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Determination of free L- and D-alanine in hydrolysed protein fertilisers by capillary electrophoresis

Luciano Cavani*, Claudio Ciavatta, Carlo Gessa

Department of Agroenvironmental Sciences and Technologies, Alma Mater Studiorum-University of Bologna, Viale Fanin 40, 40127 Bologna, Italy

Abstract

The objective of this study was to determine the degree of racemisation of hydrolysed protein fertilisers (HPFs) using an inexpensive and easy to handle analytical method for qualitative control of the products. Using a polyacrylamide coated capillary and a run buffer containing 0.1 *M* Tris–borate+2.5 m*M* EDTA-Na_2+0.1% sodium dodecylsulfate+10 m*M* β-cyclodextrin a quantitative separation of D- and L-alanine (Ala) was made from an not treated HPF sample derivatised with dansyl chlorine by capillary electrophoresis. The D-Ala:[D-Ala+L-Ala] ratio, called degree of racemisation (RD), was calculated. The analysis of ten commercial HPFs has shown that more than 60% of HPFs have an RD ≥40%, while only one product has shown an RD <5%. These results showed that most of the HPFs on the market are obtained with strong hydrolytic processes and high contents of D-amino acids are probably less effective as plant nutrients or even potentially dangerous to plants.

Keywords: Enantiomer separation; Degree of racemisation; Fertilisers; Alanine; Amino acids; Proteins

1. Introduction

The hydrolysed protein fertilisers (HPFs) are fertilisers obtained by chemical and/or enzymatic hydrolysis of different organic matrices (i.e. leather, skin, hair, etc.) and they typically contain free amino acids, oligopeptides and polypeptides. The HPFs are currently used mainly for foliar application to stimulate the plant metabolism. The content of free amino acids in HPFs (typically 5–40%) is an important parameter for their agronomic evaluation; this fraction shows the biostimulant properties [1]. The hydrolytic process, mostly when made at high temperature and with concentrate base or acid solutions, should produce high racemisation of the free amino acids [2]. Several negative or toxic effects of D-amino acids have been reported for living organism [3]. Therefore, the presence of D-amino acids in HPFs may be considered as a negative indicator of the quality of the fertiliser. The objective of this study was to introduce a useful and inexpensive analytical method for qualitative control of HPFs. Chiral separation of amino acids (AA) has been obtained using gas chromatography (GC) and high-performance liquid chromatography (HPLC). Recently, capillary electrophoresis (CE) has attracted increased attention on this topic [4]. The CE produces rapid and very high resolution of complex mixtures using small amounts of sample and volume of buffers. Chiral separation of amino acids has been

*Corresponding author. Tel.: +39-51-209-6209; fax: +39-51-209-6203.
E-mail address: l_cavani@hotmail.com (L. Cavani).

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obtained by CE with direct (enantiomeric) and indirect (diastereomeric) separation [4,5]. The direct approach is performed by the interaction of enantiomers with a chiral selector added to the background electrolyte (i.e. cyclodextrin, chiral chelator, crown ether, bile salts). Using CE it is possible to determine directly the chiral forms of some amino acids in several organic matrices without any preventive purification of the sample (i.e. biological fluids [6], pharmaceutical products [7], food products [8]).

Nevertheless, the determination of the chiral form of all amino acids is extremely complex and exceeds our objective. It is reasonable to characterise the degree of racemisation using only one representative amino acid in HPFs. Alanine is contained in HPFs and is very stable during the racemisation process under normal conditions (at 25 °C and pH 7.6 the racemisation half-life of Ala is 12 000 years [2]) and at high temperatures (a 100 °C and pH 7.6 the racemisation half-life is 120 days [2]). Furthermore, Ala is only partially degraded during hydrolysis compared to Trp, Asn, Gln and Ser [9]. Then, it is reasonable to think that the degree of racemisation of Ala is sufficiently representative of the strength of the racemisation process.

