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Evaluation of the impurity profile of amino acids by means of CE

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Abstract

The aim of this study was to develop a general approach for characterizing the impurity profile of amino acids at level 0.1% by means of capillary electrophoresis (CE). Checking a variety of labeling reagents revealed 9-fluorenylmethyl chloroformate to be favorable, due to the high stability of its derivatives and the fact that the reagent peaks do not interfere with the peaks of the impurities. After optimization, the method was sufficiently sensitive to evaluate impurities at a 0.1% level by UV detection. The method was representatively validated for phenylalanine (Phe) with regard to selectivity, precision, linearity and accuracy using model mixtures of potential impurities. The CE analyzes method was applied to Phe samples of different manufacturers and the capabilities of the strategy was also demonstrated by samples of tryptophan and serine. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Amino acid impurities; Capillary electrophoresis; 9-Fluorenylmethyl chloroformate-derivatization

1. Introduction

The technique of capillary electrophoresis (CE) will shortly be introduced to most of the international Pharmacopoeiae. Whereas, the CE is well established in the chiral analysis since a couple of years [1–11], there is still an ongoing discussion on the use of CE in the quantification of impurities described in the related substances section of drug monographs [12–14]. General strategies for method development inclusive sample pretreatment and method validation are collected, catalogued and considered by Wätzig et al. [15]. Due to the lack of capillary sample capacity and the short path length of the detection window, the UV-detection of impurities of low concentration (0.1%) remains still a challenge in CE. On the other hand, CE has a very high resolving power and is, additionally, an inexpensive and rapid method with environmental benefits. Thus, it is worth to check whether reliable impurity tests can be developed. Some examples were already accepted by official authorities, e.g. the stability and content uniformity of a cholesterol-lowering agent, BMS-188494, checked by micellar electrokinetic chromatography (MEKC) have been accepted as part of a regulatory submission to the

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Federal Drug Agency (FDA) [16]. A MEKC method was utilized and fully validated in order to determine ibuprofen, codeine phosphate, and their degradation products and impurities for submission to the regulatory authorities [17]. Capillary zone electrophoresis was used in order to quantify mitrazapine and five structurally related substances in tablets [18]. A CE method has been worked out and validated for quantitative determination of histamine acid phosphate and/or benzalkonium chloride [19]. This method is in routine use within the laboratories and will be submitted to the British Pharmacopoeia. Additional examples for applications of CE in drug analysis are given by Nishi [20].

Since a couple of years amino acids are produced by biotechnological processes and, thus, consist of other amino acids and corresponding substances as impurities. Till today, all amino acid monographs in the European Pharmacopoeia describe a TLC method which is able to limit the impurities detectable by ninhydrin to 0.5 percent. Only in the cases of acetylcysteine, *N*-acetyltryptophan and tryptophan (Trp) HPLC methods with direct UV-detection at 220 nm are used to quantify the related substances. CE has been applied to separate and determine *N*-acetylcystein and related impurities below 0.1% [21,22] and trace enantiomeric impurities of Trp [3,23].

The 'Trp affair' occurring in the late eightieth of the last century unraveled the entire problem of the switch of production methods from chemical synthesis to fermentation processes. In 1989, the first notification linking the eosinophilia–myalgia syndrome with the use of Trp-containing products was made in USA. A number of case reports from USA, Europe, and Japan prompted systematic investigations by the Center for Disease Control, FDA, and other laboratories to elucidate the nature and mechanism of the pathology. Many trace contaminants associated with the syndrome [24] have been found in batches of Trp originated from a single manufacturer in Japan [25–27]. One contaminant 'peak E' has been identified as 1,1-ethylidenebis(tryptophan) [28] and a second identified impurity associated with eosinophilia-myalgia syndrome, 'peak UV-5', [29] as 3-phenylamino-l-alanin [30,31].

The aim of this study on amino acids produced by fermentation was to show the applicability of CE to the evaluation of the impurity at level 0.1%. Four amino acids phenylalanine (Phe), serine (Ser), and Trp were representatively studied in the first step.

2. Experimental

2.1. Instrumentation

The experiments were performed using a Beckman P/ACE system MDQ (Fullerton, CA), equipped with UV detector used at 254 nm or a LIF detector with an excitation by argon ion laser at a wavelength of 488 nm and an emission wavelength of 520 nm. The fused-silica capillaries purchased from Beckman (Fullerton) were of 50, 75 or 100 μm internal diameter and an effective length of 50, 60 and 70 cm (respectively, a total length of 60, 70 and 80 cm). Samples were loaded by pressured injection at 3448–6895 Pa (0.5–1.0 p.s.i.) for 5–10 s. The electrophoresis were carried out at 25 °C and a voltage between 12 and 25 kV. The capillaries were 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

Unless otherwise stated, all chemicals used were of analytical-reagent grade. The labeling reagents 9-fluorenylmethyl chloroformate (FMOC), fluorescein isothiocyanate Isomer I (FITC) and 4-fluoro-nitro-2,1,3-benzoxadiazole (NBD-F) were purchased from Sigma (St. Louis, MO), l-amino acids used for the method development, 6-aminocaproic acid (6-ACA) and sodium dodecyl sulfate (SDS) from Fluka (Buchs, Switzerland). A number of amino acids produced by fermentation from several manufacturers were used to demonstrate the usefulness of method in its practical aspects.

