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Strategies for in-capillary derivatization of amino acids in capillary electrophoresis using 1,2-naphthoquinone-4-sulfonate as a labeling reagent

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Abstract

This paper examines the potentiality of in-capillary derivatization for improving the sensitivity of the spectrophotometric detection of amino acids in capillary zone electrophoresis. 1,2-Naphthoquinone-4-sulfonate was selected as the labeling agent of amino acids. The underivatized sample and the reagent solution segments are injected by pressure into the capillary prior to applying the running voltage. The corresponding derivatization reaction occurs inside the capillary once the potential is applied, as it induces mixing of the sample with the reagent. Several introduction modes consisting of tandem or sandwich configuration have been evaluated. These techniques result in a straightforward and automated way of carrying out a derivatization. Furthermore, in-capillary procedures may become much more attractive than conventional pre-capillary derivatization in terms of sensitivity and reproducibility. The optimum operation mode found consists of a sandwich system where the sample is injected in between two reagent segments. The method was applied to the determination of amino acids in feed samples. Results show a good concordance with those given by a standard amino acid analyzer. © 2001 Published by Elsevier Science B.V.

Keywords: Derivatization, electrophoresis; In-capillary derivatization; Injection methods; Sandwich injection; Food analysis; Amino acids; Naphthoquinonesulfonate

1. Introduction

Amino acids are generally determined using derivatization procedures to increase the sensitivity of the detection. Apart from more conventional procedures involving pre- or post-capillary [1–3] derivatization, new approaches based on in-capillary derivatization are investigated [3]. In-capillary derivatization consists of developing the reaction inside the capillary by promoting the mixing of analytes with the reagent. A remarkable feature of this technique is the small reaction chamber volume, which means that the sample consumption and dilution are minimal.

In-capillary derivatization can be accomplished by different strategies which may be classified into three groups: zone-passing, at-inlet, and throughout capillary derivatization.

Zone-passing derivatization is carried out by applying the separation potential just after the intro-

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duction of the reagent solution and the sample solution [4]. This strategy is appropriate for fast kinetic processes where high reaction yields are achieved in a few seconds. The zone-passing procedure has shown itself to be suitable for capillary zone electrophoresis (CZE) [5] and micellar electrokinetic chromatography (MEKC) [6]. Tirvesten and Folestad proved the potentiality of the combination of in-capillary derivatization with stacking by introducing a running buffer after the injection of the sample and reagent [6]. Taga et al. [4] tried a sample/reagent introduction mode, injecting a running buffer between the reagent and the sample plugs.

At-inlet derivatization comprises those techniques that let the sample and reagent segment stand for specified times to develop the reaction before applying the running voltage. This procedure is especially suitable for slow kinetic reactions which take several minutes to develop completely. During this period, the two zones are mixed by diffusion so that they start to react. The resulting derivatives are immediately determined by applying a running voltage. In this technique, various sample/reagent introduction modes can be used. For instance, Taga and Honda compared tandem and sandwich introduction modes in at-inlet derivatization [7].

In throughout-capillary derivatization, the capillary is filled with a running buffer which contains the reagent. When the separation potential is applied the components of the sample migrate and mix the reagent, and the corresponding reactions occur [8]. This technique has been utilized by Regnier and co-workers [9,10] to study the kinetics of some enzymatic reactions. Oguri et al. applied throughout derivatization to the determination of amino acids [11] and amines [12]. A good comparison of all derivatization modes discussed above has been reported elsewhere [8].

The derivatization of amines and amino acids following in-capillary techniques has been reported using *o*-phthaldialdehyde (OPA) [4,7,8,10–12] and naphthalene-2,3-dicarboxaldehyde (NDA) [13]. In a previous paper, a CZE method for the determination of amino acids based on pre-capillary derivatization with 1,2-naphthoquinone-4-sulfonate (NQS) was proposed [14]. NQS was applied to the quantification of amines and amino acids analysis using flow injection analysis [15,16], stopped-flow techniques [16] and liquid chromatography [17,18]. NQS reacts with primary and secondary amino groups and aromatic primary amino groups to yield the corresponding 4-dialquilamino, 4-alquilamino and 4-arylamino derivatives, respectively (see scheme in Fig. 1). These compounds are detectable spectrophotometrically. In general, the reaction is developed in basic medium under mild experimental conditions.

