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Journal of Chromatography A, 836 (1999) 137–146

JOURNAL OF CHROMATOGRAPHY A

Chiral separation of aromatic amino acids by capillary electrophoresis

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Abstract

3-Carboxyphenyl-substituted amino acids co-occurring with various types of other aromatic amino acids in plants have been investigated with respect to the chirality at the α-carbon atom. A free zone capillary electrophoresis (FZCE) method using cyclodextrin as chiral selector has been developed and found to be suitable for this purpose. Phenylalanine, tyrosine, tryptophan, and all of the 3-carboxyphenyl-substituted amino acids (*m*-carboxytyrosine, *m*-carboxyphenylalanine, *m*-carboxyphenylglycine and *m*-carboxy-*p*-hydroxyphenylglycine) separated well into their enantiomers and from other naturally occurring amino acids using the developed FZCE method. Identification of the separated enantiomers has been confirmed by use of authentic reference compounds, by spiking and use of stereospecific aromatic amino acid decarboxylase, L- and D-amino acid oxidases. The influence of various parameters on the separation efficiency has been investigated and optimized. Theoretical plate numbers of up to 460 000 $N \cdot m^{-1}$ have been obtained, and resolutions between D- and L-forms in the range from 0.70 to 1.26 (SD<0.10, $n=10$) of the investigated compounds have been found. The method has been found to be efficient for the determination of the naturally occurring amounts of D- and L-forms of the four *meta*-carboxy substituted amino acids, with the possibility of the racemisation of these compounds, and as a highly specific and efficient technique for use in amino acid oxidase and decarboxylase assay.

Keywords: Enantiomer separation; *Resedaceae*; Amino acids; Enzymes

1. Introduction

A range of plants including plants used for food and feed contain a wide array of free, non-protein amino acids [1–5]. In fact this group of natural products comprise ca. 900 compounds and these non-protein amino acids may be present in fairly large amounts in some plants. The study of their presence, concentration and potential physiological effects upon use for food and feed raises the need for efficient analytical methods [5–8]. The potential physiological effects may be caused by the structural analogy with the essential amino acids, thus e.g. causing competition in protein synthesis or amino acid transport. Analogy of non-protein amino acids with neurotransmitter derivatives may, for example, cause aberrations in brain metabolism as aromatic amino acids are precursors for catecholamines; dopa may be involved in Favism and the neurotoxin present in species of Lathyrus, β-N-oxalyl-α,β-diaminopropionic acid has been found to be responsible for Lathyrism [9–11].

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00065-5

The presence of naturally occurring D-amino acids has also been discussed, as have also the possible racemization of L-amino acids during processing of food and feed [12–14]. With the current focus on quality of food and feed and thus the preservation of naturally occurring L-amino acids, it is also important to have methods for identification of the different enantiomers. In addition, efficient methods of analysis for non-protein amino acids is valuable with respect to chemotaxonomic identification of different plants.

3-Carboxyphenyl-substituted amino acids are a group of non-protein amino acids with a rather narrow occurrence in the plant kingdom, i.e. the families Iridaceae, Cruciferae, Resedaceae, Iridaceae, Cucurbitaceae, Leguminosae and Combretaceae [15]. The evidence for the presence in Capparidaceae is still unpublished. The compounds detected in seeds of the five hitherto examined cruciferous plants are 3-(3-carboxyphenyl)alanine **1**, 3-(3-carboxy-4-hydroxyphenyl)alanine **3** and 3-carboxyphenylglycine **2**. These compounds are also detected in seeds from Reseda species together with 3-carboxy-4-hydroxyphenylglycine **4**, and leaves, roots and flowers of Reseda species have likewise been reported to contain **1–4** simultaneously [16]. These amino acids are biosynthetically formed from shikimic acid in a pathway closely analogous to the shikimic acid pathway generally used for biosynthesis of aromatic amino acids [2,17]. The close link to the metabolic pathway for essential amino acids also impairs another aspect to the need for methods of analysis. This aspect is related to the possible interference in the metabolic pathways of amino acids in genetically modified plants after genetic regulation of enzyme systems related to these pathways.