2.3. Buffers and reagents

Buffers were daily prepared in high purity water generated with a Milli-Q Water System (Millipore, Bedford, MA) and filtered through 0.22 μm membrane filter (Roth, Karlsruhe, Germany). The separation buffer was a borate buffer (pH 9.0–9.5, 20 mM), containing 25 mM SDS. All samples and model mixtures (main component; main component plus 'impurities' at level 0.05–0.15% and/or internal standard (IS); 'impurities' at level 0.05–0.15% with/without IS) were dissolved in boric acid 0.4 M–sodium hydroxide, pH 9.3 buffer. FMOC was dissolved in acetonitrile as a 150–350 mM solution.

2.4. Derivatization procedure

The samples were derivatized with FMOC by a procedure previously described by Chan et al. [32] and adapted: to 1.2 ml sample solution 0.8 ml FMOC solution in molar ratio FMOC/main component from 2 to 15 were added. After gentle shaking, the reaction was allowed to stand for 2 min. The excess FMOC and its hydrolyzed products were extracted with pentane. The aqueous portion was diluted to the appropriate concentration.

With FITC and NBD-F, the samples were derivatized at different concentration levels as described by Takizawa et al. [33] and by Hu and Li [34], respectively.

3. Results and discussion

3.1. Method development

3.1.1. Derivatization

With exception of the three aromatic amino

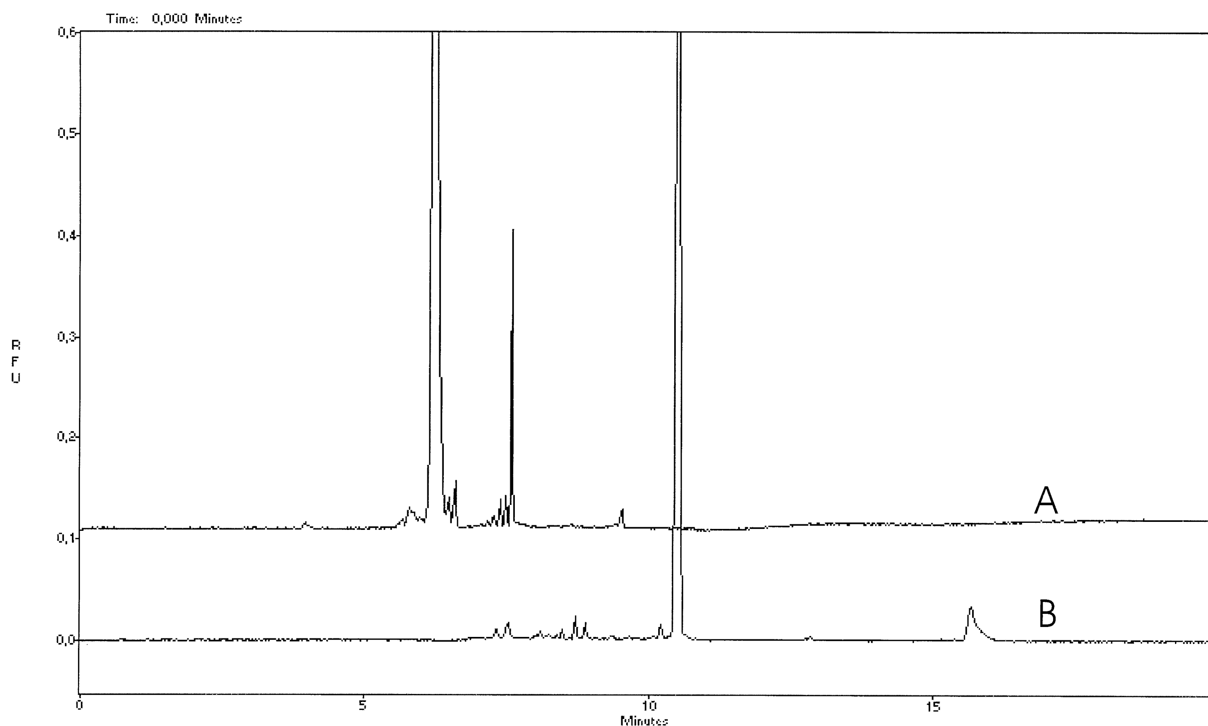

Fig. 1. Electropherograms of blank samples (A) FITC 2.5×10^{-7} M and (B) NBD-F 5×10^{-4} M. CE: running buffer, borate (pH 9.3, 20 mM) containing 25 mM SDS; Capillary: fused-silica 60.2/50 cm, 50 μm i.d. (75 μm for NBD-F); Voltage, 20 kV; Temperature, 25 °C; Injection, 3448 Pa, 5 s; Detection LIF 488/520 nm.

