Ana Belén Martínez-Girón
Carmen García-Ruiz
Antonio L. Crego
Maria Luisa Marina

Department of Analytical Chemistry, Faculty of Chemistry, University of Alcalá. Ctra. Madrid-Barcelona, Alcalá de Henares, Madrid, Spain

Received July 23, 2008
Revised September 11, 2008
Accepted September 11, 2008

Research Article

Development of an in-capillary derivatization method by CE for the determination of chiral amino acids in dietary supplements and wines

A fast in-capillary derivatization method by CE with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate was developed for the first time for the determination of amino acid enantiomers (arginine, lysine, and ornithine) in dietary supplements and wines. Because of the initial current problems due to the formation of precipitates into the capillary during the derivatization reaction, a washing step with an organic solvent as DMSO between injections was necessary. Different approaches were also investigated to enhance the sensitivity of detection. A derivatization procedure, where plugs of ACN, derivatizing agent (10 mM 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate), and sample in borate (1:1 v/v) were injected in tandem (2, 3, and 6 s, respectively, at 50 mbar), was selected because it enabled to obtain the most sensitive and reproducible results. Appropriate analytical characteristics (linearity, LOD and LOQ, precision, absence of matrix interferences, and accuracy) were obtained for this method. Finally, the optimized method was successfully applied to the determination of the enantiomers of arginine, lysine, and ornithine in food samples of different complexities (dietary supplements and wines).

Keywords:
Amino acid / 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate / CE / Chiral method / In-capillary derivatization
DOI 10.1002/elps.200800481

1 Introduction

The analysis of free amino acids and their enantiomers by CE has become an increasingly active area of research in the food field [1, 2]. It is well known that amino acids in nature occur in L-forms. However, D-forms in foods may occur during food processing (high temperatures or pHs) or fermentation processes [3]. Thus, the presence of D-amino acids has been found in wines [4–7]. Among amino acids present in wines, ornithine (Orn), lysine (Lys), and arginine (Arg) are especially relevant due to their influence on the organoleptic properties of wines. Orn and Lys are precursors of compounds responsible for the undesirable mouse flavor in wines [8]. Orn and Arg are also precursors of biogenic amines, which, in addition to originate organoleptic defects in wines, at high concentrations are toxic [9] and their levels have been regulated by the Food and Drug Administration.

Unfortunately for the analyst, most of the amino acids (except aromatic) present structures without bulky organic groups that hinder the absorption of ultraviolet radiation and their interaction with chiral selectors as CDs. Derivatization is a modification of analytes to give suitable structural features to increase the detection sensitivity and to make the inclusion and interaction of the derivatized amino acids with CDs possible [10, 11]. Derivatization procedures by CE can be classified in three different modes based on the analytical stage where derivatization takes place: before (pre-capillary), during (in-capillary), or after (post-capillary) the electrophoretic separation.

In-capillary derivatization procedures offer many advantages over conventional pre- and post-capillary derivatization modes. They can allow a full automatization of the derivatization step without additional equipment, minimize sample preparation, sample dilution is reduced to a minimum, and a low consumption of sample and derivatizing reagent is required. Thus, in recent years, the use of in-capillary derivatization procedures as an alternative to conventional derivatization techniques has been reviewed [12, 13].

Correspondence: Dr. Maria Luisa Marina, Department of Analytical Chemistry, Faculty of Chemistry, University of Alcalá, Ctra. Madrid-Barcelona Km. 33.600, 28871 Alcalá de Henares, Madrid, Spain
E-mail: mluisa.marina@uah.es
Fax: +34-91-8854971

Abbreviations: AQC, 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate**; Arg,** arginine**; DS,** dietary supplements**; HS-β-CD,** highly sulfated-β-CD**; Lys,** lysine**; Orn,** ornithine**; RRFs,** relative response factors

Although a large number of different derivatizing reagents have been applied for amino acid analysis, only some of them have been employed using in-capillary derivatization in CE, such as *o*-phthalaldehyde [14–20], naphthalene-2,3-dicarboxaldehyde [21, 22], 1,2-naphthoquinone-4-sulfonate [23], 3-(2-furoyl)-quinoline-2-carboxaldehyde [24], and 5-(4,6-dichloro-*s*-triazin-2-ylamino)fluorescein [25]. Other derivatizing reagents have been widely used in chiral separations of amino acids by CE, but they require long reaction times for derivatization and cannot be employed in the in-capillary technique. As examples, FITC [26, 27], 9- fluorenylmethyl-chloroformate [28], 4-fluoro-7-nitro-2,1,3-benzoxadiazole [29], and dansyl chloride [27] can be cited. To our knowledge, only *o*-phthalaldehyde has been employed using in-capillary derivatization in CE for chiral analysis of amino acids, such as serine [13], or Arg, alanine, and aspartic acid [20, 30].

The use of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) as derivatizing agent to develop an in-capillary method for the determination of chiral amino acids presents several advantages such as a good stability of the derivatized amino acids, and its rapid derivatization time, in a matter of seconds. In addition, a previous CE separation method using pre-capillary derivatization with AQC has demonstrated a huge separation potential for amino acids enantiomers [31].

There is no reference about the use of in-capillary derivatization with AQC as a derivatizing agent for the determination of amino acid enantiomers in foods. Only an in-capillary derivatization procedure using AQC for the determination of insulin and two glycated forms by CE with LIF detection has been published up to date [32].

In this work, an in-capillary derivatization method using AQC as the derivatizing agent was developed in order to increase the automatization in the chiral determination of amino acids by CE. The analytical characteristics of the method were evaluated and its applicability to the analysis of food samples was established.

