**Chiral micellar electrokinetic chromatography-laser induced fluorescence
of amino acids in foods: analysis of vinegars**

*Davinia Carlavilla¹, M. Victoria Moreno-Arribas¹, Salvatore Fanali²,
Alejandro Cifuentes^{1*}*

¹Department of Food Analysis, Institute of Industrial Fermentations (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain.

²Institute of Chemical Methodologies, CNR, Area della Ricerca di Roma 1, Via Salaria Km 29,300, I-00016 Monterotondo, Roma, Italy.

Running Title: Chiral analysis of amino acids in vinegars by MEKC-LIF

Corresponding author: Dr. A. Cifuentes, acifuentes@ifi.csic.es; **Fax:** +34 91 5644853; **Tel:** +34 91 5622900

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Abbreviations: D-aa, D-amino acid; GABA, γ -aminobutyric acid.

SUMMARY

The formation of D-amino acids (D-aa) in many fermented foods depends, among other factors, on the particular fermentation conditions, the action and autolysis of the microorganisms involved. In this sense, the analysis of chiral amino acids is an interesting analytical strategy for food scientists, since these compounds can be used as bacterial markers and can help e.g., to detect adulterations, microbiological contaminations, etc. In this work, a fast and sensitive method based on micellar electrokinetic chromatography with laser induced fluorescence (MEKC-LIF) has been developed to analyze and quantitate L- and D-aa in vinegars. The chiral-MEKC-LIF procedure uses 100 mM sodium tetraborate, 30 mM SDS and 20 mM β -CD at pH 9.7 as running buffer, obtaining a good separation of the main vinegar L/D amino acids previously derivatized with fluorescein isothiocyanate. Namely, L/D proline, alanine, arginine, glutamic and aspartic acid, plus the non chiral amino acid γ -aminobutyric acid are separated in less than 20 min with high efficiency (up to 720,000 plates/m) and good sensitivity (LODs lower than 16.6 nM were achieved). Several D-aa were detected and quantified in balsamic, sherry, white wine, and cider vinegars using this MEKC-LIF procedure, observing interesting differences in their L- and D-aa profiles and contents.

1. INTRODUCTION

Analysis of chiral molecules is of special importance in different areas of knowledge including pharmaceutical, clinical or environmental fields. Enantioselective separations can also be very helpful in food science where they can be used to identify adulterated foods and beverages, to control and monitor fermentation processes, to evaluate age, to study treatment and storage effects and/or to analyze some flavor components [1].

In this sense, chiral analysis of amino acids is a remarkable methodology that can provide important information on adulteration and quality of foods [2, 3]. For instance, analysis of chiral amino acids in vinegars has been shown to be a powerful method to detect different adulterations including the detection of synthetic vinegars [4, 5]. Up to now, HPLC and GC have been the techniques of choice to carry out this type of enantiomeric separations [4-6], providing in some cases unequivocal results. However, these techniques may exhibit some drawbacks because can use expensive chiral-columns, the procedures for sample preparation are frequently laborious and time consuming and separations can be lengthy (in some cases upto 50 min) [4,5]. Also, in some GC methods the derivatizing procedure does not work for some basic amino acids such as arginine [4, 5]. Considering these drawbacks and taking into account the economic impact derived

from the chiral analysis of many foods, including vinegars, new analytical procedures able to overcome these limitations would be very useful.