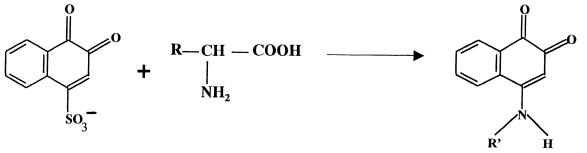

Fig. 1. Scheme of the derivatization reaction between NQS and amino acids.

Here, the possibilities of NQS for in-capillary derivatization are examined. The reactivity of NQS may be suitable for in-capillary derivatization as high yields of amino acid derivatives can be obtained inside the capillary. This study is also aimed at comparing different in-capillary reaction strategies and sample/reagent introduction modes. Furthermore, the performance of in-capillary derivatization for quantifying in complex matrix samples is evaluated from examples dealing with the determination of amino acids in feed samples.

2. Experimental

2.1. Reagents and solutions

All solutions were prepared with Milli-Q (Millipore, Milford, MA, USA) water. Sodium tetraborate (analytical grade, Carlo Erba, Milan, Italy) and isopropanol (HPLC grade, Panreac, Barcelona, Spain) were used for preparing the running buffer which consists of 40 m*M* aqueous sodium tetraborate aqueous solution–isopropanol (4:1, v/v). NQS (analytical grade, Carlo Erba) and 37% (w/w) hydrochloric acid solution (analytical grade, Merck) were used to prepare the reagent solution composed of 0.07 *M* NQS in 0.1 *M* HCl. Amino acids were purchased from Merck (all analytical grade). A $2{\cdot}10^{-3}$ *M* norleucine solution (from Sigma, analytical grade)

was used as internal standard in the autoanalyzer standard method for amino acid determination. Feed samples for animal nutrition were kindly provided by Cooperativa Agropecuaria de Guissona, Lleida, Spain.

2.2. Apparatus

A P/ACE Beckman capillary electropherograph with a diode array spectrophotometric detector was used. The multi-channel monitoring of amino acid derivatives was performed at 230, 305, 360 and 480 nm. Electropherograms were processed with a compatible computer using the Beckman P/ACE station (version 1.0) software. Fused-silica capillaries (supplied by Tecknokroma) of 67 cm (effective length 58.7 cm)×75 μm I.D.×375 μm O.D. were used. A Pharmacia LKB autoanalyzer (Model Alpha Plus, series 2) was used for the standard determination of amino acids.

2.3. Zone passing in-capillary derivatization

Amino acid derivatives were obtained by zone passing in-capillary derivatization with sandwich introduction mode as follows: (i) $7 \cdot 10^{-2}$ *M* NQS solution injected hydrodynamically at 0.5 p.s.i. for 10 s, (ii) hydrodynamic sample injection at 0.5 p.s.i. for 10 s and, finally, (iii) $7 \cdot 10^{-2}$ NQS *M* injected hydrodynamically for 10 s (1 p.s.i.=6894.76 Pa). Subsequently, the separation voltage was applied and the reagent thus mixed with the amino acids. This reaction occurs through the amino group to yield the corresponding derivatives which are detectable spectrophotometrically (see Fig. 1).

2.4. Capillary electrophoresis conditions

The separation of amino acid derivatives was performed at 20 kV and 25°C using a running buffer consisting of 40 m*M* aqueous sodium tetraborate solution (pH 9.2)–isopropanol (4:1, v/v). A forward pressure of 5 p.s.i. was applied 35 min after injection in order to accelerate the migration of the acidic amino acids (glutamic acid, aspartic acid and cysteine) of samples.

All new capillaries were activated with 0.1 *M* NaOH solution for 20 min. Subsequently, the capillaries were rinsed hydrodynamically with water at 20 p.s.i. for 5 min and conditioned with the running buffer solution at 20 kV. Before each series of analyses, the capillary was washed by introducing hydrodynamically at high pressure, 0.1 *M* NaOH for 5 min, water for 3 min and, finally, equilibrated at 20 kV with the buffer solution for 30 min. Before each run, the capillary was rinsed at high pressure with water for 2 min and buffer solution for 3 min.

2.5. Sample treatment

The sample treatment for the recovery of the free amino acids in the feed samples consisted of a lixiviation procedure of 1 g of sample with 15 ml of 0.01 *M* HCl for 1 h with the aid of magnetic stirring. The resulting extracts were filtered through a 45-μm pore size cellulose acetate membrane (Schleicher & Schuell, Dassel, Germany) before injection into the CE system.