This study presents a method for chiral analysis of Ala in HPF without time consuming prepurification of samples. The free Ala were derivatised with dansyl chlorine (Dns-Cl) and the chiral separation was obtained by CE adding β-cyclodextrin to the background electrolyte.

2. Experimental

2.1. Reagents and product samples

Standard L-, D- and D,L-alanine were obtained from Sigma (St. Louis, MO, USA). All others reagents, at HPLC grade, were purchased from Fluka (Buchs, Switzerland).The HPFs are commercially available, their chemical characteristics are reported in Table 1.

2.2. Derivatisation

Derivatisation was carried out in a 0.5-ml Eppendorf microvial with pressure plug. The sample was properly diluted in Millipore water (normally 50 mg ml^{-1}), and an aliquot (50–100 μl) was dissolved in 50 μl of carbonate buffer (0.5 *M*, pH 8.5) and mixed with 150 μl of reagent (4 mg of Dns-Cl dissolved in 1 ml of acetonitrile). The mixture was homogenised and maintained at 65 °C in the dark for 45 min. Then, the reaction mixture was cooled, filtered at 0.45 μm (PTFE, Millipore, USA) and analysed.

2.3. Capillary electrophoresis

Experiments were performed using a Biofocus 3000 capillary electrophoresis system (Bio-Rad, Hercules, CA, USA) equipped with a UV detector. A polyacrylamide coated capillary silica tube (BioCap XL, Bio-Rad) cut to a total length of 50 cm (effective separation length of 45.4 cm)×50 μm I.D.

Table 1
Characteristics of HPF samples

HPF	Declared matrix	pH	EC[a] (dS/m)	Ash (%)	Water (%)	TOC[b] (%)	TNK[c] (%)	NH_4-N (%)	Na (mg/g)	K (mg/g)
1	Animal skin	6.9	5	8.0	32.0	29.5	9.0	1.3	27.0	0.48
2	Collagen and elastine	6.1	27	13.9	46.2	21.8	6.0	0.2	55.2	0.55
3	Animal skin	6.6	9	2.8	42.8	25.9	9.5	0.9	8.27	0.09
4	Animal skin	5.8	4	0.2	53.2	23.4	8.3	0.2	0.51	0.02
5	Animal meat and skin	7.5	22	15.9	38.0	22.2	6.8	0.3	53.5	0.60
6	Animal hair and coat	5.2	49	11.1	49.3	17.6	6.7	2.2	40.2	0.49
7	Alfalfa meal	6.2	22	10.6	47.1	19.8	4.8	0.3	28.9	13.4
8	Animal skin	4.8	1	4.4	49.4	21.4	7.7	0.6	4.67	0.11
9	Animal skin	7.9	22	14.8	42.8	17.0	3.4	0.1	44.6	0.41
10	Animal meat and skin	5.4	10	11.4	50.0	23.4	6.7	0.3	27.4	0.51

[a] Electrical conductivity.
[b] Total organic carbon.
[c] Total nitrogen Kjeldahl.

was used. Background electrolytes (BGEs) consisted of 0.1 *M* Tris–borate+2.5 m*M* EDTA-Na_2+0.1% sodium dodecylsulfate+7 *M* urea, pH 8.6 (Fluka). Sample injections were made at the cathode using a positive pressure of 2 p.s.i.×s (1 p.s.i.=6894.76 Pa). Separations were conducted at a field strength of 300 V cm^{-1} (15 kV overall) and the temperature of the capillary was maintained at 25 °C. Detection was achieved by UV absorbance at 240 nm. After detection, the area of each peak was integrated using BioFocus 3000 CE system integration software. All analyses were performed at least in triplicate.