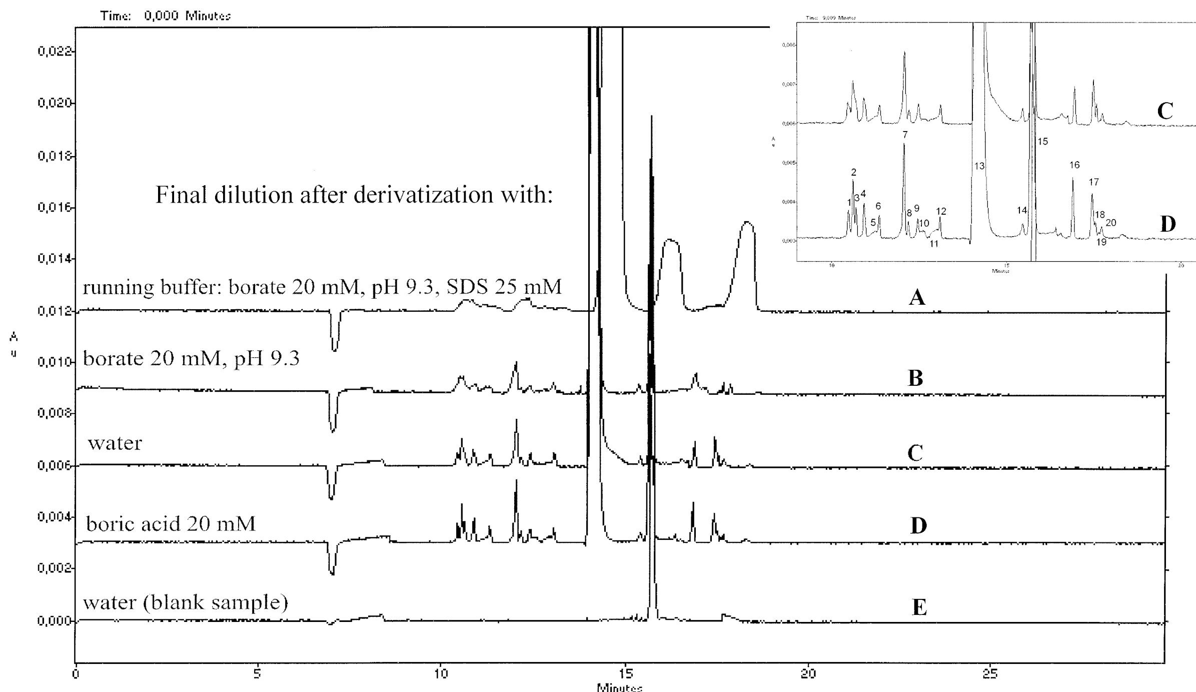

Fig. 2. Electropherograms after final sample dilutions with different solvents: (A) running buffer, (B) borate buffer (pH 9.3, 25 mM), (C) water, (D) boric acid (20 mM). Sample: Phe 0.175 mg/ml spiked with amino acids 0.1% individual. CE: running buffer, borate (pH 9.3, 25 mM) containing 25 mM SDS; Capillary: fused-silica 70/60 cm, 75 µm i.d.; Voltage, 15 kV; Temperature, 25 °C; Injection, 6895 Pa, 10 s; Detection UV-254 nm. Peak identity: 1, Ser; 2, Thr, Asn, Gln; 3, Ala; 4, Gly; 5, Pro; 6, Val; 7, 6-ACA (IS); 8, Met; 9, Ile; 10, Glu; 11, Asp; 12, Leu; 13, Phe; 14, Trp; 15, FMOC excess; 16, $(Cys\text{-}S)_2$; 17, reaction degradation product; 18, $(Cys\text{-}S)_2$; 19, Arg; 20, His, Lys.

acids, Trp, Phe and Tyr, the direct UV-detection without sample labeling as performed in [3,21–23], was not successful because most of the amino acids and corresponding impurities are lacking of groups causing a strong UV absorption. Thus, a derivatization is necessary to increase the sensitivity and to make a detection possible covering three orders of magnitude without excessive sample overloading.