Prior to sample injection into amino acid autoanalyzer, 300 μl of sample extract was mixed with 25 μl of $2 \cdot 10^{-3}$ *M* norleucine solution (internal standard) and filtered through an ultrafree MC low-binding cellulose membrane (10 000 Dalton pore size) from Millipore.

2.6. Standard method for amino acid determination

The standard method was carried out in an LKB amino acid autoanalyzer which is based on a post-column derivatization with ninhydrin [19]. Amino acids were separated with an Ultropac 7 (20 cm×4 mm I.D., 8 μm particle size) analytical column using a pH and ionic strength gradient. The derivatization process was accomplished on-line in a PTFE reaction coil of 0.3 mm I.D. heated at 135°C in which amino acids reacted with ninhydrin. The spectrophotometric detection was at 570 and 440 nm using a flow cell of 8 μl of dead volume and a path length of 15 mm.

3. Results and discussion

3.1. Optimization of in-capillary conditions

3.1.1. Strategies for in-capillary derivatization

The first studies were addressed to the selection of

the optimum strategy for the in-capillary derivatization of amino acids with NQS. In particular, the following derivatization modes were examined: (i) the zone-passing with tandem or sandwich injection modes, (ii) throughout-capillary and (iii) the at-inlet techniques.

Fig. 2 summarizes the results corresponding to each derivatization strategy for various amino acids chosen as models. In this figure, S, R and B refer to the sample, reagent and buffer solutions, respectively. Among the various zone-passing introduction modes, the R–B–S configuration gave the lowest responses. The R–S tandem mode reached high sensitivities for some amino acids. The best zone-passing approach was the R–S–R sandwich mode, as it provided the highest peak areas for all amino acids tested.

As regards the throughout-capillary technique, signals were significantly lower than those given by zone-passing techniques. This finding was attributed to the instability of NQS in basic medium and quickly decomposes yielding the corresponding hydrolysis side product. Such a product is not reactive in front of amino acids and, thus, the yield of NQS-amino acid derivatives may decreases with respect to other in-capillary formats as a consequence of this degradation.

The in-capillary derivatization by the at-inlet technique did not contribute to the increase in the sensitivity of the detection. This finding was attributed to the fact that the kinetic development of the reaction was sufficient under the dynamic conditions of the zone-passing mode. Thus, increasing the time of contact between reagent and analytes did not lead to a higher derivatization performance.

As a conclusion, a zone-passing technique with a R–S–R sandwich introduction mode was chosen as the optimum approach for the in-capillary derivatization of amino acids with NQS.

3.1.2. Effect of organic modifiers

The effect of the addition of isopropanol to the running electrolyte solution on the resolution of the amino acid derivatives was studied. The percentage of solvent was varied in the range from 0 to 20%. This percentage should be limited to a 20% in order to prevent the precipitation of sodium tetraborate which may occur at a higher content of organic solvent. A continuous increase in the resolution of peaks of amino acid derivatives with the percentage of isopropanol was observed but the analysis times were significantly longer (see Fig. 3). This increase in the migration time was more noticeable for those derivatives with smaller mobility (e.g., Asp) while the variation was more gradual for faster mobility derivatives (e.g., Lys).