3. Results

3.1. Optimisation of the separation method

The characterisation of free amino acids in HPF samples by CE was obtained using Dns-Cl as derivatising agent and 0.1 *M* Tris–borate+2.5 m*M* EDTA-Na+0.1% SDS+7 *M* urea (pH 8.6) as BGE in a previous work [10]. This method has still to be optimised: some amino acids comigrate and long run time (Fig. 1). It was possible though to characterise free amino acids in HPF samples with a satisfactory precision for quantitative analyses (Table 2). HPF samples are characterised by a high content in free glycine (Gly), alanine (Ala), proline (Pro) and hydroxyproline (Hyp) (Table 3), in agreement with the declared bioproduct matrix (i.e. collagen contains high quantity of Gly, Ala, Pro and Hyp).

The chiral separation of Dns-amino acids was obtained using the direct approach with cyclodextrin as chiral selector [11,12]. We have tested different BGEs obtained by adding 5, 10, 15 and 20 m*M* β-cyclodextrin (β-CD) to the BGE used for the free amino acids characterisation. An increasing β-CD concentration in BGE increased the resolution factor of chiral separation of D/L-Ala (Fig. 2). The resolution factor was expressed as $R' = 100(H - H')/H$, where H and H' are the height of the first peak

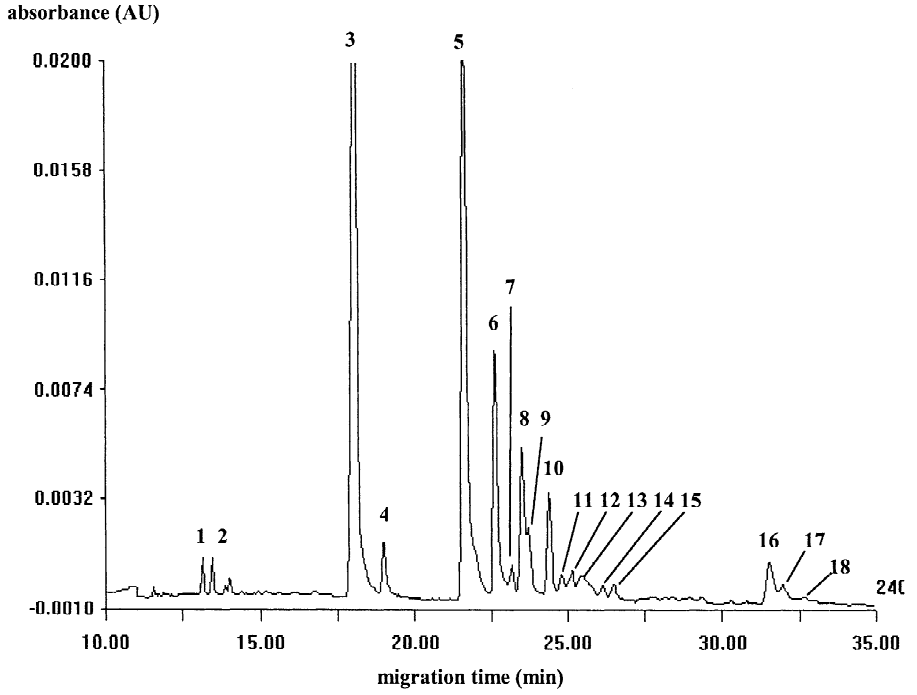

Fig. 1. Characterisation of free amino acids in a HPF sample. 1, Asp; 2, Glu; 3–4, Dns-Cl; 5, Gly; 6, Ala; 7, Ser; 8, Pro; 9, Thr; 10, Hyp+Met+Val+Asn; 11, Phe+leu; 12, Ile+Gln; 13, Unknown; 14, Tyr; 15, Trp; 16, Lys; 17, His; 18, Hyl. Capillary, 50 cm (45.4 cm effective separation length)×50 μm I.D., coated; applied voltage, 15 kV; BGE, 0.1 *M* Tris–borate+2.5 m*M* EDTA-Na_2+7 *M* urea+0.1% SDS (pH 8.6); detection, 240 nm.