The remarkable possibilities of capillary electrophoresis (CE) for the separation of chiral compounds were first demonstrated by Gasmman et al. in 1985 [7], and this technique has been applied to multiple chiral separations [3, 8-9]. However, one of the main limitations of CE is the low sensitivity that the common detectors based on UV absorbance provide, mainly due to the narrow optical path-length used as detection cell (i.e., the capillary internal diameter). Although some other strategies such as chiral-CE-MS have been proposed [10], the use of laser-induced fluorescence (LIF) together with derivatizing reagents (e.g., *o*-Phthaldialdehyde, OPA, or Fluorescein isothiocyanate, FITC) seems to be one of the best alternatives to overcome the sensitivity limitation of CE [11-15]. FITC has been frequently chosen as fluorescent label for amino acids since its excitation wavelength matches the 488 nm light of the argon laser, the derivatives are easily formed, have good electrophoretic properties and generate strong fluorescence signals [11]. In spite of these advantages, few papers have been devoted to the chiral separation of FITC-amino acids by CE [16-20]. However, to our knowledge, no MEKC-LIF method has been developed so far to separate and quantitate the derivatized L- and D-amino acids usually found in vinegars. The goal of this work was to develop a new MEKC-LIF method for the easy, sensitive and fast determination of chiral amino acids in vinegars.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. Dextrin 10, β -cyclodextrin (β -CD), from Fluka (Buchs, Switzerland), hydroxypropyl- β -cyclodextrin and dimethyl- β -cyclodextrin (both from Beckman Instruments, California, U.S.A.) were used as chiral selectors. One of these compounds together with sodium dodecyl sulphate (SDS) from Acros Organics (New Jersey, U.S.A.) and boric acid from Riedel-De Hen (Seelze, Germany) were used for the MEKC running buffer. A water solution containing 1M sodium hydroxide from Panreac Qumica S.A. (Barcelona, Spain) was used to adjust the pH of the buffers and 0.1M was used to rinse the capillary. The buffers were stored at 4 C and warmed at room temperature before use. Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA). Fluorescein isothiocyanate (FITC, from Fluka, Buchs Switzerland), dissolved in acetone analytical grade (Merck, Darmstadt, Germany). L- and D-amino acids and GABA (γ -aminobutyric acid) used for the standard were from Sigma (St. Louis MO, USA).

2.2 Samples

Twelve different vinegars were studied in this work (Table 1). They were purchased in local markets. These vinegars were selected according to the

information provided on the bottle label, in relation to their origin, characteristics and ageing in barrels. All samples were used after filtration (0.5 mm filter) to remove any solid matter.

2.3 Standard solutions

Quantitative values were assessed by injecting (in triplicate) five different concentrations of the standard solutions containing L- and D-amino acids and GABA at different concentrations. The calibration curves were calculated plotting corrected peak area (i.e., area/time) versus amino acid concentration (expressed as $\mu\text{g/ml}$) and the results are given in Table 2.

2.4 Derivatization procedure.

The FITC derivatization procedure [19] consisted of mixing an aliquot of 625 μl of the sample (or the amino acid standard solution) with 1375 μl of water and 7 ml of 355 mM sodium tetraborate buffer at pH 10. This mixture was adjusted to pH 10 by adding 1 M sodium hydroxide. Water was added till a final volume equal to 10 ml. 100 μl of this final solution were mixed with 200 μl of a 3.75 mM FITC solution in acetone. The reaction took place overnight in darkness at room temperature. After derivatization, samples were diluted with water (balsamic vinegars (1:20, v/v), sherry vinegars (1:15, v/v) and the other types of vinegars investigated (1:10, v/v)), prior to their injection in the MEKC-LIF.

2.5 MEKC-LIF conditions

Analysis of L- and D-amino acids was carried out in triplicate in P/ACE 2100 CE apparatus Beckman Instruments (Fullerton, CA, USA) equipped with an Ar⁺ laser at 488nm (excitation wavelength) and 520 nm (emission wavelength) from Beckman Instruments (Fullerton, CA, USA) to detect FITC-amino acids. Bare fused-silica capillary was purchased from Composite Metal Services (Worcester, England). The dimensions of capillary were 50 cm to the detection window, 57 cm total length and 50 µm i.d. and was thermostated at 25.0 °C. Injections were made at the anodic end using N₂ pressure of 0.5 p.s.i. (3.45 kPa) for 5 s and the applied voltage was 20 kV. The P/ACE 2100 CE instrument was controlled by a PC running the System GOLD software from Beckman.

Before first use, new capillaries were preconditioned by rising with 0.1 M NaOH for 30 min. Washing protocol between runs and separation buffer were optimized. Thus, the conditions that provided better resolution and reproducibility were as follows: at the beginning of each run, the capillary was rinsed with 0.1 M NaOH for 1 min, followed by 2 min with Milli-Q water, and then equilibrated with the run buffer (100 mM sodium tetraborate, 30 mM SDS and 20 mM β-CD adjusted to pH 9.7 with 1M NaOH) for 5 min. At the end of the day, the capillary was rinsed with Milli-Q water for 5 min and then nitrogen was passed for 2 min. The capillary was stored overnight without water inside.