2 Materials and methods

2.1 Reagents and samples

All reagents employed were of analytical grade. Highly sulfated-β-CD (HS-β-CD) from Fluka (Buchs, Switzerland) and acetylated-β-CD (Ac-β-CD) from Cyclolab (Budapest, Hungary) were used as chiral selectors. Orthophosphoric acid from Merck (Darmstadt, Germany) was used for the preparation of the buffer solutions. Pure triethanolamine solution from Riedel-de Haën (Seelze, Germany) was used to adjust the buffer pH. Sodium hydroxide from Merck and DMSO from Fluka were used to rinse the capillary.

Buffer solutions were prepared diluting the appropriate volume of orthophosphoric acid with Milli-Q water, adjusting the pH to the desired value with pure trietanolamine before completing the volume with water to get the desired buffer concentration. Finally, BGEs were prepared dissolving the appropriate amount of CD in the buffer solution.

Amino acids, DL-Orn, DL-Arg, and DL-Lys were supplied by Fluka. The derivatizing reagent AQC was purchased from Waters (Mildford, MA, USA) in a reagent kit containing borate buffer, AQC, and anhydride ACN. The dietary supplements (DS) and wine samples analyzed were commercially available and acquired in several markets of Madrid, Spain.

Solutions of racemic mixtures of amino acids and DS were directly prepared by dissolving them in Milli-Q water up to the desired concentration. Wine solutions were only filtered and used for derivatization. All solutions (buffers, standards, and samples) were filtered prior use through 0.45 mm pore size disposable nylon filters from Titan (Eatontown, NJ, USA).

2.2 Derivatization procedure

2.2.1 Preparation of the AQC solution

The AQC derivatizing solution was prepared by adding 1 mL of anhydride ACN to the AQC reagent (~10 mM AQC) and introducing it in a bath pre-heated to 55°C for 20 min. This derivatizing solution was stored at room temperature in a desiccator up to use.

2.2.2 In-capillary derivatization procedure

Sample solutions were mixed with 200 mM borate buffer (pH 8.8) in a 1:1 volume ratio for amino acid standards and DS, and in a 1:3 volume ratio for wines. The in-capillary derivatization procedure was performed by injecting (50 mbar) ACN, AQC solution, sample solution, and BGE for 2, 3, 6, and 5 s, respectively.

2.2.3 Pre-capillary derivatization procedure

For the preparation of the AQC-amino acids by the pre-capillary derivatization procedure, 100 μL of the amino acid solution with 100 μL of 200 mM borate buffer (pH 8.8) and 100 μL of the AQC solution were mixed. The resulting solutions were sonicated for 1 min and injected in the CE system.

2.3 CE conditions

CE experiments were carried out with an HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with an on-column DAD working at 260 nm with a bandwidth of 5 nm. The instrument was controlled by a PC running the 3D-CE ChemStation from Agilent Technologies. Separation was carried out in a common uncoated fused-silica capillary 72.5 cm total length (64 cm to the detector) × 50 μm id (375 μm od) from Composite Metal

Services (Worcester, England). The electrophoretic separation was achieved with a voltage of −25 kV (reverse-polarity mode). The temperature of the capillary was kept constant at 15°C. The BGE consisted of a mixture of HS-β-CD (5% m/v) and Ac-γ-CD (2% m/v) as chiral selectors in acidic media (50 mM phosphate buffer at pH 2.0).

Before the first use, the capillary was conditioned by flushing at 1 bar with 1 M NaOH for 30 min, with water for 5 min, and with the running buffer for 60 min. At the beginning of each day, the capillary was pre-washed at 1 bar for 10 min with the running buffer. After each run, the capillary was rinsed for 4 min with DMSO, followed with 2 min water, and 4 min BGE, at a pressure of 1 bar to maintain an adequate repeatability of run-to-run injections and to prevent obstructions in the capillary.

2.4 Data treatment

Calibration curves were established by considering the corrected peak areas (Ac was calculated by dividing the peak area by the corresponding migration time) to compensate fluctuations in electrophoretic conditions. Because peak areas are related to both concentrations and migration times [33], if there is a shift in the migration times, normalization using Ac can be used to improve the precision of quantitative data [34].

Experimental data analysis and composition of graphs with different electropherograms were carried out using Excel Microsoft XP® and Origin® version 6.0 software.

3 Results and discussion

3.1 Development of the in-capillary derivatization method by CE

A CE method in the EKC separation mode using HS-β-CD and Ac-γ-CD as chiral selectors in acidic media has recently been developed by our research team for the chiral separation of amino acids [31]. This method demonstrated a huge enantiomeric separation potential for chiral protein amino acids including Arg and Lys, and the chiral non-protein amino acid Orn. However, this method required a pre-capillary derivatization of amino acids to allow their optical detection and to obtain larger molecules capable of interacting with the CDs used as chiral selectors. Because derivatization implies an undesirable additional step, in this work, an in-capillary derivatization methodology using AQC as the labeling reagent was investigated to increase the automatization of the chiral CE method. Orn, Lys, and Arg were chosen as representative amino acids usually present in food supplements. In addition, Orn is an important non-protein amino acid in fermented foods because it is the precursor of compounds responsible for bad taste (mousy off-flavor) in wines [8] and of biogenic amines (*i.e.* putrescine) in fermented foods such as wines [9].

