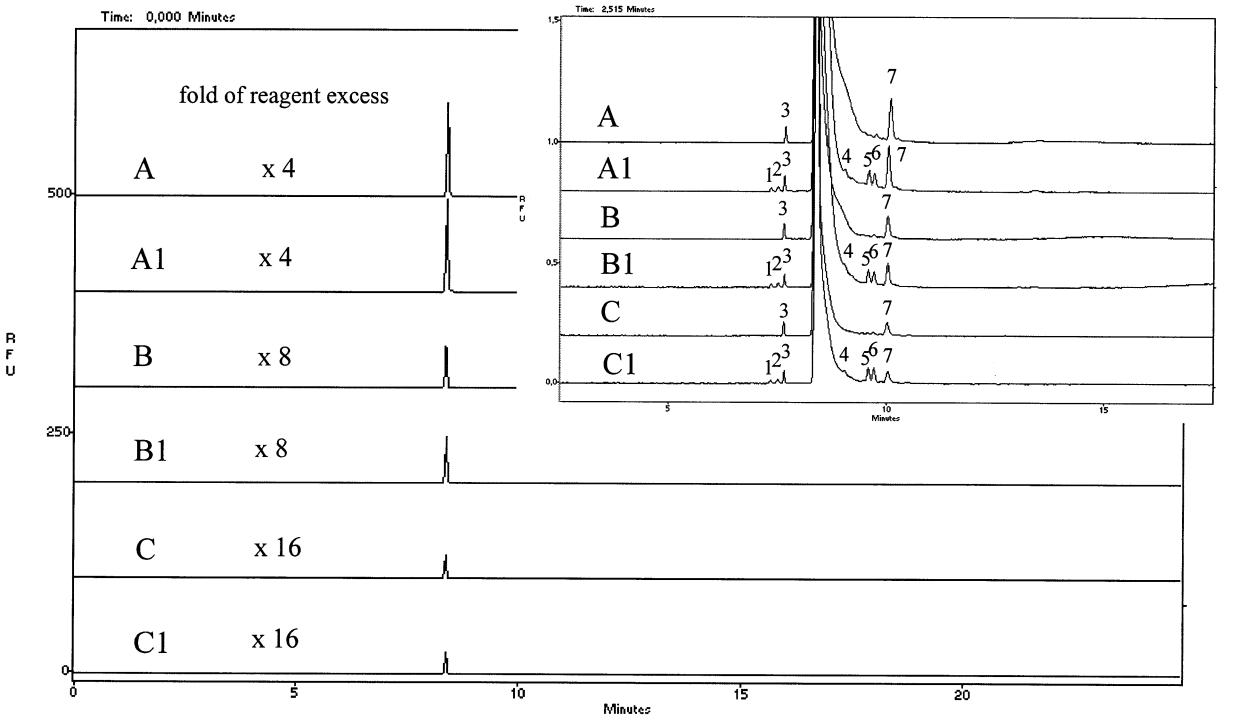

Fig. 8. Electropherograms of CBQCA derivatized samples: (A) Arg 0.45 mg/ml; A1, Agr 0.45 mg/ml, spiked with 'impurities' 0.00045 mg/ml each; (B) Arg 0.225 mg/ml; B1, Agr 0.225 mg/ml, spiked with 'impurities' 0.00045 mg/ml each; (C) Arg 0.1125 mg/ml; C1, Agr 0.1125 mg/ml, spiked with 'impurities' 0.00045 mg/ml each. CE conditions as described in Fig. 1. Peak identity as described in Fig. 6.

Table 3
Accuracy of the method expressed as recovery at three levels—50, 100 and 200% from the target

Component	Replicate	Recovery of expected content at level (%)			Confidence ($\alpha = 0.05$, $n = 9$)
		50	100	200	
D(+)-glucose	1	98.6	98.6	97.3	1.58
	2	101.2	103.2	98.8	
	3	102.6	102.7	103.6	
D(+)-galactose	1	100.4	97.4	104.5	2.12
	2	104.3	101.3	105.1	
	3	98.6	98.1	97.3	
Phe	1	104.2	105.6	103.3	2.36
	2	102.4	102.3	96.1	
	3	96.8	98.6	105.5	
Ser	1	99.3	101.9	100.6	2.06
	2	96.8	96.8	95.9	
	3	103.4	104.7	97.6	

respective not cover the potential impurity peaks at the level of interest. A respective sample concentration of 0.00045 mg/ml was found to be appropriate and was taken as '0.1% w/w'. The corresponding electropherograms are shown in Fig. 6. In turn, the '100% level' of this constituted as 0.1% concentration would be 0.45 mg/ml, which would correspond to a range of 6 mM for Gly and approximately 1.9 mM for $(CysS)_2$. This cover the order of required excesses for the reagent and the cyanide catalyst reported by Novotny et al. [14].