3. RESULTS AND DISCUSSION

A group of five chiral amino acids (proline, alanine, arginine, glutamic and aspartic acid) plus the non-chiral amino acid GABA was selected to carry out this study. This selection was based on the fact that these six amino acids are usually responsible for more than 90% of the amino acidic content in vinegars [4, 5]. Moreover, since a total of ten enantiomers plus GABA were included in this study, it can be expected that the chiral-MEKC-LIF profiles obtained from these eleven compounds can provide enough information to characterize any vinegar [4, 5].

3.1 Chiral-MEKC-LIF of amino acids: method development and figures of merit.

As mentioned above, to our best knowledge, no chiral-MEKC-LIF analysis has been reported so far to analyze the main chiral amino acids from vinegars. In order to find suitable conditions to achieve their analysis, the conditions already developed by our group for orange juices amino acids, including the washing routine procedure between injections [19], were initially tested in some of the vinegars of Table 1. However, due to the high concentration of Ala in vinegars compared to that in orange juices, no suitable separation could be achieved observing an important overlapping between Ala and one of the FITC peaks. Moreover, the washing routine was also insufficient to get reproducible

results between injections probably due to a higher matrix effect from vinegars compared to orange juice samples. Namely, the use of a 5 min washing step with water followed by a 5 min washing with the running buffer [19] provided relative standard deviations (%RSD_{n=5}) for analysis times as high as 8 % for vinegars. In order to overcome these limitations different washing routines (including sodium hydroxide) and background electrolytes (BGE, 100 mM sodium tetraborate, 30 mM SDS and 20 mM β -CD as chiral selector adjusted to pH values of 9.1, 9.4, 9.7 and 10.0) were tested. Although the best resolution was obtained with the BGE at pH 10, the total analysis time was too long (ca. 40 min). Therefore, the selected BGE was 100 mM sodium tetraborate, 30 mM SDS and 20 mM β -CD at pH 9.7 since this running buffer provided adequate resolutions within much shorter analysis times (ca. 20 min). Moreover, the use of the following washing conditions: 0.1 M NaOH for 1 min, followed by water for 2 min and a last rinse with running buffer for 5 min provided %RSD_{n=5} values lower than 1% for analysis time repeatability. An example of the electropherograms typically obtained using these experimental conditions is given in Figure 1. As can be seen, the eleven chiral standard amino acids plus the FITC peaks (marked with an asterisk) can be separated in less than 20 min by using this procedure, being L- and D-Pro (peaks 3 and 4, respectively, in Figure 1), the compounds separated with a lower resolution. Namely, a resolution value (R_s) equal to 0.6 (calculated as $(t_{D-Pro} - t_{L-Pro})/w_{D-Pro}$, where t_{D-Pro} and t_{L-Pro} are the migration times for D-Pro and L-Pro, and w_{D-Pro} is the baseline peak width for the last migrating peak, i.e.,

D-Pro in this case) was obtained. Although different concentrations of β -CD were tested as well as other chiral selectors such as hydroxypropyl- β -CD, dimethyl- β -CD and Dextrin 10, no improvement of enantiomeric resolution was observed. Therefore, the mentioned buffer (i.e., 100 mM sodium tetraborate, 30 mM SDS and 20 mM β -CD at pH 9.7) was selected for all the next experiments. Moreover, it can be also seen in Figure 1 that the peak from FITC migrating at 14 min (marked with an asterisk) does not interfere with L-Ala (peak 7) using this new chiral BGE. Interestingly, these MEKC-LIF conditions also provided very high efficiencies with values ranging from 254,000 plates/m for L-Asp to 720,000 plates/m for L-Arg. Also, the limits of detection (LOD) were calculated for a signal/noise ratio equal to three, obtaining LOD values ranging from 45.7 nM for L-Asp to 16.6 nM for GABA under these conditions.

3.2 Quantitative determination of L- and D-amino acids in vinegars.

Once the figures of merit of the MEKC-LIF method were determined, the procedure was applied to determine chiral amino acids in vinegars. Thus, Figure 2 shows some representative electropherograms obtained from three vinegars. Namely, the electropherograms correspond to a balsamic vinegar (Figure 2A), a sherry vinegar (Figure 2B) and a vinegar from white wine with extracts of herbs (Figure 2C). Characterization of the different L- and D- amino acids was carried out by adding increasing amounts of standard amino acids and subsequent MEKC-LIF analysis as shown in Figure 3.