First, the possibility of introducing the bands of the amino acid sample (AA) and AQC by either the tandem or the sandwich modes, and the order of these bands, was investigated. Initially, the AA solution (diluted 1:1 with borate buffer at pH 8.8) for 5 s and the AQC solution for 3 s were introduced at 50 mbar in the capillary. In addition, systematically, a last plug of BGE (50 mbar × 5 s) was also introduced to prevent the loss of AA or AQC when the separation voltage was applied to the capillary. The first problem was to avoid contamination of the AQC solution with the aqueous solution of AA or BGE during the sequence described because AQC decomposed (hydrolyzed) rapidly in the presence of water. To do so, a plug of ACN (2 s at 50 mbar) was introduced before the band of AQC.

Figure 1 shows the schemes illustrating the different bands injected for the optimization of the in-capillary derivatization reaction and the electropherograms obtained in each case. Curiously, the best results were obtained when only a band of AQC was injected in tandem before or after the AA band, while the sandwich mode (AQC-AA-AQC) produced broad peaks and loss in resolution. Taking into account the high negative charge of HS-β-CD, and the pK_as of AQC and amino acids studied (see Table 1), the results observed could be explained as follows. When the AQC band was injected after the AA band (Fig. 1A), the inclusion complexes formed between the negatively charged HS-β-CD and the neutral AQC are responsible for the migration toward the anode of the AQC reagent, reaching the positively charged AA and giving rise to the derivatization reaction. When they are derivatized, the AQC-AA becomes neutral. However, when the AQC band was injected before the AA band (Fig. 1B), the ion pairs formed by electrostatic interactions between the highly negatively charged HS-β-CD and the positively charged amino acids are responsible for the migration toward the anode of the amino acids, reaching the AQC band and giving rise to the derivatization reaction. In this latter case, an increase in peak areas was obtained (∼threefolds), which could be explained for the less availability of AQC in Fig. 1A, when it is included into the CD core. Finally, when the AA band was injected in a sandwich of AQC bands (Fig. 1C), both phenomena occur simultaneously at both ends of the AA band, leading a splitting of the derivative band (AQC-AA) formed as it is observed in Fig. 1. Because the best sensitivity without loss in resolution was obtained when the introduction of AA was made after the AQC solution (Fig. 1B), this in-capillary derivatization strategy was selected for further experiments. This derivatization reaction was based on the mobility differences between the analyte and the derivatizing reagent, which are mixed in the capillary taking place the derivatization reaction during the separation process (derivatization technique named ''zone-passing'') [17, 35].

Once the derivatization protocol was selected, experiments showed that a loss in current intensity usually happened with this and other strategies when long analysis sequences were programmed. As a consequence, a method

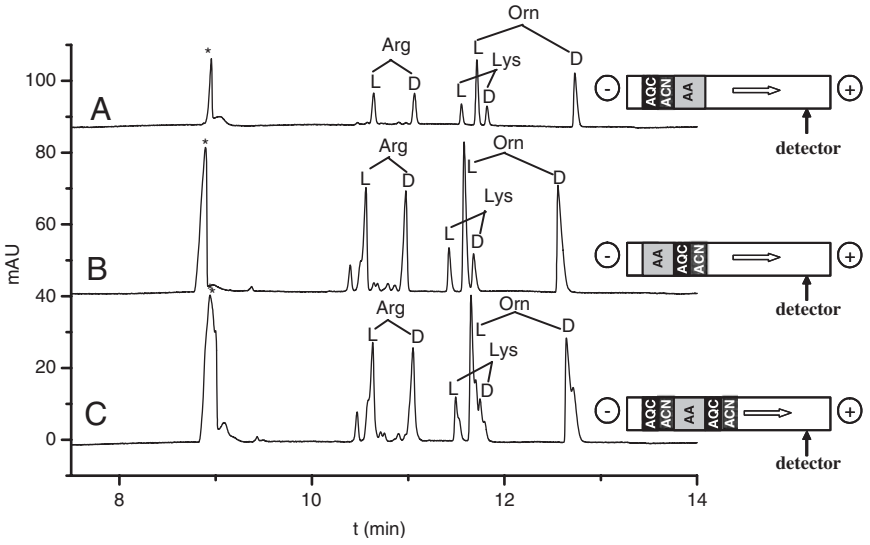
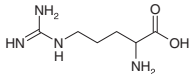
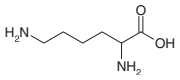
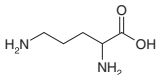
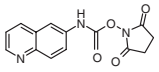


Figure 1. Strategies employed for the in-capillary derivatization procedures studied: (A and B) tandem mode; (C) sandwich mode. Experimental conditions: BGE, 50 mM phosphate buffer at pH 2.0 containing 5% m/v HS-β-CD and 2% m/v Ac-γ-CD; capillary, 50 μm × 72.5 cm uncoated fused-silica; injection, 50 mbar for 5 s of analyte (AA), 2 s of ACN, and 3 s of AQC reagent (plugs order indicated in the figure); AA concentration, 2 mM for DL-Arg and DL-Orn and 0.6 mM for DL-Lys; run voltage, −25 kV; temperature, 15°C; detection wavelength, 260 nm with a bandwidth of 5 nm. *Peak corresponding to the AQC hydrolysis product.