During an investigation of the amino acid pattern of seeds, roots, leaves and flowers of some cruciferous and related plants it was observed that especially **3** was widely distributed and predominant in the roots and green material [16]. In addition, it has previously been stated, that Reseda species contain both L- and D- forms of the *m*-carboxy substituted amino acids **2** and **4**, but the possibilities of racemisation occurring during comprehensive purification procedures could not be excluded as an explanation of the co-occurrence of both of the enantiomers [12,13]. We hereby report the results of possibilities for use of free zone capillary electrophoretic (FZCE) chiral separation as a reliable, fast and simple method of analyses for studies of these compounds and other structurally related compounds following gentle extraction procedures [6,8].

Previously, a range of enantiomeric separation methods has been developed for derivatized amino acids. However, the aromatic nature of the analytes considered here together with the need for gentle methods of analyses of complex samples favours the development and use of a FZCE method omitting the need for derivatization. In addition, the possible racemization of the amino acids during purification procedures has previously made it difficult to determine the content of the native individual enantiomers of *m*-carboxyphenylglycine amino acids, problems that have been overcome with this work.

2. Experimental

2.1. HPCE

Analyses were performed using a Hewlett-Packard HP^3D CE capillary electrophoresis system (Hewlett-Packard, Waldbronn, Germany) equipped with diode-array detector and a 645 mm×0.05 mm I.D. fused-silica capillary. Detection was performed on-column at 214 nm at a position 560 mm from the inlet.

Chiral separation was investigated using α-cyclodextrin (cyclohexaamylose, C-4642, Sigma, St. Louis, MO, USA), β-cyclodextrin (cycloheptaamylose, C-4767, Sigma) and β-dimethylcyclodextrin [heptakis(2,6-di-*o*-methyl)-β-cyclodextrin, H-0513, Sigma] as chiral selectors. The background buffer used contained trizma phosphate {mono[tris-(hydroxymethyl)aminomethane]-phosphate, T-1758, Sigma, St. Louis}.

DL-Tryptophan (T-3300), DL-phenylalanine (P-1876) and DL-tyrosine (T-3379) were all purchased from Sigma. The non-protein aromatic amino acids and their derivatives and isolated D- and L-enantiomers of the different compounds were isolated from plant material or synthesised in our laboratories. L-Amino acid oxidase (T-4629) and L-tyrosine decarboxylase (A-9553) were purchased from Sigma.

The resulting buffer for chiral separation of **1–4** contained 40 m*M* trizmaphosphate, 60 m*M* β-di-

methylcyclodextrin and 25 μl 4 *M* phosphoric acid per ml buffer. The temperature was 30°C, and the samples were run at 15 kV. Buffers were filtered through a 0.20 μm membrane filter before use. For separation of the aromatic amino acids and their corresponding decarboxylated amines, the method was modified to 12°C and 20 kV. The electrophoreses were run for 50 min and between each run the capillary was washed with 1 *M* NaOH for 5 min, water for 2 min and buffer for 5 min.

2.2. Amino acid decarboxylation

Amino acid sample (25 μl; 18 m*M*) and L-tyrosine decarboxylase (20 μl; 33.5 mg ml^{-1}) were added to substrate (140 μl; 43 m*M* citric acid, 114 m*M* Na_2HPO_4, 0.4 m*M* pyridoxal-5-phosphate (PLP) in water) and left for 10 min at 37°C followed by centrifugation (2000 *g*).

2.3. Amino acid oxidation

L-Amino acid oxidase [20 μl; 30 mg ml^{-1} in Tris–HCl buffer (0.1 *M*, pH 7.8)] was mixed with buffer (200 μl Tris–HCl) and amino acid sample (50 μl, ca. 1 mg ml^{-1}) and left for 15 min at room temperature (**1**, **3** and **4**) or at 37°C for 10 min and then left overnight at room temperature (**2**).

2.4. Extraction of Reseda inflorescence

Inflorescence (0.5 *g*) of *R. lutea* was subjected to three rounds of extraction by Ultra Turrax (Janke & Kunkel, Darmstadt, Germany) homogenization for 2 min in 3 ml boiling 70% methanol followed by centrifugation for 2 min at 2500 *g* [18]. The supernatants were pooled and evaporated to dryness and then redissolved in 3 ml Milli-Q water (Millipore, Milford, MA, USA).

