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OCCURRENCE OF D-AMINO ACIDS IN FOOD

DETECTION BY CAPILLARY GAS CHROMATOGRAPHY AND BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH L-PHENYLALANINAMIDES AS CHIRAL SELECTORS

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SUMMARY

The presence of D-amino acids was investigated in dairy products, in raw ham and in roasted coffee. The analysis was carried on by capillary gas chromatography, using a very stable tetraamidic selector derived from L-phenylalanine, which allowed the detection of D-alanine, D-aspartic acid, D-glutamic acid and other D-amino acids. Moreover, D-glutamic acid and D-alanine were detected also by reversed-phase high-performance liquid chromatography, using L-phenylalaninamide and copper(II) acetate as chiral additives to the eluent. The presence of D-amino acids may be ascribed to thermal racemization or to microbial activity.

INTRODUCTION

Amino acids in proteins are generally assumed to occur as L-enantiomers. However, D-amino acids are quite common in nature as constituents of bacterial cell walls (D-alanine, D-glutamate) and of several antibiotics[1]. In addition, heat and alkali treatments, used for food processing, have been shown to produce racemization of "natural" L-amino acids[2–5], thus affecting the food quality by decreasing the nutritional value, or producing xenobiotics of unknown biological properties.

In the last few years we have been involved in the study of the mechanism of chiral recognition by both gas chromatography (GC)[6] and high-performance liquid chromatography (HPLC)[7]. In particular, we synthesized a novel series of chiral tetraamidic selectors containing L-phenylalanine, which enabled good separations of N-trifluoroacetyl (TFA) alkyl esters of D,L-amino acids by GC[8], and devised new copper(II) complexes as chiral additives to the eluent, which yielded good separations of D,L-dansyl (Dns) amino acids in HPLC on a C_{18} column[9,10].

In a general project aimed at studying the presence of xenobiotics in food, we have investigated the occurrence of D-amino acids in processed foods, in particular those which undergo a severe heat treatment such as roasted coffee, ultra-high temperature (UHT) milk and in products obtained by fermentation processes (yoghurt

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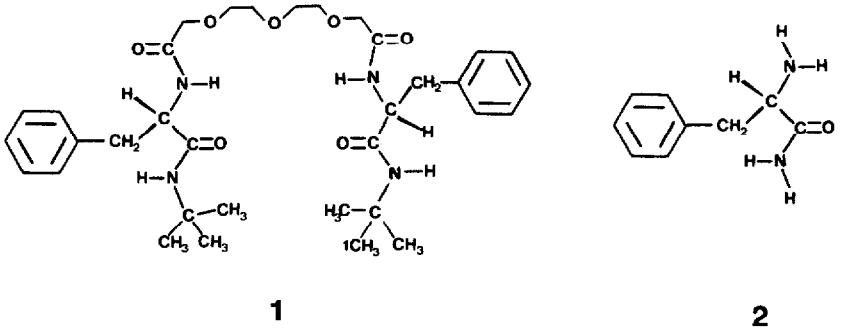

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Fig. 1. Structure of the chiral selectors L-Phe-3-O-TA (**1**) and L-Phe-A (**2**).

and cheese). For this work we used the selector trioxaundecanoyl tetramide, **1** (L-Phe-3-O-TA), as a stationary phase for GC analysis, and L-phenylalaninamide, **2** (L-Phe-A), with copper acetate as a chiral additive for the HPLC eluent (Fig. 1).