Table 1. Structure and pK_as of the amino acids studied [29] and AQC [30]

Compounds	Structure	pK_a		
Arg		2.17	9.04	12.48
Lys		2.18	8.95	10.53
Orn		1.71	8.69	10.76
AQC		4.5		

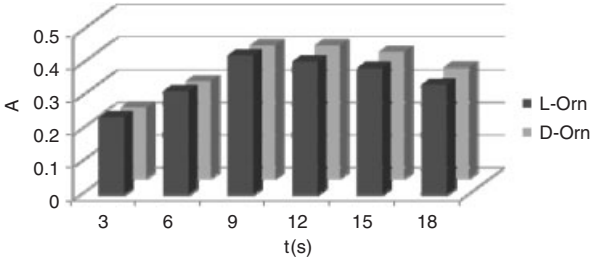


Figure 2. Influence of the injection time for the analyte (Orn enantiomers) in the corrected peak area obtained using a tandem mode with ACN-AQC-AA for 2, 3, and ''x'' s, respectively. Other experimental conditions are as in Fig. 1.

for pre-conditioning the capillary walls between injections was optimized to prevent obstruction in the capillary and maintain a stable current. Thus, a washing step with an organic solvent to avoid precipitations originated into the capillary during each run was explored. Because a large number of organic compounds are soluble in DMSO, and it is miscible with water, this solvent was employed. Good results were obtained when a pre-conditioning method consisting of flushing the capillary at 1 bar with DMSO during 4 min followed by water for 2 min, and BGE for 4 min was used between injections.

Second, different band lengths of AA and AQC solutions were injected when using the previously selected strategy (Fig. 1B) in order to obtain the best sensitivity and precision. Thus, the introduction time of the AA solution was varied from 3 to 18 s when maintaining constant the time of the AQC plug at 3 s. As shown in Fig. 2, considering Orn as example, an increment in the corrected peak areas for both enantiomers was obtained when increasing the injection time of the analyte until 9 s. Over this injection time, a decrease in the corrected peak areas was observed. However, because an analyte injection time of 6 s instead of 9 s gave a precision significantly better ($RSD_{6\,s} = 9.8\%$ and $RSD_{9\,s} = 16.5\%$ for six repeated injections), this time was selected to study the effect of varying the time of introduction of AQC band from 3 to 6 s. Owing to the negligible changes appreciated when varying this time, 3 s were used for the injection of the AQC band.

Figure 3 shows the electropherograms obtained when pre-capillary and in-capillary derivatization procedures, using the same injection time for analytes (6 s for the amino acids mixture), were performed. Both techniques gave sharp and symmetrical peaks for the three amino acids analyzed (Arg, Orn, and Lys enantiomers), and all peaks were baseline resolved. However, in-capillary derivatization gave worse sensitivity in comparison with pre-capillary derivatization. These results can be explained on the basis of the best reaction performance when the derivatization reaction was achieved outside of the CE capillary. Nevertheless, the in-capillary derivatization method developed in this work shows the potential benefits of automating the analysis (which represents a significant

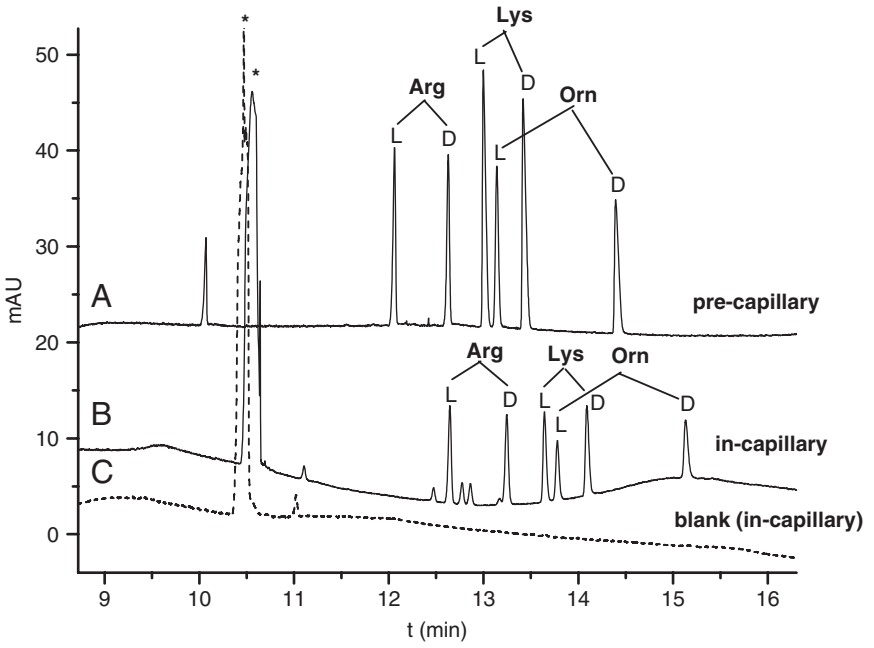


Figure 3. Electropherograms obtained using pre- and in-capillary derivatization corresponding to injections of racemic mixtures of Arg, Orn, and Lys enantiomers. (A) Pre-capillary derivatization, 1:1:1: AA:borate:AQC. Injection; analytes derivatized in 200 mM borate at pH 8.8 (6 s) and BGE (5 s). (B) In-capillary derivatization of amino acids using a tandem mode with ACN (2 s)-AQC (3 s)-AA (6 s). (C) Blank using the same in-capillary sequence as in (B) but with borate instead of AA in borate. Amino acids concentration: 0.4 mM DL-Arg and DL-Lys, and 0.2 mM DL-Orn. Other experimental conditions are as in Fig. 1. *Peaks corresponding to the AQC hydrolysis product.

saving of time) as well as the significant reduction in the derivatizing reagent consumption (high cost) and samples as advantages.

3.2 Analytical characteristics of the in-capillary derivatization method by CE

The analytical performance of the developed method was studied to demonstrate its applicability to the enantiomeric determination of amino acids in foods. Thus, the chiral method was validated in terms of selectivity, linearity, repeatability and intermediate precision, accuracy, and LOD and LOQ.