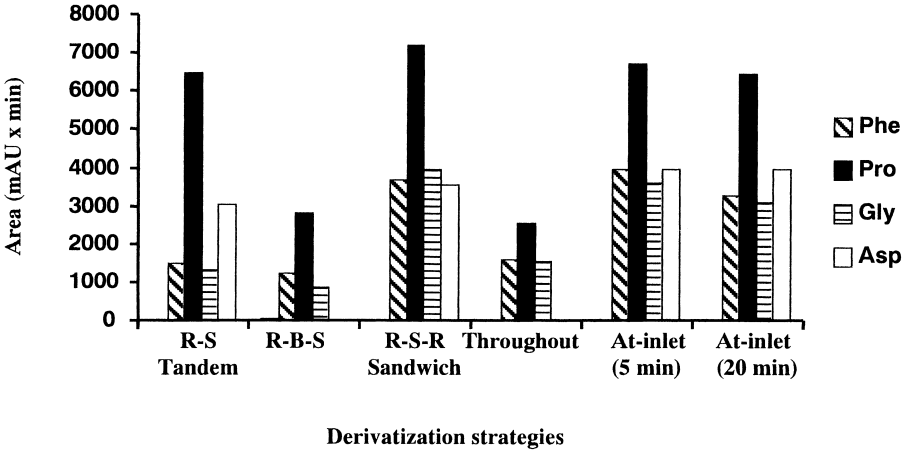

Fig. 2. Comparison of strategies for in-capillary derivatization. Conditions: zone-passing and at-inlet derivatization: reagent solution=0.03 *M* NQS+0.1 *M* HCl; running buffer solution=40 m*M* sodium tetraborate; reagent injection time=10 s at 0.5 p.s.i.; sample introduction time=10 s at 0.5 p.s.i. Throughout derivatization: running buffer solution=40 m*M* sodium tetraborate+0.03 *M* NQS; sample introduction time=10 s at 0.5 p.s.i.; potential=20 kV. Segment assignment: S, sample; R, reagent; B, buffer.

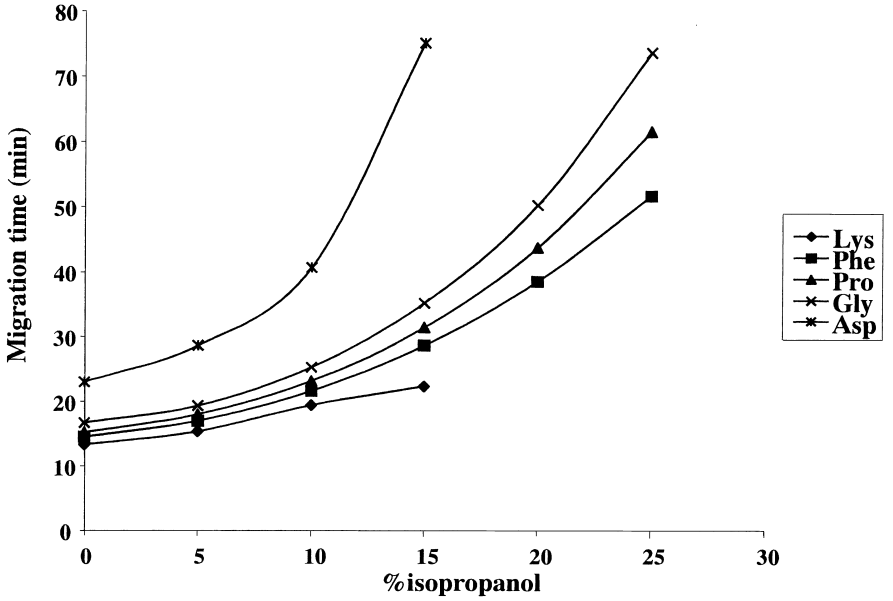

Fig. 3. Influence of the isopropanol percentage on the migration of amino acid derivatives. Conditions: zone-passing derivatization with a R–S–R sandwich introduction mode; reagent solution=0.03 *M* NQS+0.1 *M* HCl; running buffer solution=40 m*M* aqueous sodium tetraborate solution–isopropanol; sample injection time=10 s at 0.5 p.s.i.; reagent injection time=10 s at 0.5 p.s.i., potential=20 kV.

The effect of the percentage of isopropanol in the running electrolyte solution on the peak area was studied as well. The results indicated that the peak response was almost unaffected by the changes in the solvent composition. As a result, a running buffer composed of 40 m*M* aqueous sodium tetraborate solution–isopropanol (4:1, v/v) was used for the resolution of derivatives. In order to accelerate the migration of acidic amino acid derivatives (Glu, Asp and Cys), a low pressure of 5 p.s.i. was applied 35 min after injection.

3.1.3. Sample/reagent introduction time

This study was aimed at optimizing the injection volumes of sample and reagent solutions in the zone-passing mode selected. These variables were studied in a univariate manner as follows.

Firstly, the introduction time of reagent solution was varied from 5 to 13 s, applying a pressure of 0.5 p.s.i., while the introduction time of sample solution was maintained constant at 5 s. The peak response of most amino acid derivatives increased when the injection volume of the reagent solution became larger. However, injection times longer than 10 s gave double peaks. Consequently, an injection time of 10 s was chosen for the reagent solution taking into account both sensitivity and resolution.

Secondly, the effect of the sample injection was evaluated in the range from 3 to 13 s (at a pressure of 0.5 p.s.i.). The reagent time injection in this series was set constant to 10 s. It was observed improvement in the sensitivity with increasing introduction time of sample solution. This variation should be approximately proportional to the amount of analyte introduced into de capillary. Finally, an injection time of 10 s was selected as a suitable compromise for all amino acids.