Table 2
Linearity, limit of detection (LOD) and precision of the method used for amino acids determination

Amino acids	Linear range (μM ml^{-1})	r^2	LOD[a] (μM l^{-1})	Peak area, RSD (%, $n=4$)
Alanine	0.5–1.5	0.9982	5.65	0.813
Aspartic acid	0.5–1.5	0.9996	5.97	0.221
Cysteine	0.5–1.5	0.9957	16.4	0.551
Glutamic acid	0.5–1.5	0.9952	36.8	1.033
Glycine	0.5–1.5	0.9957	7.45	0.262
Histidine	0.5–1.5	0.9985	4.90	0.744
Hydroxyleucine	0.5–1.5	0.9968	3.30	1.276
Hydroxyproline + Val + Met + Asp[b]	0.5–1.5	0.9995	2.37	1.361
Isoleucine + glutamine[b]	0.5–1.5	0.9970	5.27	0.978
Lysine	0.5–1.5	0.9979	4.59	1.311
Phenylalanine + leucine[b]	0.5–1.5	0.9970	5.70	0.964
Proline	0.5–1.5	0.9999	3.44	0.870
Serine	0.5–1.5	0.9991	4.54	0.845
Threonine	0.5–1.5	0.9946	7.15	0.921
Tryptophan	0.5–1.5	0.9997	8.47	0.101
Tyrosine	0.2–0.6	0.9999	12.0	0.368

[a] The LOD was evaluated at a signal-to-noise ratio of 3.
[b] Equimolar concentrations of each amino acid.

and that of the valley between the two peaks, respectively [12]. In this definition, the greater the R' value, the better resolution, and $R' = 100$ represent a baseline separation of the two peaks. The BGE with 5 mM β-CD was rejected ($R' < 20\%$), while the other three ($R' > 90\%$) were tested to separate the D/L-Ala in HPF samples. The better resolution of D/L-ala in HPF samples were obtained using BGE with 10 mM of β-CD (Fig. 3). In the 15–20 mM β-CD BGE, the peaks of D/L-ala comigrated with the

Table 3
Free amino acids concentration (μM g^{-1}, RSD<5%) in HPF samples

Amino acid	HPF									
	1	2	3	4	5	6	7	8	9	10
Alanine	323	132	97	385	134	393	153	77	128	228
Aspartic acid	25	17	13	18	18	83	38	10	18	63
Cysteine	<1[a]	<1[a]	<1[a]	<1[a]	<1[a]	<1[a]	<1[a]	<1[a]	<1[a]	<1[a]
Glutamic acid	36	20	14	61	20	277	25	9	26	23
Glycine	1225	54	294	719	126	543	370	251	88	584
Histidine	32	16	12	31	15	250	17	9	25	12
Hydroxyleucine	7	4	9	<1[a]	4	<1[a]	2	<1[a]	8	3
Hydroxyproline + Val + Met + Asp	138	84	51	145	67	289	72	39	111	80
Isoleucine + glutamine	27	31	23	79	36	87	47	59	44	26
Lysine	82	11	21	115	10	10	17	12	9	23
Phenylalanine + leucine	25	21	102	121	26	141	22	10	49	26
Proline	171	29	38	157	38	563	43	36	35	70
Serine	29	22	14	<3[a]	21	593	12	17	25	43
Threonine	92	18	21	17	13	316	17	18	18	39
Tryptophan	38	14	12	28	7	10	8	17	15	7
Tyrosine	18	5	7	6	<1[a]	16	<1[a]	18	20	11
Total	2268	478	728	1882	535	3488	843	582	619	1238

[a] LOD.