A suitable reagent for the labeling the impurities of low quantity has to fulfil several requirements. First, the labeling reagent must have either a strong UV absorption or a strong fluorescence. Second, the reagent has to be available in a high degree of purity or–as a compromise–should have only few impurities and by-products from the derivatization reaction of low amounts, both good separable from the labeled analytes. The purity of the labeling reagent is of high importance because an excess of the reagent ten times of the main component concentration is a ten times excess in comparison to the amount of impurities at level 0.1%. Thus, the impurities of the amino acid tested and the impurities of the labeling reagent are of comparable amount, or even worse, the peaks of interest are smaller than the ones of the reagent. Moreover, when all reagent peaks cover large portions of the baseline, the recognition of the peaks of interest is extremely difficult or impossible. Third, the labeling reagent should be stable and should give a rapid reaction in quantitative yields at low temperatures. Fourth, the reaction products have to be sufficiently stable.

In initial experiments, three often used reagents for derivatization of primary and secondary

amines were tested: the fluorescein compound FITC and benzoxadiazole derivative NBD-F, both combined with LIF-detection ($\lambda_{ex/em} = 488/520$ nm), and the fluorenyl compound FMOC with UV-detection ($\lambda = 254$ nm). Although FITC and NBD-F provides a very high detection sensitivity for amino acids when excited by an argon ion laser at 488 nm, both are not suitable for the characterization of the impurities. Due to many additional peaks originating from the reagents, the evaluation of impurity profile of the amino acids was impossible. Neither a purification of the reagent by means of re-crystallization nor the modification of the separation conditions led to satisfying separations of the impurities of the amino acid tested and the impurities of the reagent. The typical electropherograms of blank samples of FITC and NBD-F displayed in Fig. 1 clearly demonstrate the problem.

FMOC was successfully applied as labeling reagent for UV-detection in chiral separation of amino acids [35–37]. It should be noted that FMOC was also used for fluorescence detection of labeled amines in order to improve the sensitivity [32,38]. Although the UV detection is less sensitive in comparison to the LIF-detection, FMOC in combination with absorption measurements at 254 nm was found to be favorable for the quantification of the impurities after a working-up procedure. The excess of FMOC and its hydrolyzed products could be extracted from a reaction solution of the derivatized samples with pentane. The small amounts of the remaining labeling reagent could be separated from the substances of interest in the electropherograms.

3.1.2. Separation method

MEKC first proposed by Terabe et al. [39] has been proved to be a good separation technique for native and derivatized amino acids and was applied in this study. Using this separation mode, a surfactant was added to the electrolyte at a concentration exceeding the critical micellar concentration. The separation of the solutes occurred