From a first sight of Figure 2, it could be tentatively concluded that the highest content of L-aa and D-aa correspond to the balsamic vinegar compared to the other two, while the sherry vinegar showed intermediate values and the vinegar from white wine showed the lowest content of L- and D- amino acids. In order to adequately determine the amount of L-aa and D-aa of the 12 vinegars studied in this work (see Table 1), calibration curves were obtained for the 11 L-aa and D-aa, injecting in triplicate five different concentrations in the range indicated in Table 3 for each amino acid. As can be seen in Table 3, in general good linearity was obtained in all cases, with correlation coefficients, r^2 , up to 0.999 for L-Glu and D-Glu. Using these calibration curves the L-aa and D- content of the eleven amino acids was determined for the 12 vinegars (also injected in triplicate) and the results are given in Table 3. As can be seen, the MEKC-LIF method allows the quantitative determination of these amino acids with RSD values lower than 5%, comparing reasonably well with the reproducibility achieved by using the most common procedure by GC [4, 5]. However, an interesting advantage of our procedure compared to that of GC is the much less labor required. Thus, the purification and derivatization of amino acids prior to GC requires more than ten different steps [4, 5], while our procedure only needs the addition of the reaction buffer and the fluorescent probe.

As can be seen in Table 3, the presence of L- and D- amino acids was detected in all the vinegars studied. The individual D- and L-amino acids values

and the percentage of D-aa of these vinegars are similar to those obtained in samples of traditional and commercial vinegars from different origins previously investigated by other methods [4, 21, 22]. From the analysis of the quantitative results given in this table, the next conclusions can be achieved: the four balsamic vinegars (samples 1 to 4 in Table 3) show the highest absolute amounts of L- and D-aa compared to the rest of vinegars studied, therefore, this could be used as a good indicator to distinguish this type of vinegar from the rest. Also, these vinegars present the higher relative percentage of D-Pro in comparison to the rest of vinegars studied. Proline together with arginine are the amino acids that suffer the most marked changes during the industrial production of vinegars [23, 24]. Furthermore, higher amounts of D-Pro can be considered a marker for maturation of balsamic vinegars [4], and they are attributed either to microbial enzymatic activity and the Maillard reaction that takes place in the course of maturation of these vinegars [5].

In comparison with balsamic vinegars, sherry vinegars contain much lower amounts of L-amino acids. However, higher relative amounts of D-aa were found in these vinegars, especially for the amino acids Arg and Ala, and to a lesser extent for Glu and Asp. Similar results could not be found in literature for D-Arg, since this enantiomer could not be previously analyzed as mentioned above [4, 5, 22, 21]. However, the major content of the D-enantiomers of the amino acids Ala, Glu and Asp in vinegars made from sherry was also detected in previous works

[4]. It may be due to the action of bacterial racemases on L-Aas and by autolysis of bacteria which contain these free D-amino acids in their cytoplasm as well as in their cell wall [25] indicating that these vinegars have been produced by microbial fermentation and not by dilution of synthetically produced acetic acid [4]. The presence of the last D-enantiomers is more evident for vinegar 5, suggesting that a higher racemization of free amino acids by acetic bacteria metabolism has occurred, probably favored by the lower pH of this vinegar (Table 1).

In the rest of the vinegars analyzed (samples 9-12 in Table 3), L-Pro also was the major amino acid. In contrast to previous studies [4], this amino acid was detected in the two white wines vinegars examined, probably due to the different grape varieties used. In general, these vinegar samples contain low amounts of the D-aa enantiomers, with exception of cider vinegar, in which higher amounts of D-Arg and of the typical amino acids for bacterial fermentation, D-Ala and D-Glu, were detected.

4. CONCLUDING REMARKS

In this work, a chiral-MEKC-LIF procedure has been developed that allows the fast and sensitive analysis of the eleven main L- and D-amino acids typically found in vinegars. The separation of the eleven compounds can be achieved in a relatively short time (less than 20 min) with efficiencies up to 720,000 plates/m and LODs down to 16.6 nM. It has been demonstrated that the

MEKC-LIF profiles obtained from these eleven compounds can be considered as a useful tool to characterize the vinegars studied in this work.