EXPERIMENTAL

Gas chromatography

GC analyses were carried out with a Dani 8500 instrument, from Dani (Monza, Italy), equipped with a flame ionization detector, using wall-coated open-tubular (WCOT) fused-silica columns (0.25 mm × 20–25 m I.D.), injector and detector at 250°C, with helium as the carrier gas at a pressure of 0.75 bar. Bare silica columns (Supelco, Bellefonte, PA, U.S.A.) were leached with 20% HCl at 120°C, deactivated with barium carbonate[11] and then statically wall-coated with a dichloromethane solution of the chiral tetraamide L-Phe-3-O-TA (0.15%) and of the commercial silicone gum OV-101 (0.15%) (Carlo Erba, Italy). Other columns were statically coated with the same procedure, using tetraamide L-Phe-3-O-TA and Carbowax 20M (Carlo Erba), instead of OV-101. Columns were conditioned for 10 h at 180°C. The numbers of effective theoretical plates per metre, N/m, were calculated under isothermal conditions at 120°C and 0.75 atm, with split injection of *n*-dodecane for OV-101 columns and of methyl dodecanoate for Carbowax 20M columns.

Derivatization of standard D,L-amino acids for GC

N-TFA-amino acid methyl, isopropyl or butyl esters were prepared as follows: 10 mg of D,L-amino acid were dissolved in 2 ml of 1 *M* HCl in alcohol (methanol, 2-propanol or butanol respectively) and kept in a sealed tube at 90°C for 1 h. The sample was evaporated, the residue was dissolved in dry dichloromethane (4 ml) and treated with trifluoroacetic anhydride (0.5 ml) in a sealed or a screw-capped tube at 70°C for 1 h. After cooling, the tube was opened with care, the solvent was evaporated and the residue was redissolved in dichloromethane and analyzed. No racemization of pure L-enantiomers was observed during this treatment.

Liquid chromatography

Chromatographic analyses were carried out with a Waters 440 Model liquid

TABLE I

ELUTION PROGRAMME USED FOR THE SEPARATION OF D,L-DNS-AMINO ACIDS BY HPLC

t (min)	*Flow-rate (ml/min)*	*Buffer A (22% acetonitrile)*	*Buffer B (50% acetonitrile)*
0	0.5	100	0
68	0.5	68	32
85	0.8	57	43
110	0.8	0	100
130	0.2	100	0

chromatograph, equipped with a UK-6 septumless injector, a 6000 A pump, a Model 420 fluorescence detector and a Waters recorder (Waters Assoc., Milford, MA, U.S.A.). A C_{18} Novapak (4 μm, 15 cm $\times$ 0.4 cm) column (Waters Assoc.) was used. Two aqueous solutions containing 4 m*M* L-phenylalaninamide, 2 m*M* copper acetate and 0.3 *M* sodium acetate were prepared, the former (A) containing 22% of acetonitrile, the latter (B) 50%. The eluent was filtered and degassed under reduced pressure. The pH was adjusted to 7.3 with concentrated sodium hydroxide. The mixing was performed by means of a Waters automated gradient apparatus, according to the program shown in Table I.

Derivatization of standard D,L-amino acids for HPLC

To 50 μl of a 10 m*M* sample of the amino acids, 150 μl of an acetonitrile solution containing dansyl chloride (Dns-Cl) (3 mg/ml) and 300 μl of an aqueous solution of Li_2CO_3 (40 m*M*, pH 9.5) were rapidly added. The mixture was gently shaken until the turbidity disappeared and then allowed to stand at room temperature for 35–40 min, in the dark. The reaction was quenched by adding 50 μl of ethylamine. The reaction mixture can either be injected directly or dried with nitrogen and redissolved in doubly distilled water.