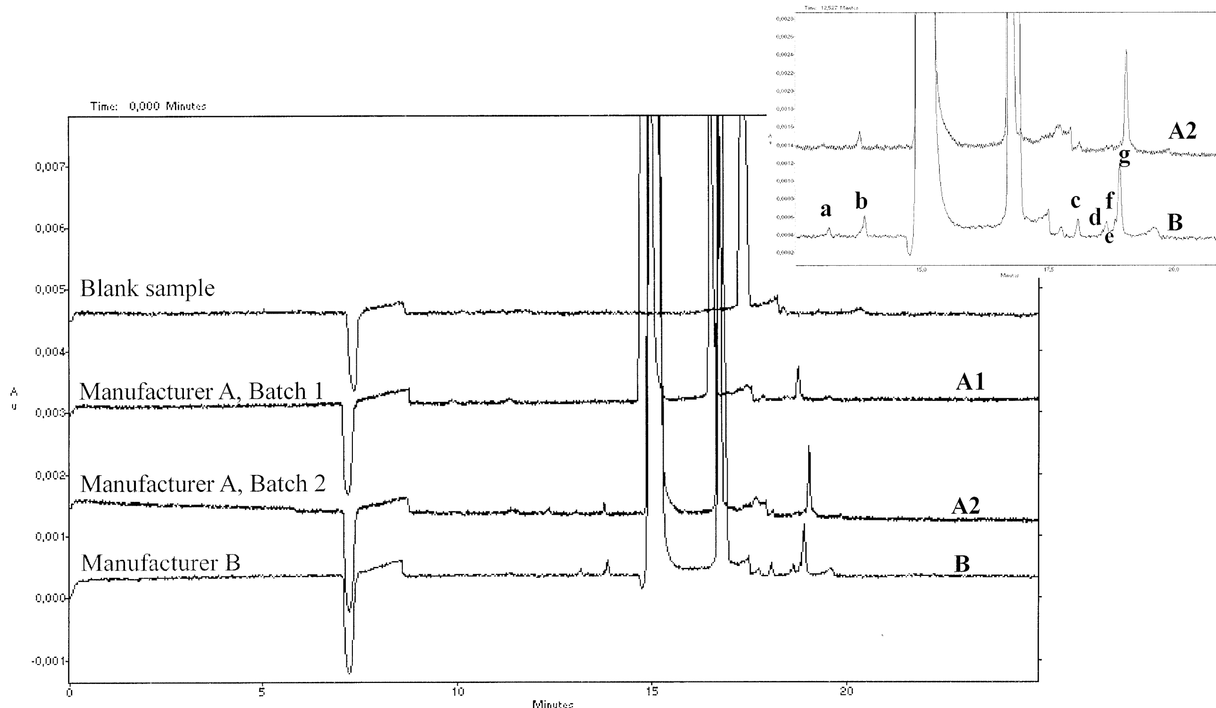

Fig. 3. Electropherograms of three samples Phe 0.175 mg/ml from two manufacturers. CE conditions as in Fig. 2.

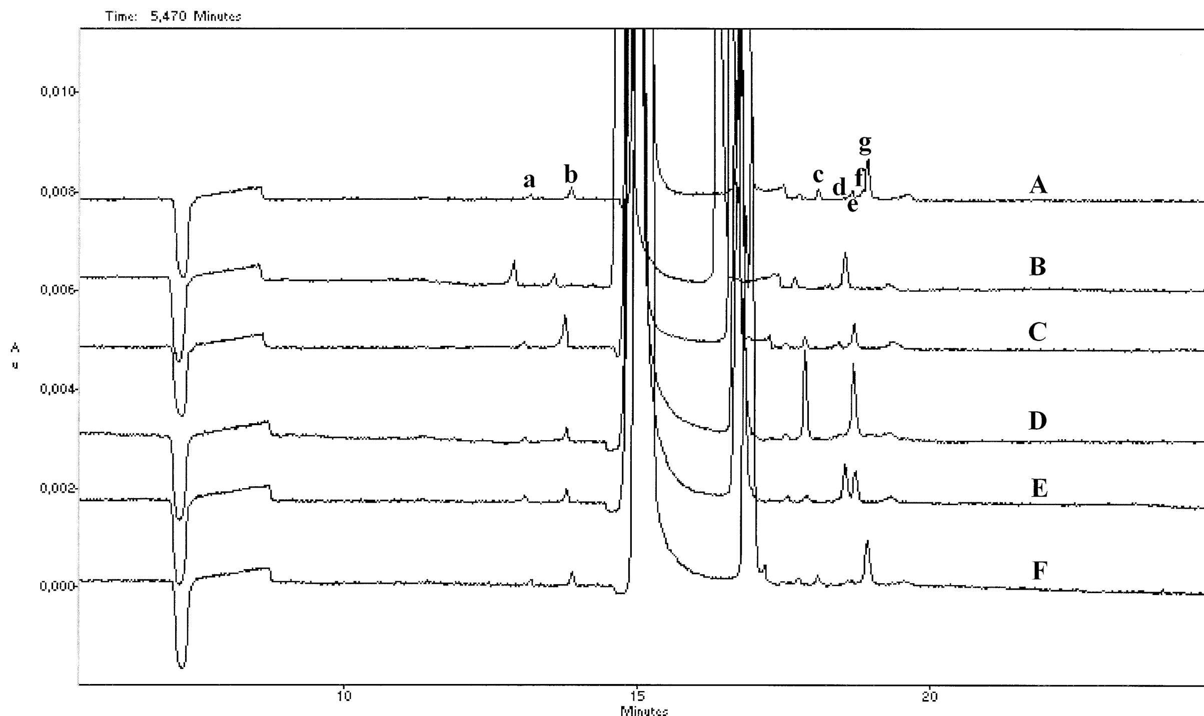

Fig. 4. Electropherograms of Phe (Manufacturer B) 0.175 mg/ml: (A) not spiked, (B) spiked with Ile 0.1%, (C) spiked with Leu 0.1%, (D) spiked with $(Cys\text{-}S)_2$ 0.1%, (E) spiked with Agr 0.1%, (F) spiked with Lys 0.1%. CE conditions as in Fig. 2.

Table 1
Precision of the method

Component	Run-to-run precision		Total precision	
	RSD of ratio (peak area/IS peak area) (%)	RSD of ratio (migr. time/IS migr. time) (%)	RSD of ratio (peak area/IS peak area) (%)	RSD of ratio (migr. time/IS migr. time) (%)
Leu	3.0	0.12	3.6	0.14
Ile	3.1	0.11	3.4	0.13
Val	2.6	0.09	3.3	0.12

on the basis of their electrophoretic behavior and partition processes between the aqueous buffer and the micelles. The most popular electrolyte used in amino acid analyzes is the borate buffer, pH 9.0–9.5 with addition of SDS as the micelle forming compound. The separation conditions reported by Chan et al. [32] were used for further optimization. The pH value and the voltage have to be varied. For each of these parameters, a series of experiments were performed to study their effect on the separation of the analytes. The optimum separation was obtained at pH 9.3 and a voltage of 15 kV.