3.1.4. Effect of the reagent concentration

The effect of NQS concentration in the reagent solution was studied in the range from 0.03 to 0.1 *M*. Amino acids such as phenylalanine, proline and glycine increased their responses with the NQS concentration. The optimum values were achieved at 0.07 *M* NQS, so that this value was chosen as optimum for further studies.

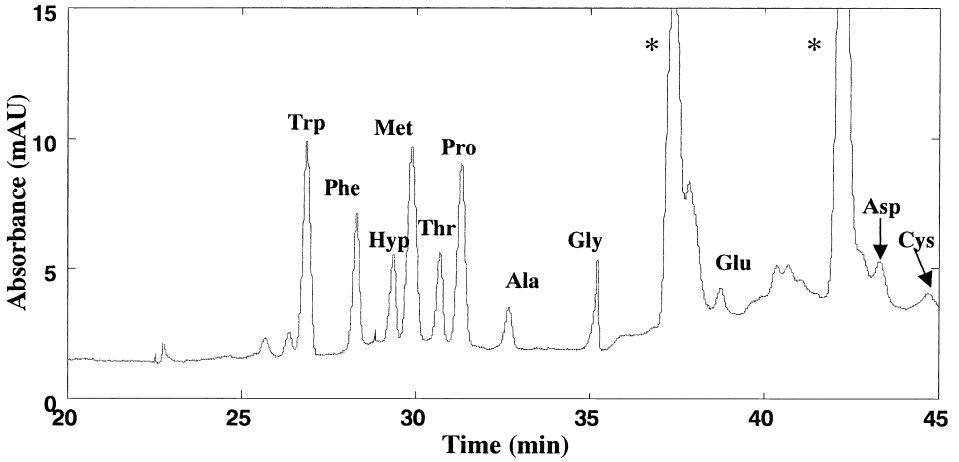

Fig. 4. Electropherogram of a standard amino acid solution under the selected experimental conditions. (*) Reagent species.

3.2. Figures of merit

As an example, Fig. 4 shows the electropherogram under the optimum experimental conditions corresponding to a standard amino acid solution. The figures of merit of the method proposed were established at 230 nm using the peak area as analytical parameter. The repeatabilities (run-to-run precision) for migration time and peak areas were calculated as the relative standard deviation (RSD) of six consecutive injections of a standard solution of $5 \cdot 10^{-4}$ *M* each amino acid. The reproducibilities (day-to-day precision) were calculated as the RSD for the injection of a standard solution of $5 \cdot 10^{-4}$ *M* each amino acid on 6 nonconsecutive days. The detection limits were estimated for a signal-to-noise ratio of 3. The linearity was tested in the range $3.5 \cdot 10^{-5}$ to $7 \cdot 10^{-4}$ *M* by injecting six standard solutions.

Results summarized in Table 1 show that run-to-run precision ranged from 3 to 12% for peak area and from 0.5 to 1.5% for migration time. Good linearities were obtained up to $7 \cdot 10^{-4}$ *M*, with correlation coefficient higher than 0.99. Detection limits were from 0.08 to 1.4 pmol when injecting 40 nl of sample.

Table 1
Figures of merit of the method proposed

Amino acid	Migration time (min)	Migration time repeatability (RSD, %, $n=6$)	Peak area repeatability (RSD, %, $n=6$)	Straight line equation[a]	Correlation coefficient	Detection limit (pmol)
Pro	29.5	1.4	11.4	$9 \cdot 10^5 c + 29$	0.996	0.14
Gly	33.0	1.5	9.4	$4 \cdot 10^5 c + 35$	0.997	0.14
Trp	25.5	1.4	6.6	$2 \cdot 10^6 c + 21$	0.999	0.08
Hyp	27.5	1.3	11.8	$5 \cdot 10^4 c + 53$	0.987	0.14
Thr	29.0	1.4	5.8	$3 \cdot 10^5 c + 34$	0.991	0.14
Lys	21.5	1.6	6.2	$7 \cdot 10^3 c + 1.2$	0.999	1.4
Phe	27.0	1.4	8.9	$5 \cdot 10^5 c + 38$	0.995	0.08
Met	28.5	1.2	4.0	$1 \cdot 10^6 c + 66$	0.992	0.08
Ala	30.5	1.5	5.0	$2 \cdot 10^5 c + 0.03$	0.999	0.3
Glu	38.0	0.5	6.5	$1 \cdot 10^5 c + 4.6$	0.993	1.4
Asp	42.5	0.6	2.8	$4 \cdot 10^5 c + 43$	0.997	1.4
Cys	43.5	0.7	3.8	$3 \cdot 10^5 c + 20$	0.995	1.4

[a] c, concentration in Mol l^{-1}; peak area expressed in μA.V.×min.