Extraction and derivatization of free amino acids from milk

A 20-ml volume of milk was diluted to 100 ml with distilled water, treated under stirring with 5 ml of trifluoroacetic acid to remove proteins and then centrifuged at 3000 *g* for 10 min. The supernatant was concentrated under vacuum to remove the trifluoroacetic acid, the residue was dissolved with 50 ml of water and extracted twice with 50 ml of diethyl ether to remove fats. The aqueous phase was filtered, evaporated to remove the organic solvent and passed through an ion-exchange column (30 cm $\times$ 1.5 cm) filled with a cation-exchange resin (Dowex 50W-X2), freshly regenerated with 60 ml of 2 *M* HCl. Amino acids were then eluted from the resin with 60 ml of 2 *M* HCl. The eluate was divided into two fractions, one for GC and the other for HPLC analysis, and evaporated to dryness. One fraction was dissolved in 1 *M* HCl in 2-propanol and treated according to the procedure reported for the derivatization of standard amino acids in GC. The fraction for HPLC analysis was dissolved in 2 ml of distilled water and ultrafiltered through a Millipore PLGC cellulose filter (Millipore, Milford, MA, U.S.A.) to remove proteins. A 1-ml volume of the ultrafiltered sample was diluted to 2 ml by adding a 30% aqueous methanol solution containing 0.1%

trifluoroacetic acid, and then passed through a Sep-Pak C_{18} cartridge (Waters Assoc.), previously conditioned by washing with 10 ml of HPLC-grade methanol, 10 ml of 0.1% trifluoroacetic acid in water and 10 ml of 0.1% trifluoroacetic acid in 20% aqueous methanol. The sample eluted from the cartridge was dried, redissolved in 0.1 *M* LiOH and 40 m*M* Li_2CO_3 to pH 7.5 and brought to a final volume of 4 ml. The derivatization procedure was performed on 50 μl of the sample at a time, by adding 150 μl of the acetonitrile solution of Dns-Cl (3 mg/ml) and 300 μl of the 40 m*M* Li_2CO_3 aqueous solution.

Extraction and derivatization of free amino acids from cheese

A 1-g of grated cheese was dissolved in 50 ml of distilled water, treated with 5 ml of trifluoroacetic acid and stirred for 2 min. The sample was centrifuged for 10 min at 3000 *g*, the supernatant was evaporated to remove trifluoroacetic acid, then diluted with 20 ml of water and extracted twice with 50 ml of diethyl ether to remove fats. The aqueous fraction was recovered, divided into two parts and evaporated to dryness. The first part was dissolved in 1 *M* HCl in methanol and treated according to the derivatization procedure for standard amino acids in GC. The second fraction was ultrafiltered and derivatized as reported for HPLC analysis of milk samples.

Extraction and derivatization of free amino acids from yoghurt

A 10-g amount of yoghurt was dissolved in 50 ml of distilled water, treated with 2 ml of trifluoroacetic acid, stirred for 2 min and centrifuged at 3000 *g* for 10 min. The supernatant was evaporated to remove the acid, diluted with water to 100 ml and extracted three times with 50 ml of dichloromethane. The aqueous fraction was evaporated to remove the organic solvent and was passed through a cation-exchange resin in the acid form, as reported for the milk treatment. Amino acids were recovered from the resin by eluting with 2 *M* HCl and were derivatized as reported for milk amino acids.

Extraction and derivatization of free amino acids from coffee

A 5-g amount of roasted coffee (Brasilian) was stirred for 2 min with 20 ml of hot water and filtered. The filtrate was evaporated to dryness and 0.5 g of residue were dissolved in 50 ml of methanol, filtered and evaporated to dryness. A 50-mg amount of the new residue was dissolved in 10 ml of 1 *M* HCl in methanol and kept at 80°C for 1 h in a screw-capped test-tube. The solution was evaporated, and the residue was dissolved in dichloromethane (3 ml) and derivatized with trifluoroacetic anhydride for GC. Green coffee was analyzed in the same way, starting with 5 g of a milled sample.

Extraction and derivatization of free amino acids from raw ham

A 5-g amount of raw ham, aged for 18 months (Parma technology), was homogenized for 5 min in 20 ml of warm water containing 2 ml of trifluoroacetic acid, and then centrifuged at 3000 *g* for 10 min. The supernatant was concentrated to dryness, dissolved in 50 ml of water and extracted with diethyl ether. The aqueous fraction was concentrated under vacuum and the residue was dissolved in 10 ml of 1 *M* HCl in methanol and derivatized for GC analysis as reported for standard amino acids.