The prepared raw extracts were purified by ion-exchange chromatography using an initial purification on CM-Sephadex C-25 (Pharmacia Biotech, Uppsala, Sweden) on acetate form followed by purification of the unbound material on Dowex 50-X8 (Sigma) [8]. The latter material was eluted with 2 *M* pyridine resulting in elution of the acidic and neutral amino acids. The eluate was evaporated to dryness and redissolved in 200 μl water.

Names and structures of the four *m*-carboxy substituted aromatic amino acids and the related compounds investigated are given in Fig. 1.

3. Results and discussion

Preliminary experiments using a standard mixture of **1**, **2**, **3** and **4** showed that α- and β-cyclodextrin were less efficient for chiral separation of the studied compounds than β-dimethylcyclodextrin. Different concentrations of β-dimethylcyclodextrin were investigated (10, 20, 40, 60 m*M*) and it was found that the high level (60 m*M*) of the cyclodextrin were most efficient for chiral separation of all the four compounds. However, the rise in cyclodextrin concentration also prolonged the migration time, but decreasing the trizmaphosphate concentration in the buffer compensated for this.

The resulting chiral separation of compounds **1–4** (Fig. 2) was found to be very sensitive to pH. For reproducible buffer preparation at the low pH values used (pH 1.8 – 1.9) it was found that addition of phosphoric acid at defined volumes resulted in the most reproducible buffer preparation. The effect of added phosphoric acid on the separation is illustrated in Fig. 2. The best resolutions both between different compounds and between the enantiomers of the individual compounds were obtained with addition of 25 μl phosphoric acid per ml buffer. Optimum enantiomeric separation conditions using CD at acidic background buffer have previously been found for weak chiral acids [19,20].

The cavity of the cyclodextrin is expected to be able to bind the hydrophobic parts of the analytes [21]. Resolution between enantiomers were found at low pH values (Fig. 3), which may imply that the analytes only are able to form complexes with the cyclodextrins when the carboxylic acid group on the aromatic ring are protonated thereby rendering the analytes more hydrophobic. The migration order of the enantiomers is maintained independent of the pH. This is in accordance with the findings of Rawjee et al. who found that for other chiral and weak acids [19], where only the non-ionic enantiomer forms are able to form complexes with the CD, the migration order of the enantiomers are independent of CD concentration and pH.