The *linearity* was assessed by analyzing six standard solutions at different concentration levels ranging from 0.2×10^{-4} to 4×10^{-4} M of each Arg and Lys enantiomers and from 0.1×10^{-4} to 2×10^{-4} M for Orn enantiomers. Three replicates were prepared at each concentration in different days, and each one was injected in triplicate. Calibration curves were established by plotting corrected peak areas *versus* molar concentrations. To evaluate the linearity, calibration lines (calculated by least-squares regression) were obtained and the correlation coefficient, *y*-intercept, and slope with their confidence intervals were calculated (see Table 2). Acceptable linearity was obtained in the concentration range studied with adequate correlation coefficients higher than 0.999 for each enantiomer in most cases. Moreover, all confidence intervals at 95% for the intercept included the zero value, *i.e.* they did not differ statistically from zero (the lack of significance of the intercept confirms the absence of systematic errors in the calibration), and all slope values differed statistically from zero (at 95% confidence level), confirming the linearity of the method.

On the other hand, to study if the response of the minority stereoisomers was equivalent to that of the main stereoisomers, the *relative response factors* (RRFs) were determined. RRFs were obtained by dividing the slopes of the calibration lines for each minority stereoisomer (D-form) and the slope obtained for the majority stereoisomer (L-form). Because RRFs obtained ranged from 0.9 to 1.0, the response for both enantiomers of all amino acids studied can be considered equivalent as established by the European Pharmacopoeia [36]. As a consequence, the percentage of the D-form can be determined from the ratio between the areas corresponding to peaks of the L- and D-forms.

To evaluate the *accuracy* of the method, a comparison between the slopes obtained using the external standard calibration method and the standard addition calibration method was performed using a *t*-test with a confidence level of 95% for determining possible *matrix effects*. Three different concentrations of amino acid enantiomer standards (1.0×10^{-4}, 2.0×10^{-4}, and 3.0×10^{-4} M for Arg and Lys enantiomers; and 0.5×10^{-4}, 1.0×10^{-4}, and 1.5×10^{-4} M for Orn enantiomers) were added to solutions of one of the DS studied (DS-1 that contained the L-forms of Arg, Lys, and Orn), which had a concentration of 1.0×10^{-4} M in L-Orn. Because the overlapping of the confidence intervals (*t*-test) of the slopes of both calibration methods was demonstrated (see Table 2), no significant differences between the slopes were obtained; hence the matrix did not produce systematic errors. Therefore, the external standard calibration method was suitable for the quantitation of these chiral amino acids in the seven commercial DS analyzed in this work. Next, *recovery studies*

Table 2. Analytical performance of the method for the determination of Arg, Lys, and Orn enantiomers

Linearity	L-Arg	D-Arg	L-Lys	D-Lys	L-Orn	D-Orn
Linear concentration range	0.2–4 × 10^{-4} M	0.2–4 × 10^{-4} M	0.2–4 × 10^{-4} M	0.2–4 × 10^{-4} M	0.1–2 × 10^{-4} M	0.1–2 × 10^{-4} M
Linear equation	$y = 0.086 + 12448x$	$y = 0.020 + 11668x$	$y = 0.038 + 10529x$	$y = 0.032 + 10561x$	$y = 0.050 + 12554x$	$y = 0.038 + 12800x$
Standard errors	$S_a = 0.052$, $S_b = 230$	$S_a = 0.023$, $S_b = 100$	$S_a = 0.060$, $S_b = 267$	$S_a = 0.046$, $S_b = 206$	$S_a = 0.027$, $S_b = 236$	$S_a = 0.020$, $S_b = 181$
Correlation coefficient (r)	0.9993	0.9998	0.998	0.9992	0.9993	0.9996
Intercept $\pm tS_a$	0.086 ± 0.144	0.020 ± 0.062	0.038 ± 0.168	0.032 ± 0.129	0.050 ± 0.074	0.038 ± 0.057
Slope $\pm tS_b$	12 448 ± 638	11 668 ± 277	10 529 ± 743	10 561 ± 571	12 554 ± 654	12 800 ± 503
Accuracy	L-Arg		L-Lys		L-Orn	
Study of matrix interferences						
Standard additions: Slope $\pm tS_b$	11 550 ± 1302		8780 ± 3758		10 140 ± 4374	
Recovery (%)						
Level 1 ($n = 3$)	107 ± 5		99 ± 12		106 ± 10	
Level 2 ($n = 3$)	101 ± 6		101 ± 11		112 ± 9	
Level 3 ($n = 3$)	90 ± 7		93 ± 12		98 ± 5	
Mean recovery ($n = 9$)	*99 ± 9*		*100 ± 7*		*105 ± 7*	
Precision	L-Arg	D-Arg	L-Lys	D-Lys	L-Orn	D-Orn
Instrumental repeatability: RSD % ($n = 6$)						
t	0.3	0.3	0.4	0.4	0.5	0.5
A_c	4.4	5.0	3.2	3.1	2.3	3.0
Method repeatability: RSD % ($n = 6$)						
t	0.7	0.7	1.0	1.0	1.0	0.9
A_c	6.6	8.2	4.2	4.9	3.5	4.0
Intermediate precision: RSD % ($n = 9$)						
t	4.0	5.0	4.1	5.2	4.1	5.3
A_c	8.1	6.8	7.2	6.0	5.2	4.8
LOD	7.5 × 10^{-6} M		8.2 × 10^{-6} M		6.4 × 10^{-6} M	
LOQ	2.5 × 10^{-5} M		2.7 × 10^{-5} M		2.1 × 10^{-5} M	

were achieved for the L-enantiomers of Arg, Lys, and Orn when one DS was spiked with a standard addition of these chiral amino acids at three different levels (1.0×10^{-4}, 2.0×10^{-4}, and 3.0×10^{-4} M for Arg and Lys enantiomers; and 0.5×10^{-4}, 1.0×10^{-4}, and 1.5×10^{-4} M for Orn enantiomers). Recovery values were obtained by comparing these concentration values with those determined by the CE method using the external standard method for calibration. Each standard addition level was performed in triplicate with two different samples. Results grouped in Table 2 show recoveries ranging from 99 to 105% with acceptable RSD ($\leq$9%).