3.2. Detection

The excitation/emission spectra of the CBQCA derivatives of Gly and D(+)-galactosamine reported by Novotny et al. [14,15] exhibit maximums at 450/550 and 456/552 nm, respectively. For the on-column fluorescence detection an excitation by 442 nm were performed with helium/cadmium laser and the induced fluorescence was measured at 550 nm. Despite the higher deviation from both the excitation and emission maximum, in our experiments an argon ion laser was applied as excitation source at 488 nm and the fluorescence emission was measured at 520 nm. Compared with the reported 240 amol as minimum detectable amount for the derivatized galactosamine, with the LIF-configuration used here a limit of detection (LOD) of 36 amol was obtained (signal-to-noise ratio equal to 3) after derivatization of galactosamine at a concentration level of 2×10^{-7} M. It should be noted that in order to enhance the sensitivity the experiments were performed using a capillary with larger diameter (75 μm) and the sample was loaded by pressured injection by 3448 Pa for 5 s.

3.3. Method validation

The linearity of calibration was assessed using four standard mixtures of the test 'impurities'

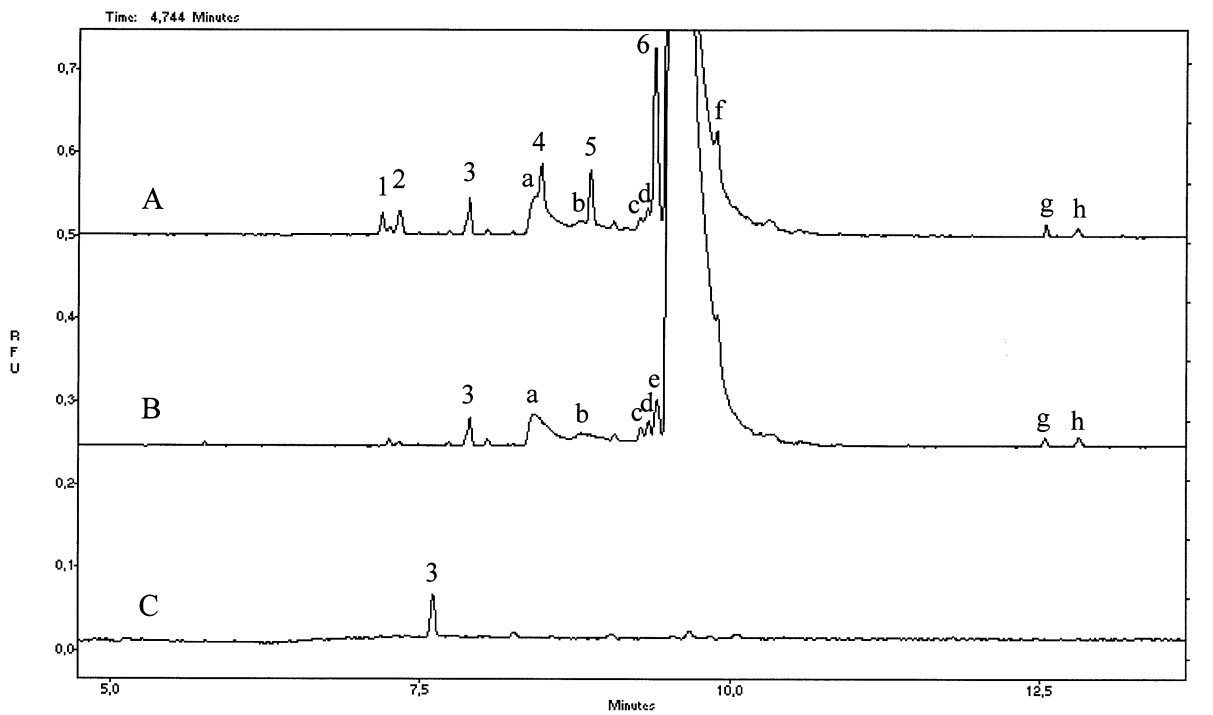

Fig. 9. Electropherograms of CBQCA derivatized samples: (A) Ser 0.45 mg/ml, spiked with 'impurities' each 0.1 %; (B) Arg 0.45 mg/ml; (C) blank. Peak identity: (1) D(+)-glucosamine; (2) D(+)-galactosamine; (3) major reagent peak; (4) Gly–Pro–Gly–Gly; (5) Gly–Gln; (6) Phe, a–h, unknown. CE conditions as described in Fig. 1.