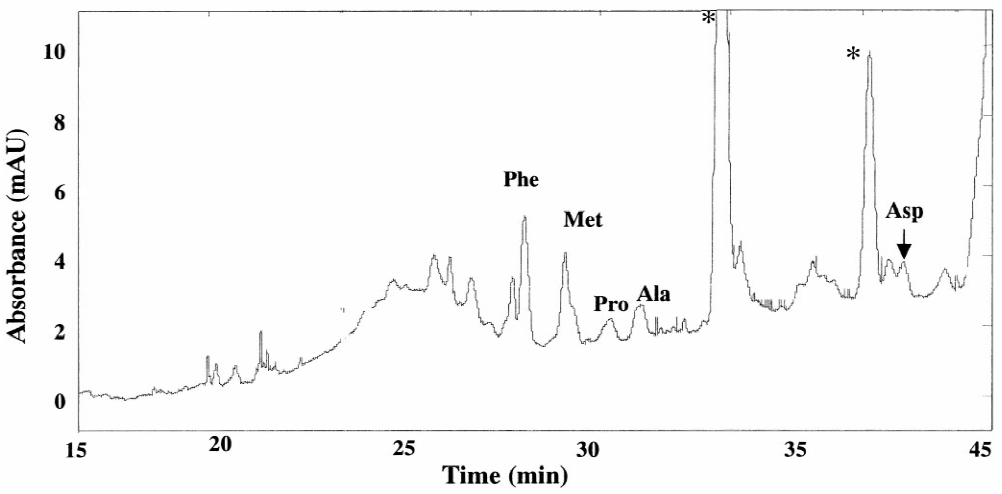

Fig. 5. Electropherogram of a feed sample under the selected experimental conditions. (*) Reagent species.

3.3. Determination of amino acids in animal feed samples

This in-capillary derivatization CZE method was applied to the analysis of free amino acids in feed samples for animal nourishing. Samples were subjected to a lixiviation procedure as described in the Experimental section. Fig. 5 shows an electropherogram of feed sample extract which indicated that samples commonly contained various amino acids such as phenylalanine, glycine, glutamic acids, etc. Each sample was analyzed in triplicate and the results obtained were compared with those given by the standard method using the amino acid autoanalyzer [19]. The amount of methionine found with the method proposed seems to be lower than that given by the standard amino acid analyzer. However, these differences may be attributed to experimental variability rather than a loss of methionine due to its oxidation to methionine sulfoxide which we do not believe possible under the working conditions. In the other cases, as shown in Table 2, a reasonable concordance between the two methods was found, with an average quantification error around 5%.

4. Conclusions

This paper examines the analytical potentiality of in-capillary derivatization techniques such as zone-passing, throughout, and at-inlet modes for CZE determination of amino acids. Among these strategies, zone-passing derivatization with a sandwich introduction mode, in which the sample is injected

Table 2
Determination of amino acids in feed samples using the proposed CZE method

Amino acid	Feed sample A*		Feed sample B*	
	CE method	Standard method	CE method	Standard method
Phe	10.9	11.5	–	–
Met	9.01	12.3	68	79
Ala	32.7	32.1	3.1	3.6
Pro	18.0	18.8	–	–
Asp	7.9	7.9	–	–

Comparison with the standard method using an amino acid autoanalyzer.
*Amounts are expressed as mg of amino acid in 100 g of feed sample.

between two reagent segments, showed the best performance. In the present study, in-capillary derivatization techniques exhibited some relevant advantages over conventional batch pre-capillary derivatization, namely: full automation of the derivatization procedure in a easy way; improvement of the detection limit (in a 10–100-fold factor) and the reproducibility of peak areas; minimization of the formation of side products dealing with hydrolysis or oxidation reactions; low consumption of samples and reagent. Some requirements of the corresponding reactions are the compatibility between the separation and reaction conditions, and the kinetics aspects of the reaction, which should be developed in a reasonable time.

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