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FIGURE LEGENDS

Figure 1. Chiral MECK-LIF separation of standard FITC-amino acids. (1) D-Arg; (2) L-Arg; (3) L-Pro; (4) D-Pro; (5) GABA; (6) D-Ala; (7) L-Ala; (8) D-Glu; (9) L-Glu; (10) D-Asp; (11) L-Asp; (*) peaks from FITC. Capillary with 50 cm of detection length, 57 cm total length and 50 μm i.d.. Temperature: 25.0 $^{\circ}\text{C}$. Running buffer: 100 mM sodium tetraborate, 30 mM SDS and 20 mM β -CD at pH 9.7. Injections were made at the anodic end using N_2 pressure at 0.5 p.s.i. (3.45 kPa) for 5 s and the applied running voltage was 20 kV. Fluorescence detection: 488 nm (excitation wavelength) and 520 nm (emission wavelength).

Figure 2. FITC-amino acids in vinegars. (A) Vinegar 1 (balsamic vinegar), (B) Vinegar 5 (Sherry vinegar), and (C) Vinegar 12 (white wine with extracts of herbs). All the conditions as in Figure 1.

Figure 3. Chiral-MEKC-LIF electrophoregrams of the vinegar 1 (A); vinegar 1 plus 10 $\mu\text{g/ml}$ of D-Asp (B); and vinegar 1 plus 40 $\mu\text{g/ml}$ of D-Asp (C). All the conditions as in Figure 1.

Table 1. Description of the vinegar samples analyzed in this work.

No.	Vinegar	Origin	Content of acetic acid (%)	pH
1	Aceto Balsámico di Modena	Italy	6	3.00
2	Aceto Balsámico di Modena (coloured with caramel)	Italy	6	3.12
3	Aceto Balsámico di Modena	Italy	6	3.06
4	Aceto Balsámico di Modena (matured in oak barrels)	Italy	6	2.89
5	Sherry vinegar (aged in oak barrels)	Spain	8	2.70
6	Sherry vinegar (aged in oak barrels)	Spain	8	2.94
7	Sherry vinegar (aged in oak barrels for 5 years)	Spain	7	2.85
8	Sherry vinegar ("Pedro Ximenez" wine)	Spain	7	2.79
9	White wine vinegar	Spain	6	2.87
10	Cava vinegar	Spain	6	2.90
11	Cider vinegar	Spain	5	3.19
12	Wine vinegar with extracts of herbs	Italy	6	2.95

Table 2. Linear range and calibration curves for the quantitative determination of D- and L- amino acids.

Amino acid	Concentration range ^{a)} [µg/ml]	Equation ^{b)}	Correlation coefficient (r ²)
D-Arg	55 - 0.1	$y = 0.018 + 0.091x$	0.997
L-Arg	400 - 1	$y = 1.181 + 0.069x$	0.993
D-Pro	150 - 0.1	$y = 0.407 + 1.675x$	0.991
L-Pro	900 - 1	$y = 0.946 + 1.287x$	0.983
GABA	200 - 0.1	$y = 1.030 + 2.479x$	0.987
D-Ala	30 - 0.1	$y = 0.326 + 3.510x$	0.990
L-Ala	220 - 1	$y = 0.676 + 2.634x$	0.989
D-Glu	50 - 0.1	$y = 0.800 + 1.640x$	0.999
L-Glu	100 - 1	$y = 0.841 + 1.330x$	0.999
D-Asp	25 - 0.1	$y = 0.325 + 1.319x$	0.985
L-Asp	150 - 1	$y = 1.355 + 1.776x$	0.993

^{a)} five different concentrations were used for each calibration curve and each concentration was injected in triplicate.

^{b)} y = amino acid peak area ; x = amino acid concentration given in µg/ml.

Table 3. L- and D-amino acids content (µg/ml) determined in the twelve vinegars analyzed in this work.