The *precision* of the whole method was evaluated in terms of repeatability and intermediate precision. First, due to the migration of the charged CD, the possibility of interchanging the inlet and outlet vials after each run was investigated to improve method precision by preventing the decrease in the CD concentration in the cathode vial during the run and its increase in the other reservoir [37]. The significantly better results obtained when interchanging the vials after each run (from RSD~10% to RSD $<$5%) lead us to implement this protocol for further analysis. Thus, *instrumental repeatability* was determined at one concentration level (2×10^{-4} M for Arg and Lys enantiomers and 1×10^{-4} M for Orn enantiomer) by means of repetitive application of the described procedure to one solution on the same day from six repeated injections. *Method repeatability* was determined at the same above concentrations, with two different standard solutions injected in triplicate on the same day. RSD values for these standard solutions were $<$8.2% for corrected peak areas and $<$1.0% for migration times (see Table 2). *Intermediate precision* was assessed for three solutions with the same concentration level analyzed to evaluate the repeatability injected in triplicate on three

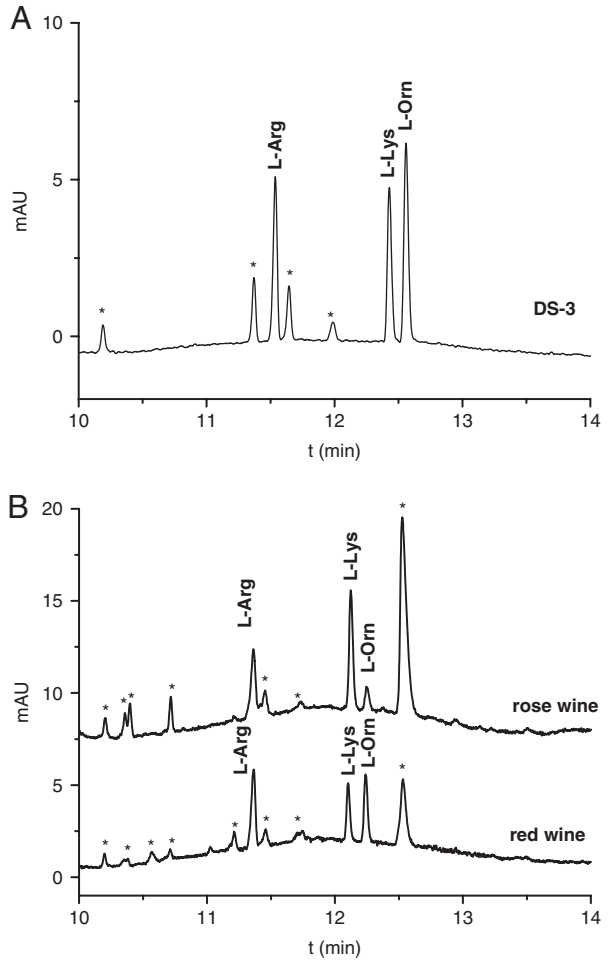


Figure 4. Electropherograms corresponding to one DS and two different wines. (A) DS-3 (sample with borate in a 1:1 volume ratio). (B) Wine samples (sample with borate in a 1:3 volume ratio). Other experimental conditions are as in Fig. 3. *Unknown peaks.

different days with the same equipment and with three different capillaries. The results for corrected peak areas, and migration times are grouped in Table 2. As it can be observed, acceptable precision (RSD $\leq$ 8.1% for corrected peak areas, RSD $\leq$ 5.3% for migration times) was obtained in all cases.

Finally, LOD and LOQ were calculated as the minimum analyte concentration in the solution previous to its derivatization yielding an S/N equal to 3 and 10, respectively. As shown in Table 2, LODs for the L-forms of Arg, Lys, and Orn (similar results were obtained for the D-enantiomers) were about 8×10^{-6} M, while LOQs were $< 3 \times 10^{-5}$ M for all amino acid enantiomers studied.

3.3 Analysis of food samples

The in-capillary derivatization method developed for the determination of Arg, Lys, and Orn enantiomers was applied to the analysis of several DS and fermented products as wines.