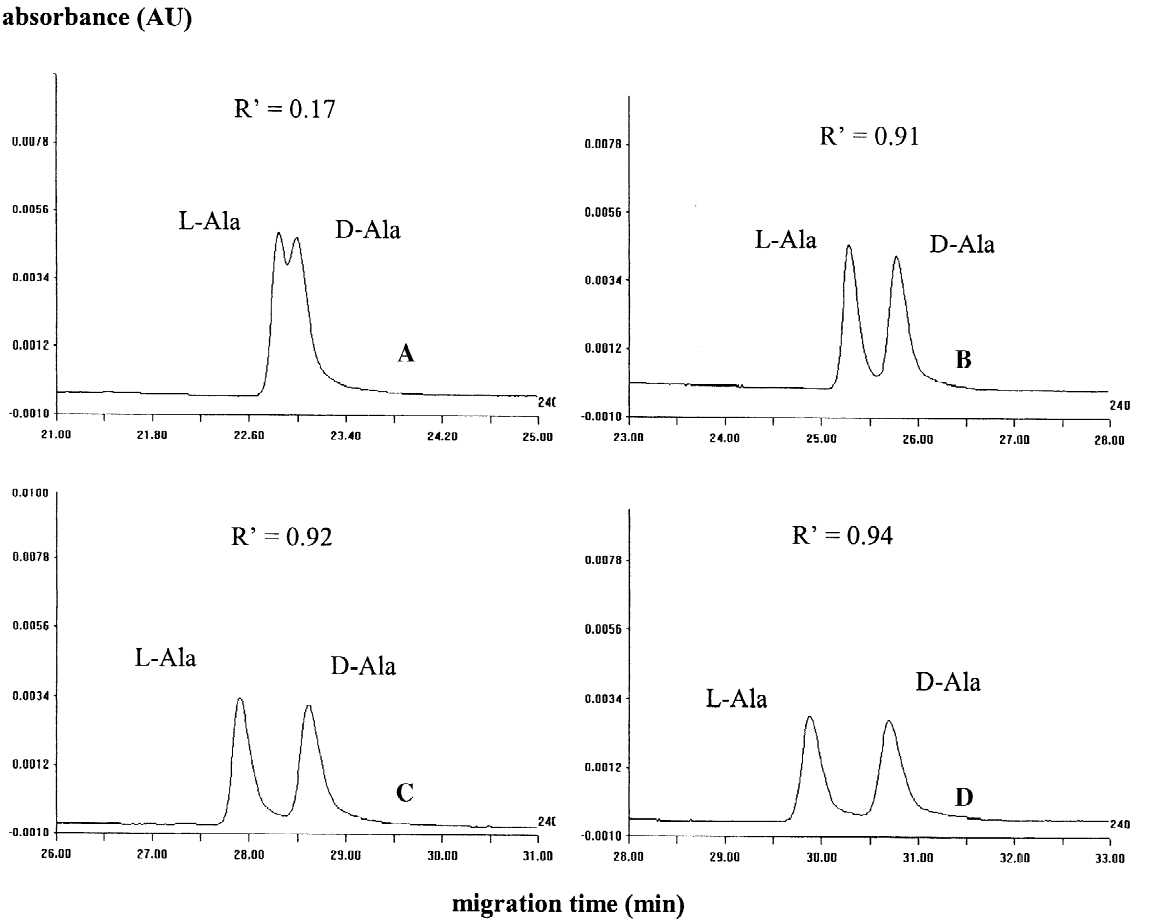

Fig. 2. Chiral separation of D/L-ala with different β-cyclodextrin concentrations. Coated capillary, 50 μm I.D.×50 cm (45.4 cm effective separation length); applied voltage, 15 kV; BGE, 0.1 *M* Tris–borate+2.5 m*M* EDTA-Na_2+7 *M* urea+0.1% SDS (pH 8.6)+5 m*M* β-CD (spot A), 10 m*M* β-CD (spot B), 15 m*M* β-CD (spot C), 20 m*M* β-CD (spot D); detection, 240 nm; *R′*=resolution factor.

peaks of others amino acids. This is due to the fact that the migration time of a specific D/L-amino acid pair is changed depending on β-CD concentration [13].