TABLE II

COLUMN CHARACTERISTICS AND RESOLUTION FACTORS FOR N-TFA-D,L-AMINO ACID METHYL ESTERS

Column	*N/m*	*Length (m)*	*I.D. (mm)*	*Film (μm)*	*Resolution factors, $r = t'_L/t'_D$*							
					Ala	*Val*	*Leu*	*Nleu*	*Asp*	*Met*	*Phe*	*Glu*
A	1200	20	0.25	0.21	1.084	1.102	1.090	1.067	1.015	1.018	1.017	1.016
B	1850	25	0.25	0.15	1.037	1.050	1.043	1.034	1.006	1.011	1.010	1.009

RESULTS AND DISCUSSION

Food analysis of D,L-amino acids by capillary GC

The two chiral columns used were characterized by different polarities: A, less polar, carrying L-Phe-3-O-TA (**1**) on OV-101 silicone gum; B, more polar with L-Phe-3-O-TA on Carbowax 20M. The column properties and performance, with resolution factors $r = t'_L/t'_D$ of N-TFA-D,L-amino acid methyl esters, are reported in Table II.

The resolution, r, was obtained at 0.75 bar helium, with a temperature programme of 110–170°C at 3°/min, after an initial hold at 110°C for 4 min. The r values obtained with columns A and B for N-TFA-D,L-valine 2-propyl ester at 90°C were 1.130 and 1.095, respectively, and were considered satisfactory. Column A shows higher resolution, but B is more efficient.

In Fig. 2 gas chromatograms of a cheese sample, obtained with columns A and B, respectively, are compared. In many cases, the reversed elution order for alanine and valine, and for glycine and leucine, recorded on the two columns, was of help for identification. The resolution of D,L-valine and D,L-alanine is higher with column B, while D,L-aspartic and D,L-glutamic acids are better resolved on column A. Both columns can easily be fitted and used on gas chromatography–mass spectrometry (GC–MS) quadrupole instruments (Finnigan 1050, Hewlett-Packard 5970B), with operating parameters similar to those used for GC.

Milk. Samples of cow's milk, new, pasteurized, ultra-high temperature treated (UHT) milk and dried milk were tested. D,L-Norleucine was used as the internal standard for quantitative analysis. Because of their low amount in milk (60–200 ppm), the free amino acids were derivatized to 2-propyl esters, thus obtaining an higher response to the flame ionization detector than the methyl esters. Several amino acids were identified by their retention volumes and by GC–MS. The presence of alanine, valine, glycine, leucine, proline, aspartic acid, methionine, glutamic acid and phenylalanine was confirmed by comparison with the fragmentation patterns reported in the NBS library (of the Finnigan 1050 quadrupole). The most abundant amino acid detected was glutamic acid (40–130 mg/l), which derives also from the hydrolysis of glutamine during the derivatization step. A typical gas chromatogram of milk amino acids (as N-TFA-2-propyl esters) is shown in Fig. 3.

The percentages of racemization of free amino acids detected in milk, cheese, yoghurt, ham and coffee samples are summarized in Table III.