3.3.1 DS

Quantitative analysis of L-Arg, L-Lys, and L-Orn enantiomers in DS was achieved using the in-capillary derivatization method developed by CE. As an example, Fig. 4A shows the electropherogram obtained for one of the seven DS analyzed, which contained the three amino acids studied. It is clear that the developed method showed adequate selectivity to determine L-Arg, L-Lys, and L-Orn enantiomers in these products. Table 3 shows the labeled and determined amounts for L-Arg, L-Lys, and L-Orn, as well as the percentage of these enantiomers with respect to the stated contents indicated on the label of the seven different DS analyzed. Fifteen out of the sixteen values obtained for this percentage ranged from 70 to

Table 3. Quantitative determination ($n = 3$) of L-Arg, L-Lys, and L-Orn enantiomers in seven DS with different amino acid composition[a)]

Samples	Determined amount (mg/capsule)[b)]	L-Arg			L-Lys			L-Orn	
		Labeled amount (mg/capsule)[c)]	% Respect to the labeled content	Determined amount (mg/capsule)[b)]	Labeled amount (mg/capsule)[c)]	% Respect to the labeled content	Determined amount (mg/capsule)[b)]	Labeled amount (mg/capsule)[c)]	% Respect to the labeled content
DS-1	172 ± 11	200	86 ± 5	151 ± 14	200	75 ± 7	221 ± 16	200	111 ± 8
DS-2	284 ± 18	400	71 ± 5	–	–	–	442 ± 24	400	110 ± 6
DS-3	211 ± 20	250	84 ± 8	235 ± 55	250	94 ± 22	265 ± 26	250	106 ± 10
DS-4	157 ± 15	189	83 ± 8	–	–	–	121 ± 9	310	39 ± 3
DS-5	350 ± 16	500	70 ± 3	–	–	–	205 ± 4	250	82 ± 2
DS-6	77 ± 5	76	102 ± 7	–	–	–	19.3 ± 0.1	19	101 ± 1
DS-7	279 ± 22	300	93 ± 7	–	–	–	316 ± 30	300	105 ± 10

a) All DS were capsules except DS-6, which was a powder form.
b) Except for DS-6 for which the concentration determined is expressed as mg/100 g.
c) Except for DS-6 for which the labeled concentration is expressed as mg/100 g.

111%, which were considered acceptable values. Only one of them reached a value of 39%. The differences between the determined amounts of analytes and the labeled ones could be attributed to an inadequate distribution of the powder in the dosage form, capsules in most cases. In fact, for the only DS commercialized as a powder (DS-6), the percentages obtained with respect to the labeled content of L-Arg and L-Orn were 102 and 101%, respectively.

3.3.2 Wines

In order to show the wide applicability of the method, other foods with more complex matrices than DS were analyzed. Fermented foods such as wines were selected because these samples can contain the amino acids studied. A slight modification of the in-capillary derivatization method developed was needed in order to obtain reproducible signals for these samples. The 1:1 volume ratio between the wine sample and the borate buffer was not enough to produce the derivatization reaction of the amino acids contained in the wine samples analyzed. This result was attributed to the fact that the sample–borate solution did not achieve the basic pH required for the derivatization reaction (pH > 8). As an example, rose wine presented a pH value of 5.4 when a 1:1 sample:borate ratio was employed, whereas a value of 8.5 was measured when the borate buffer volume was increased up to a 1:3 ratio. As a consequence, a 1:3 volume ratio between the wine sample and the borate buffer was used for complex samples such as wines. Figure 4B shows the electropherograms obtained for two wines analyzed. In both cases, the three amino acids (L-Arg, L-Lys, and L-Orn) were detected, being identified by spiking the samples with a racemic mixture of each amino acid. As an example, Fig. 5 shows the electropherograms obtained for a non-spiked red wine sample and a spiked one with a racemic mixture of Arg, Lys, and Orn. The results showed that D-enantiomers that could be present in the wines analyzed were below the detection limit concentrations of the developed method.

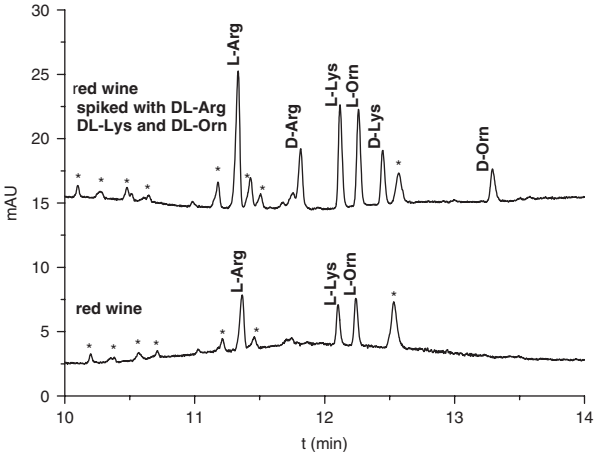


Figure 5. Electropherograms corresponding to a non-spiked red wine sample and a spiked one with DL-Arg (1×10^{-4} M), DL-Lys (2×10^{-4} M) and DL-Orn (2×10^{-4} M). Other experimental conditions are as in Fig. 4. *Unknown peaks.

The enantiomeric determination of these amino acids showed that higher concentrations of L-Lys were present in the rose wine (1.6×10^{-4} M) than in the red wine (6.6×10^{-5} M). Similar concentrations of L-Arg were obtained in both wines (6.6×10^{-5} and 7.5×10^{-5} M, respectively). However, the highest concentrations of L-Orn were found in the red wine (6.2×10^{-5} M) in comparison with the rose wine (2.0×10^{-5} M).

4 Concluding remarks

A fast in-capillary derivatization method by CE allowing the full automatization of the derivatization and separation steps was developed for the first time for the determination of amino acid enantiomers. The appropriate conditioning run to run of the capillary, using DMSO as the solvent, was a crucial factor to solve the current problems produced due to the formation of precipitates into the capillary during the derivatization reaction. The study of different band injection approaches allowed enhancing the sensitivity of detection previously to the application of the method to food samples. Analytical performance of the method was evaluated showing appropriate characteristics (linearity, absence of matrix interferences, accuracy, precision, and acceptable LOD and LOQ) for its application to food samples. The applicability of the developed method to samples of different complexities (DS and wines) has been demonstrated showing its usefulness for the chiral determination of amino acids in food matrices.