3.1.3. Enhancement of the sensitivity

In order to achieve the sensitivity of the method over a concentration range of three orders of magnitude, the following conditions were varied: decrease of the final sample dilution, decrease of the FMOC/main component molar ratio for the

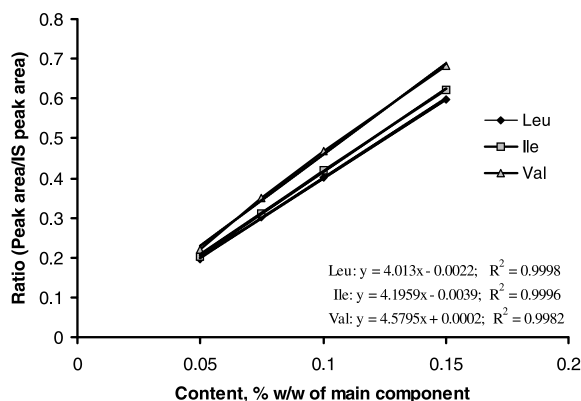

Fig. 5. Linearity of response for Leu, Ile and Val as plots of the ratio of the peak area to the area of IS peak versus the content in % w/w of main component.

labeling reaction, increase of the capillary diameter, and sample 'stacking' after loading a high sample volume.

First, after derivatization the sample has to be diluted with water by a factor from 2 to 20. Dilutions lower than 10 cannot be used because the otherwise obtained high concentrations of acetonitrile in sample zone resulted in a low separation efficiency. Second, a series of derivatization with decreasing FMOC/main component molar ratio from 15 to 2 was performed with model mixtures consisting of Phe spiked with 0.1% of each amino acids as 'impurities'. At ratios lower than 5, the peak areas of the 'impurities' were irreproducible compared to the results obtained with higher ratios. Thus, a ratio of 10 was applied in the further experiments. Third, increasing the capillary diameter enhanced the injected sample volume and, along with that, the length of the light pathway in the capillary at the detector window. Increasing the diameter from 50 to 75 μm resulted in an approximately three times increased peak area without considerably affecting the separation efficiency. Fourth, an enhancement of the signal area by increasing sample volume and without diminishing the separation efficiency is possible by sample 'stacking'. After derivatization, the samples were diluted with different solvents and loaded by pressure injection at 3448–6895 Pa for 5–10 s. Electropherograms obtained after sample injection at 6895 Pa for 10 s are shown in Fig. 2. Only the sample diluted with boric acid solution remained the separation efficiency unaffected. An enhancement of the peak areas of 'impurities' by approximately a factor of 3.5 was obtained with a fourfold increase of the injection volume (the pressure from 3448 to 6895 Pa and the duration from 5 to 10 s).

In order to compensate injection errors, dilution errors, errors occurring at sample pretreatment and migration time variations, an IS is required which should be similar to the structure of the analytes, i.e. with at least one amino and one acidic group. In addition, the mobility of the IS should be similar but not identical to the substances of interest. 6-ACA was found to fulfil all requirements. The corresponding electropherogram is depicted in Fig. 2(D) (peak 7).

Table 2
Accuracy of the method expressed as recovery at three levels 75, 100, 125% from the expected level of 0.1% w/w

Component	Replicate	Recovery of theoretical content at level (%)			Confidence ($\alpha = 0.05$, $n = 9$)
		75	100	125	
Leu	1	104.2	97.4	103.4	2.73
	2	98.7	104.6	98.2	
	3	103.1	102.3	101.6	
Ile	1	94.6	98.3	98.2	3.1
	2	104.1	103.5	102.4	
	3	102.0	95.1	100.1	
Val	1	97.1	104.6	100.4	3.5
	2	97.3	99.3	93.1	
	3	103.1	103.1	97.6	