The presence of D-glutamic acid (3–5%) and D-aspartic acids (1–3%) was ob-

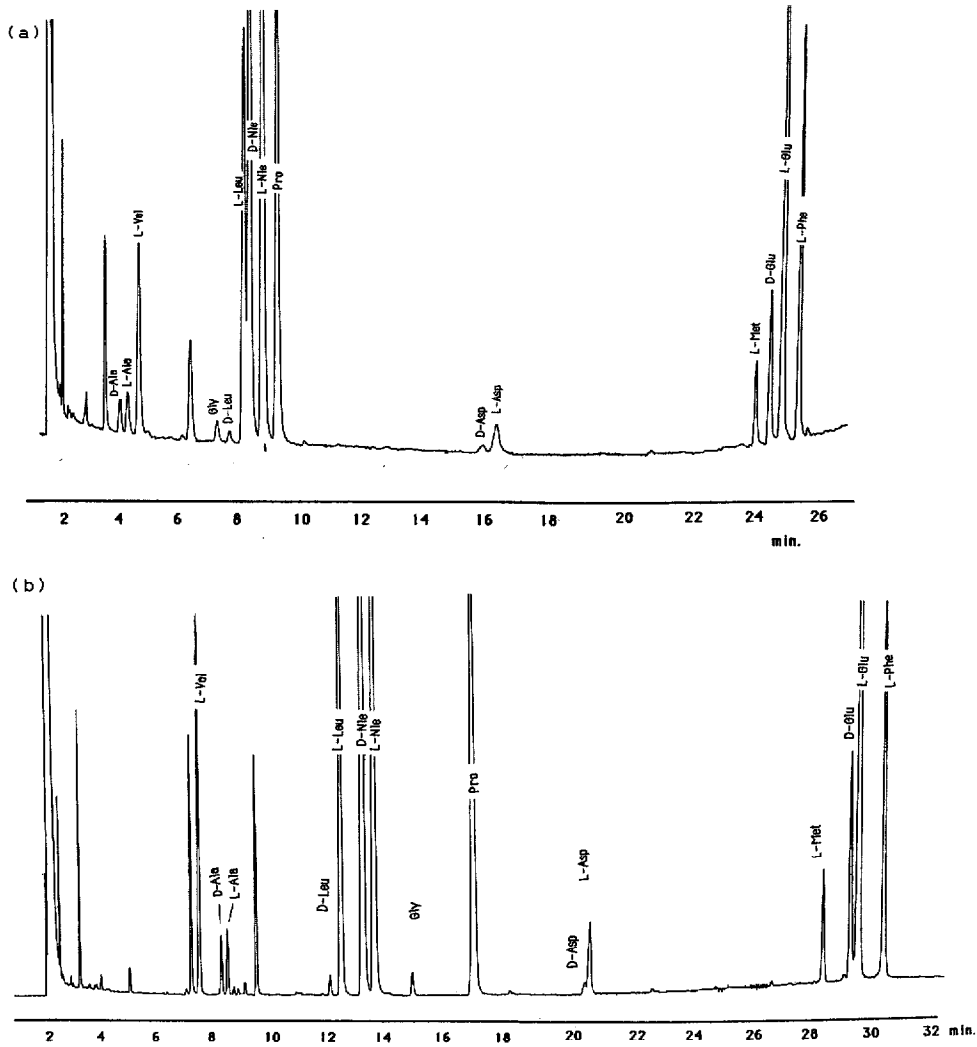

Fig. 2. Gas chromatograms of the enantiomeric separation of free amino acids (as N-TFA methyl esters) from aged cheese, recorded with a 20 m × 0.25 mm I.D. fused-silica column wall-coated with the chiral phase Phe-3-O-TA/OV-101 (a) and with a 25 m × 0.25 mm I.D. fused-silica column wall-coated with Phe-3-O-TA/CW (b). Temperature programme: 100–180°C at 4°/min after an initial hold at 100°C for 4 min.

served in each sample of new, pasteurized and UHT milk examined (twelve samples). Surprisingly, the percentage of D-derivatives was not influenced by the temperature of the thermal treatment. Only samples of dried milk showed more D-aspartic acid (4–5%), and D-alanine (8–12%). This is in contrast to previous reports on heated (60°C) dried milk[2–5]. In order to determine whether D-amino acids were also present in milk proteins, a sample of casein was precipitated with trifluoroacetic acid, and hydrolyzed with 6 *M* HCl at 100°C for 6 h. The hydrolyzate was derivatized and analyzed as reported for standard D,L-amino acids. In this case, the amounts (< 1%)