3.2. Determination of D/L-ala in HPF samples

This method was applied to 10 HPF samples commercially available. Fig. 3 shows a typical chiral separation of Ala in a HPF sample. The peak areas of L-Ala and D-Ala were calculated and the data were used to calculate the racemisation degree (RD)=area D-Ala/[L-Ala+D-Ala]. The results obtained are reported in Table 4. The RD value ranged from a minimum of 4.5% (sample HPF 4) to a maximum of 50.2% (sample HPF 1). This great difference in RD values might be due to the different hydrolytic processes: a low RD value is probably associated with enzymatic hydrolysis at relative low temperatures, while a high RD value to chemical hydrolysis at high temperatures. If this finding is true, only HPF 4 is obtained by an enzymatic process, while the other HPF samples (RD>30%) were obtained by chemical hydrolysis. This suggestion is in agreement with chemical analysis of HPF (Table 1): the HPF 4 has very low ash, Na and K contents in comparison with other HPF samples (NaOH and KOH is the

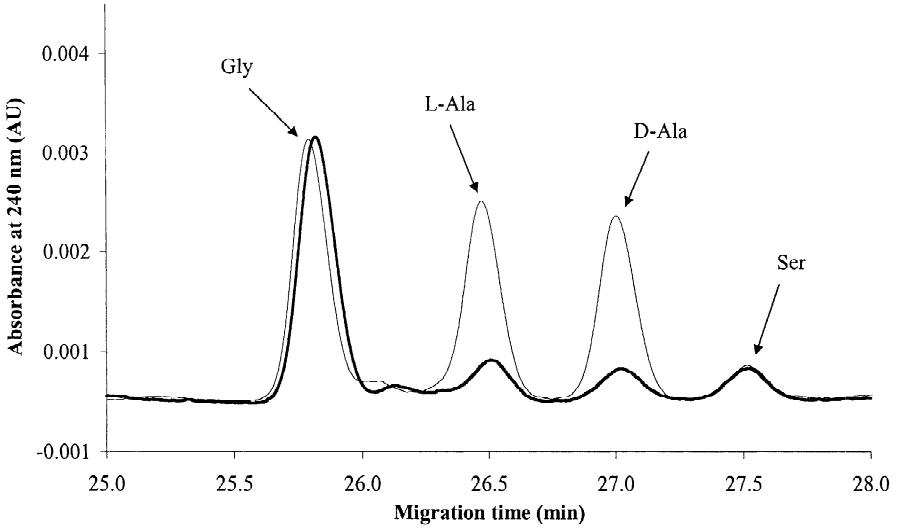

Fig. 3. Chiral separation of D/L-ala of HPF sample (thick line) and HPF sample + D,L-Ala (thin line). Coated capillary, 50 cm (45.4 cm effective separation length) × 50 μm I.D.; applied voltage, 15 kV; BGE, 0.1 *M* Tris–borate + 2.5 m*M* EDTA-Na_2 + 7 *M* urea + 0.1% SDS + 10 m*M* β-cyclodextrin (pH 8.6); detection, 240 nm.

most widely used alkaline solutions for chemical hydrolysis).

4. Conclusions

The chiral separation of Ala in HPF samples was obtained by CE without any initial purification of the sample. The analysis of ten commercially available HPFs has shown that more than 60% of HPF samples have an RD ≥ 40% (close to the raceme mixture), while only one product had an RD < 5%.

Table 4
Racemisation degree (RD) of HPF samples

HPF	Declared matrix	RD[a] (%)	RSD[b] (%, $n=3$)
1	Animal skin	50.2	1.92
2	Collagen and elastine	40.6	1.15
3	Animal skin	33.1	2.60
4	Animal skin	4.5	0.89
5	Animal meat and skin	39.3	3.52
6	Animal hair and coat	27.7	4.82
7	Alfalfa meal	32.0	2.45
8	Animal skin	39.8	1.80
9	Animal skin	38.9	1.14
10	Animal meat and skin	50.1	2.69

[a] Average of three determinations.
[b] Relative standard deviation.

These results clearly show that most of the HPF samples available on the market are obtained by strong hydrolytic processes (normally chemical hydrolysis at high temperatures) and with a high content in D-amino acids probably less effective to plant nutrition or even potentially dangerous to plants.

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