The authors thank the Ministry of Education and Science (Spain) and the Comunidad Autónoma de Madrid (Spain) for research projects CTQ2006-03849/BQU and S-0505/AGR-0312, respectively. Carmen García-Ruiz thanks the Ministry of Science and Technology (Spain) for the Ramón y Cajal *program (RYC-2003-001). A.B.M.-G. thanks the University of Alcalá for her pre-doctoral contract.*

The authors have declared no conflict of interest.

5 References

[1] Frazier, R. A., Ames, J. M., Nursten, H. E., *Electrophoresis* 1999, *20*, 3156–3180.

[2] García-Cañas, V., Cifuentes, A., *Electrophoresis* 2008, *29*, 294–309.

[3] Simó, C., Barbas, C., Cifuentes, A., *Electrophoresis* 2003, *24*, 2431–2441.

[4] Brükner, H., Hausch, M., *Chromatographia* 1989, *28*, 487–492.

[5] Brückner, H., Langer, M., Lüpke, M., Westhauser, T., Godel, H., *J. Chromatogr. A* 1995, *697*, 229–245.

[6] Brückner, H., Westhauser, T., *Chromatographia* 1994, *39*, 419–426.

[7] Brückner, H., Haasmann, S., Langer, M., Westhauser, T., Wittner, R., *J. Chromatogr. A* 1994, *666*, 259–273.

[8] Snowdon, E. M., Bowyer, M. C., Grbin, P. R., Bowyer, P. K., *J. Agric. Food Chem.* 2006, *54*, 6465–6474.

[9] Ardö, Y., *Biotechnol. Adv.* 2006, *24*, 238–242.

[10] Wan, H., Blomberg, L. G., *J. Chromatogr. A* 2000, *875*, 43–88.

[11] Cifuentes, A., *Electrophoresis* 2006, *27*, 283–303.

[12] Underberg, W. J. M., Waterval, J. C. M., *Electrophoresis* 2002, *23*, 3922–3933.

[13] Poinsot, V., Rodat, A., Gavard, P., Feurer, B., Couderc, F., *Electrophoresis* 2008, *29*, 207–223.

[14] Taga, A., Honda, S., *J. Chromatogr. A* 1996, *742*, 243–250.

[15] Oguri, S., Yokoi, K., Motohase, Y., *J. Chromatogr. A* 1997, *787*, 253–260.

[16] Oguri, S., Watanabe, S., Abe, S., *J. Chromatogr. A* 1997, *790*, 177–183.

[17] Taga, A., Sugimura, M., Honda, S., *J. Chromatogr. A* 1998, *802*, 243–248.

[18] Taga, A., Nishino, A., Honda S., *J. Chromatogr. A* 1998, *822*, 271–279.

[19] Oguri, S., Yoneya, Y., Mizunuma, M., Fujiki, Y. *et al*., *Anal. Chem.* 2002, *74*, 3463–3469.

[20] Oguri, S., Hibino, M., Mizunuma, M., *Electrophoresis* 2004, *25*, 1810–1816.

[21] Oilman, S. D., Ewing, A. G., *Anal. Chem.* 1995, *67*, 58–64.

[22] Lillard, S. J., Chiu, D. T., Scheller, R. H., Zare, R. N., *Anal. Chem.* 1998, *70*, 3517–3524.

[23] Latorre, R. M., Hernandez-Cassou, S., Saurina, J., *J. Chromatogr. A* 2001, *934*, 105–112.

[24] Veledo, M. T., Frutos, M., Diez-Masa, J. C., *J. Chromatogr. A* 2005, *1079*, 335–343.

[25] Molina, M., Silva, M., *Electrophoresis* 2002, *23*, 2333–2340.

[26] Nouadje, G., Couderc, F., Puig, P., Hernández, L., *J. Capillary Electrophor.* 1995, *2*, 117–124.

[27] Simó, C., Rizzi, A., Barbas, C., Cifuentes, A., *Electrophoresis* 2005, *26*, 1432–1441.

[28] Boniglia, C., Carratu, B., Sanzini, E., *J. Food Sci.* 2002, *67*, 1352–1355.

[29] Tsunoda, M., Kato, M.; Fukushima, T., Santa, T. *et al*., *Chromatography* 1997, *18*, 21–25.

[30] Oguri, S., Kumazaki, M., Kitou, R., Nonoyama, H., Tooda, N., *Biochim. Biophys. Acta* 1999, *1472*, 107–114.

[31] Martínez-Girón, A. B., Domínguez-Vega, E., García-Ruiz, C., Crego, A. L., Marina, M. L., *J. Chromatogr. B* 2008, *875*, 254–259.

[32] Le Potier, I., Franck, G., Smadja, C., Varlet, S., Taverna, M., *J. Chromatogr. A* 2004, *1046*, 271–276.

[33] Altria, K. D., *Chromatographia* 1993, *35*, 177–182.

[34] Altria, K. D., Fabre, H., *Chromatographia* 1995, *40*, 313–320.

[35] Zhang, H., Le Potier, I., Smaja, C., Zhang, J., Taverna, M., *Anal. Bioanal. Chem.* 2006, *386*, 1387–1394.

[36] European Pharmacopoeia, 4th ed. *European Directorate for the Quality of Medicines (Council of Europe)*, Strasbourg 2004, pp. 3843–3849.

[37] Sokoließ, T., Köller, G., *Electrophoresis* 2005, *26*, 2330–2341.