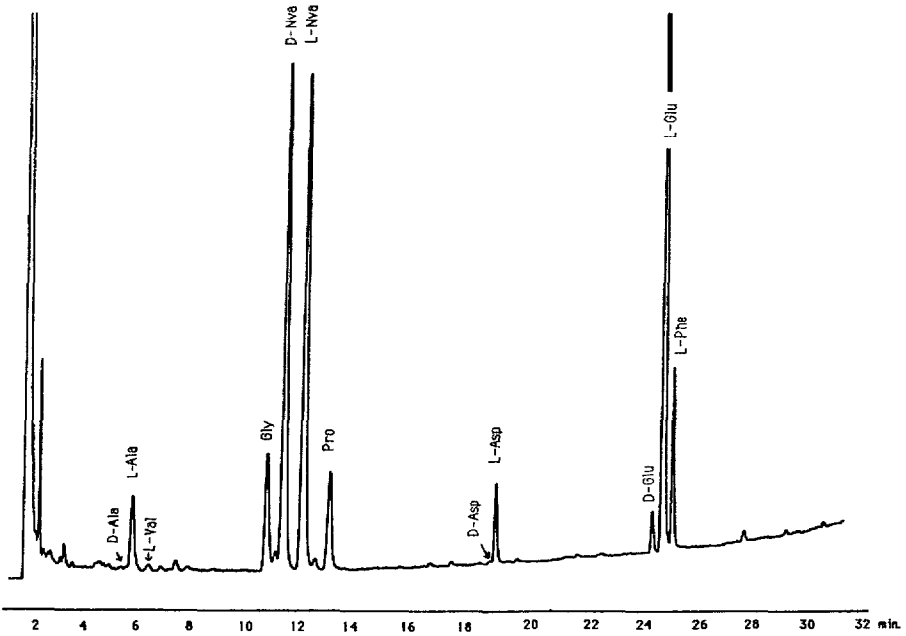

Fig. 3. Gas chromatogram of the enantiomeric separation of free amino acids (as N-TFA 2-propyl esters) from dried milk, recorded with a 20 m × 0.25 mm I.D. fused-silica column wall-coated with the chiral phase Phe-3-O-TA/OV-101. Temperature programme: as in Fig. 2.

of D-glutamic and D-aspartic acids detected were minimal, probably arising from the racemization induced by hydrolysis.

Yoghurt. An higher content of D-amino acids was detected in yoghurt samples, where the amount of D-alanine and D-glutamic acid was in some cases higher than that of the L-isomer (Table III). Amino acids were isolated by the same procedure as

TABLE III

RACEMIZATION OF FREE AMINO ACIDS IN COW'S MILK AND OTHER FOODS, DETECTED BY CAPILLARY GC WITH THE CHIRAL PHASE L-Phe-3-O-TA

Food	*Degree of racemization, D/D+L (%)*				
	Ala	*Leu*	*Asp*	*Glu*	*Phe*
New milk	3–4		2–3	2–3	
Pasteurized milk	3–4		1–2	3–5	
UHT milk	4–6		2–3	3–5	
Dried milk	8–12		4–5	3–4	
Yoghurt	64–68		20–32	53–66	
Aged cheese	20–45	2–7	8–35	5–22	2–13
Raw ham	0–1	<0.2		0–0.5	0–0.3
Roasted coffee			23–38	32–41	9–12
Green coffee				<0.2	

reported for milk, and derivatized to N-TFA 2-propyl esters. The amino acid pattern and the percentage of the D-enantiomers changed from sample to sample, probably on account of different strains of microorganisms.

Cheese. Microbial action is probably responsible for the presence of D-amino acids in aged cheese, which shows considerable amount of D-alanine, D-glutamic and D-aspartic acid. Minor quantities of D-leucine and D-phenylalanine were also detected, as shown in Table III.

Raw ham. Free amino acids, isolated from raw ham (Parma ham), were derivatized with 1 *M* HCl in methanol and then with trifluoroacetic anhydride. Only trace amounts of D-glutamic acid and D-phenylalanine (less than 0.5% as D/D+L) and about 1% of D-alanine were detected (Table III).