Vinegar No.	Arg			Pro			GABA	Ala			Glu			Asp		
	L- (RSD)	D- (RSD)	% D	L- (RSD)	D- (RSD)	% D		L- (RSD)	D- (RSD)	% D	L- (RSD)	D- (RSD)	% D	L- (RSD)	D- (RSD)	% D
1	192.0 ^{a)} (3.3) ^{b)}	4.1 ^{a)} (1.8) ^{b)}	2.1 ^{c)}	857.2 (3.3)	58.4 (0.3)	6.3	78.9 (4.2)	192.5 (1.4)	13.5 (0.4)	6.5	17.6 (4.4)	2.7 (0.6)	13.3	113.5 (0.4)	14.9 (5.2)	11.6
2	361.7 (4.6)	10.2 (3.6)	2.7	669.5 (2.4)	83.2 (1.5)	11.0	62.9 (3.4)	186.1 (1.2)	4.6 (1.8)	2.4	13.7 (0.7)	2.4 (5.3)	14.9	111.2 (0.7)	21.0 (2.0)	15.9
3	373.3 (1.4)	6.2 (4.5)	1.6	373.4 (3.0)	116.6 (3.5)	23.8	118.6 (2.6)	115.0 (2.1)	6.7 (0.3)	5.5	27.4 (2.1)	1.6 (1.8)	5.5	53.9 (1.6)	5.2 (3.6)	8.8
4	340.7 (3.5)	10.2 (4.6)	2.9	712.8 (2.1)	48.4 (1.7)	6.4	159.6 (1.3)	82.0 (4.6)	9.1 (0.2)	9.9	67.6 (0.2)	2.9 (3.2)	4.2	50.8 (0.3)	6.9 (3.1)	11.9
5	136.7 (3.2)	9.3 (2.6)	6.3	68.2 (3.8)	1.2 (3.8)	3.4	9.9 (4.8)	8.6 (3.3)	2.1 (1.2)	19.7	13.4 (1.4)	3.5 (3.0)	20.5	9.4 (3.7)	2.3 (1.0)	19.7
6	33.2 (4.7)	3.3 (3.6)	8.9	40.9 (1.8)	2.2 (2.8)	2.8	9.9 (0.9)	4.9 (4.4)	1.8 (0.8)	27.1	15.0 (2.0)	1.2 (2.8)	7.4	25.2 (1.9)	3.2 (4.1)	11.3
7	37.5 (2.6)	6.0 (4.9)	13.9	57.7 (1.2)	0.9 (3.9)	3.9	13.4 (2.1)	3.9 (1.8)	0.8 (0.9)	17.4	15.8 (1.6)	0.5 (1.1)	3.0	17.2 (1.4)	1.1 (3.4)	6.1
8	132.9 (1.6)	20.3 (3.3)	13.2	38.7 (2.7)	1.4 (3.4)	3.4	21.0 (1.0)	8.4 (4.1)	0.3 (4.5)	3.4	22.8 (2.9)	0.3 (2.4)	1.3	8.2 (1.4)	1.4 (2.6)	14.9
9	54.4 (2.3)	0.8 (1.8)	1.4	18.9 (4.2)	0.1 (0.1)	0.3	7.9 (1.5)	27.9 (1.4)	2.9 (0.2)	9.5	8.2 (4.0)	0.1 (3.5)	1.5	9.9 (1.3)	0.3 (3.5)	2.7
10	6.7 (4.2)	0.4 (3.9)	5.9	31.6 (1.5)	0.6 (1.5)	1.8	1.6 (2.4)	10.5 (4.3)	0.1 (1.9)	1.0	13.3 (2.8)	0.1 (1.1)	0.5	7.5 (4.9)	0.2 (3.1)	2.3
11	37.7 (3.9)	2.8 (1.1)	7.0	68.7 (2.2)	0.4 (1.7)	0.5	2.3 (1.5)	36.4 (0.9)	8.5 (4.8)	19.0	26.9 (1.7)	1.4 (0.8)	4.9	16.5 (1.7)	0.6 (1.7)	3.9
12	18.6 (4.9)	0.6 (1.9)	3.0	14.8 (3.3)	0.6 (4.7)	3.7	0.8 (2.6)	1.0 (1.7)	0.2 (2.4)	13.9	8.5 (4.9)	0.16 (1.1)	1.9	11.5 (0.7)	0.5 (1.0)	3.9

^{a)} Average values of three replicates given in µg/ml.^{b)} Relative standard deviation (% RSD_{n=3}).^{c)} Relative percentage of D-AA calculated as 100 × D-aa/(D-aa+L-aa)

Figure 1

Figure 2

Figure 3