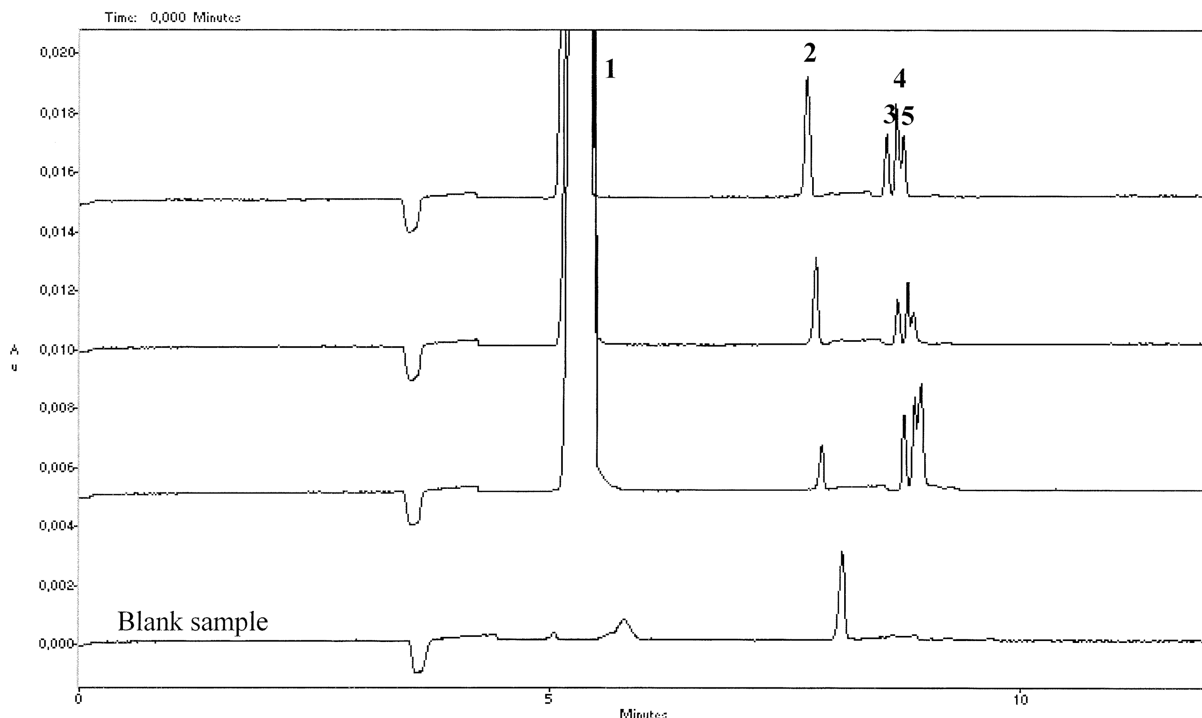

Fig. 6. Electropherograms of Ser 0.175 mg/ml from three manufacturers. CE: running buffer, borate (pH 9.3, 25 mM) containing 25 mM SDS; Capillary: fused-silica 60.2/50 cm, 75 μm i.d.; Voltage, 20 kV; Temperature, 25 °C; Injection, 6895 Pa, 10 s; Detection UV-254 nm. Peak identity: 1, Ser; 2, FMOC excess; 3, reaction degradation product; 4–5, unidentified impurities.

3.1.4. Application to samples of two manufactures

After assignment of each peak of the electropherogram by spiking the sample with the individual amino acids, the method was applied to three batches of Phe from two manufacturers A and B. The electropherograms are presented in Fig. 3. Six impurity peaks a, b, c, d, e, and g could be detected in batches of Phe from both manufacturers. Since the migration times of Val, Met, Glu, Ile, Asp, Leu were similar to the migration times of substances corresponding to peaks a and b and the migration times of Cys, $(CysS)_2$, Arg, Lys and His were similar to the migration times of substances corresponding to peaks b, c, d, f and g, a sample from manufacturer B were either spiked with these amino acids in order to assign the impurity peaks a–g. The results are shown in Fig. 4. The amino acids Ile and Leu were found to cover peaks a and b, $(CysS)_2$-peaks c and f or g, Arg-peak d or e or f, Lys-peak f or g. Thus, it is likely that the impurities observed in the batches are of amino acid origin. Even though an independent analytical method is necessary to make a final decision about the assignment, the example clearly shows the suitability of the method with regard to the characterization of the impurity profile.

The total impurity content of Phe e.g., from manufacturer B could be estimated to be below 0.5% (w/w) and the contents of Ile and Leu, corresponding to peaks a and b, were found to be below 0.03 and 0.05% (w/w), respectively. However, in the following step, the methods have to be validated for Phe.

3.2. Method validation

The selectivity of the method was demonstrated by a sample of Phe spiked with the other eighteen amino acids. As can be seen in the electrophero-

gram D in Fig. 2, Phe is well separated from all other added amino acids and the IS. At concentration levels higher than 0.00004 mg/ml all these eighteen amino acids are detectable as impurities in Phe. This concentration was found to be the limit of quantification (ratio signal/noise above 10) for the amino acids with low response factors.

In order to evaluate the precision, the accuracy and the linearity of method samples of Phe from manufacturer A, Batch 1, free from impurities before main peak (Fig. 3) was spiked with Val, Ile, Leu at levels ranging from 0.05 to 0.15% (w/w) of main component as representative potential impurities. 6-ACA as IS was also added at a concentration of 0.5% (w/w) of main component. Run-to-run precision of migration time and peak area for 'impurity' peaks at level 0.1% expressed as relative standard deviation (RSD) of six replicate injections are depicted in Table 1.