Roasted coffee. Chromatograms of roasted coffee were very much complicated by the presence of aroma components, and only aspartic acid, glutamic acid and phenylalanine were dependably determined, with D/D+L % values ranging from 10 to 40%. Several methods were employed in attempts to obtain clean GC profiles, including chromatography on ion exchangers, absorption with active carbons and solvent extraction. The best method was the simplest one; it is described in the Experimental section.

Analysis by HPLC with chiral eluents

D-Amino acids were determined as dansyl derivatives by HPLC on a C_{18} reversed-phase column, using L-phenylalaninamide/Cu(II) as a chiral eluent. Cheese, milk and yoghurt have been examined so far. A chromatogram of a cheese extract is presented in Fig. 4.

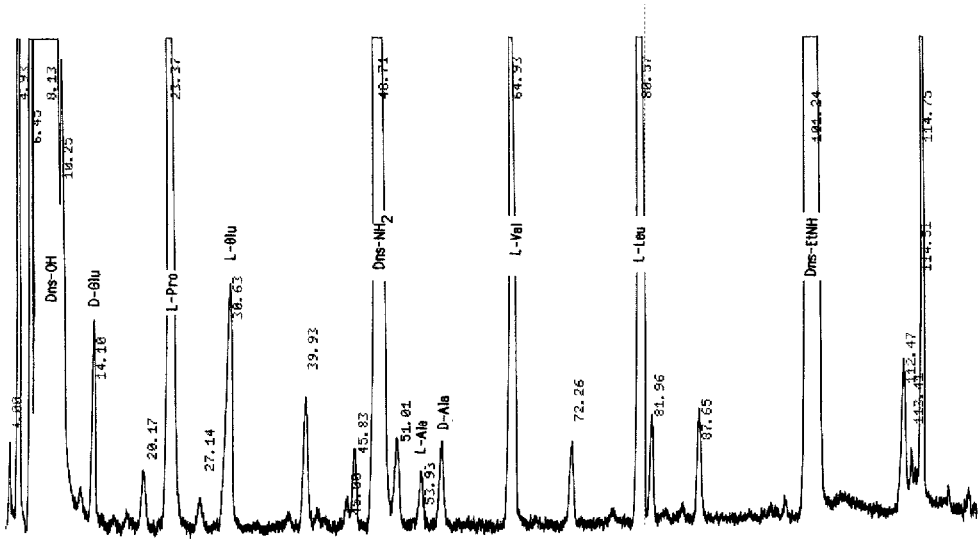

Fig. 4. HPLC chromatogram of the enantiomeric separation of free amino acids (as dansyl derivatives) from aged cheese, with the chiral additive L-Phe-A/Cu(II). Conditions: 4 m*M* L-Phe-A, 2 m*M* copper acetate, 0.3 *M* sodium acetate, pH 7.3 in acetonitrile and water; Novapak C_{18} column; gradient as reported in the Experimental.

Although many interfering substances were present in the matrix, which complicated the chromatograms, D-amino acids were detected and identified by comparison with authentic samples. In cheese, D-alanine and D-glutamic acid were quantified by HPLC and the results were found to be consistent with the GC data. In milk and yoghurt the analyses are still being carried out.

Undoubtedly, the present results raise questions concerning the quality of the food products examined and their sensory, nutritional and toxicological properties[12], which will be discussed in a more suitable context. In the present paper, we wish to point out that D-amino acids are surprisingly present not only in harshly treated foods, such as roasted coffee, but also in fermentation products, such as cheese and yoghurt, while high-temperature short-time treatments do not affect the D/D+L values, *cf.*, *e.g.*, UHT milk. Finally, it must be stressed that, when approaching the analysis of complex mixtures such as those present in biological systems, the combination of different chromatographic techniques allows cross-checks of the results so as to avoid pitfalls of single methods or derivatization procedures.

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