covering the ranges of target concentration shown in Table 1. In individual experiments, samples of Ser and of Phe ranging from 0.00045 to 0.45 mg/ml were derivatized with CBQCA in order to study the labeling reaction over three orders of magnitude. For both amino acids correlation coefficients above 0.999 were obtained. The precision of the system was evaluated with six successive analyses of Arg samples spiked with D(+)-glucosamine, D(+)-galactosamine, Gly–Gln, Gly–Pro–Gly–Gly, Phe and Ser at a target level of 0.1%. Corresponding electropherograms are shown in Fig. 6. The peak of the Gly–Pro–Gly–Gly peptide was not separated from the Arg peak and for this reason not presented in Table 2, which is summarizing the results. The unknown peak 7 is definitely higher than 0.1%; this peak is present at the same level in other Arg batches of the same origin and in batches of other manufacturers (see Fig. 7).

It was examined whether peak 7 increases when the labeling reaction is carried out longer than 10 h but no significant increasing was observed. In order to find out whether peak 7 results from a real impurity or from a second reaction product, e.g. double derivatized Arg, labeling reactions with different reagent mol excesses were performed. The samples consisting of Arg 0.45 mg/ml (4-fold reagent mol excess), Arg 0.225 mg/ml (8-fold reagent mol excess) and Arg 0.1125 mg/ml (16-fold reagent mol excess), each unspiked and spiked with 'impurities' 0.00045 mg/ml, respectively, were derivatized according to the procedure, described above. The corresponding electropherograms are presented in Fig. 8. In all cases, independent of the reagent excess, the corrected area of peak 7 is found to be 0.2% of the corrected area of the Arg peak. Thus, peak 7 is likely to be a real impurity. It should be mentioned that such a peak at same level was not found when the Arg samples were derivatized with FMOC and analyzed as previously described [6]; however, it may be possible that the peak remains masked (not resolved from Arg and FMOC peaks) or the corresponding substance was not labeled (data not shown).

Recovery studies were performed in order to assess the accuracy of the analytical system. Arg was spiked with D(+)-glucosamine, D(+)-galactosamine, Phe and Ser at three levels: 50, 100 and 200% of the target concentration of 0.00045 mg/ml, e.g. 'impurities' of 0.05, 0.1 and 0.2%. At each level three analyses were carried out. The results as confidence intervals are presented in Table 3. Linearity, precision and accuracy of the method were found to be sufficient for quantitative evaluation of purity of Arg at level 0.1%. The same analytical system was applied to estimate the impurity profiles of other amino acids. The corresponding electropherograms for Ser are shown in Fig. 9.

4. Conclusions

CBQCA has been proven to be an appropriate labeling reagent for the evaluation of impurities of biotechnologically produced amino acids. Amino sugars, low molecular weight peptides and other amino acids could be determined in amino acids as impurities at a 0.1% level. The validation aspects of the analytical system have been estimated with model mixtures, consisting of Arg spiked with representative 'impurities'. The results obtained from the studies of linearity, precision and accuracy of method are acceptable with regard to quantification criterion and demonstrate the applicability of system to quantitative studies of impurity profiles of amino acids. In comparison to the recently reported method utilizing derivatization of amino acids with FMOC and UV detection, the method reported in this paper show a broader spectrum of detectable substances. Both analytical systems have an equal sensitivity with respect to their possibility to evaluate impurities at 0.1% level and for this reason could be applied mutually in amino acids analysis. With respect to the higher sensitivity of LIF-detection mode should be mentioned that the concentrations of the samples analyzed after labeling with CBQCA were 8-fold lower compared with the concentrations required for UV-detection after derivatization with FMOC.

In our future work, the impurity profile of other biotechnologically produced amino acids will be studied. In addition, other potential group

impurities such as nucleic acids and carbohydrates are also object of our interest.

Acknowledgements

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