In a series of six separate labeling experiments, a total precision of the method, including a labeling reproducibility, was studied to assess all variations across an experiment. The results expressed as RSD of migration time and peak area for the three representative potential 'impurities' at level 0.1% are also depicted in Table 1.

The linearity was studied at four concentration levels for Leu, Ile, Val in a range from 0.05 to 0.15%. Plots of the ratio of the peak area to the area of IS peak versus the different concentrations for all these amino acids are presented in Fig. 5.

The accuracy of method for representative 'impurities' was assessed as percent recovery with the confidence intervals at three levels in three replicates each. A sample of Phe from manufacturer A, Batch 1 spiked with Leu, Ile and Val each 0.1% (w/w) was used as 100% level to calculate the recovery at levels 75, 100, and 125%. The results are presented in Table 2. Precision, linearity and accuracy of the method appear adequate for quantification of impurities in Phe at level 0.1% (w/w).

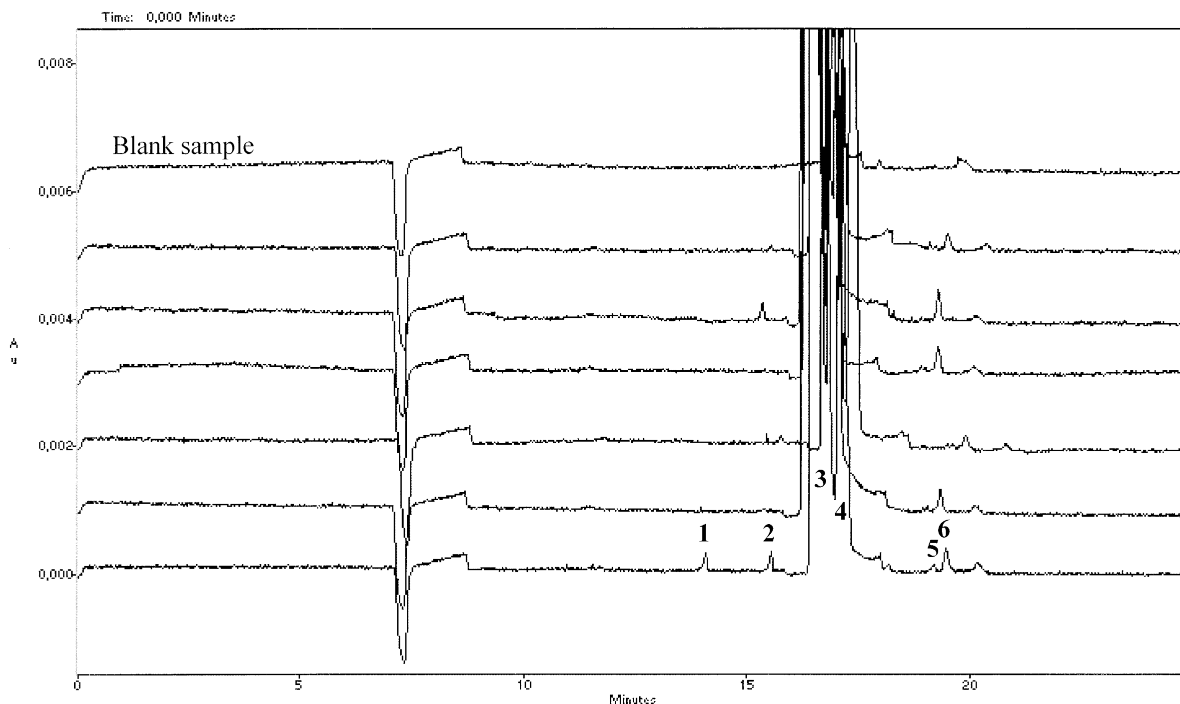

Fig. 7. Electropherograms of Trp 0.175 mg/ml from five manufacturers. CE as in Fig. 2. Peak identity: 1, 2, 5, 6, unidentified impurities; 3, Trp; 4, FMOC excess.

Typical electropherograms of Ser and Trp from several manufacturers derivatized with FMOC are presented in Figs. 6 and 7.

4. Conclusions

An evaluation of the impurity profile of amino acids by means of CE has been presented. A method for quantification of other amino acids and amino containing substances at level 0.1% in Phe after labeling with FMOC and UV-detection by 254 nm has been validated for representative potential impurities regarding selectivity, precision, linearity and accuracy. The validation data are acceptable which makes the method suitable for purity control of Phe.

In future work, the impurity profile of other biotechnologically produced amino acids will be studied. In addition, other potential group impurities such as nucleic acids, carbohydrates and peptides are also object of our interest.

Acknowledgements

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