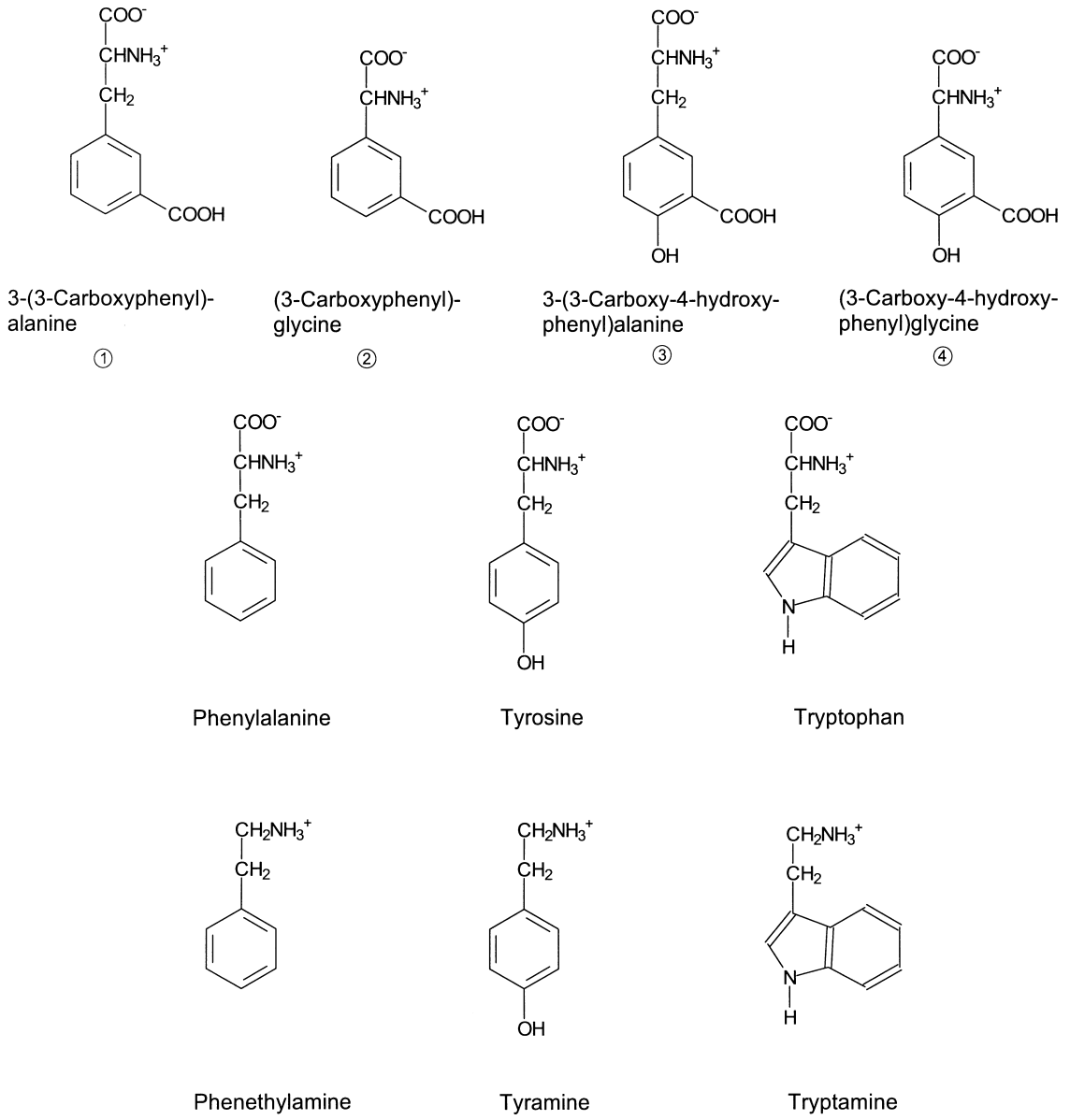

Fig. 1. Names and structures of non-protein aromatic amino acids, aromatic amino acids and their decarboxylated amine forms.

The great influence of buffer pH on the migration times are due to the pK_a' values of **1–4**. At pH values of about 2, the aromatic carboxylic acid groups are largely on their protonated forms [22]. The pK_a' values [8] given in this paper are for **1**: 2.4 and 4.0; for **2**: 2.4 and 3.8; for **3**: 2.4 and 3.5; and for **4**: undetermined and 3.1 [22]. These previously reported pK_{a1}' values [22] are obviously different from the pK_a' values for the compounds in the buffer system applied here. The low pH values in the applied buffer are close to the pK_a' values of the α-carboxyl group of the amino acids and in the FZCE system employed, protonation of this carboxylate group results in decreased migration times

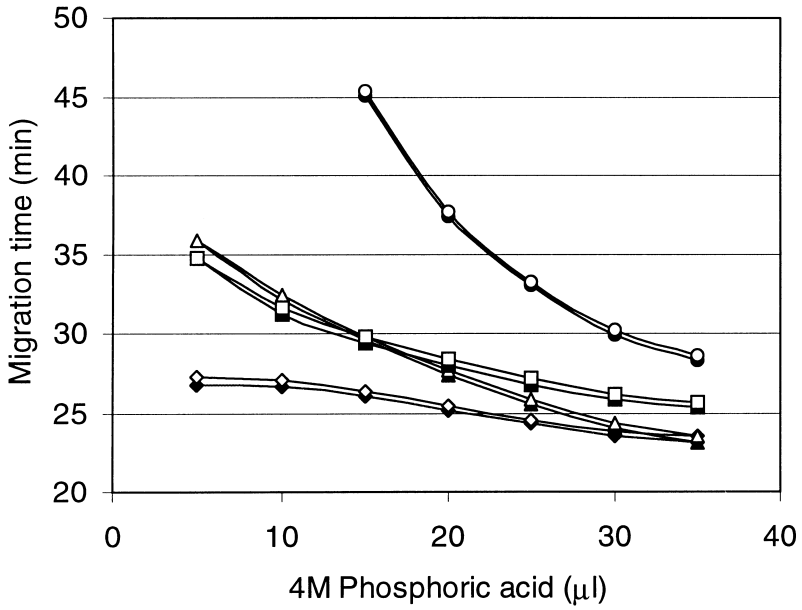

Fig. 2. Influence on migration times of buffer pH and ion strength investigated by addition of varying amounts of 4 *M* (H_3PO_4) per ml of buffer (40 m*M* trizmaphosphate; 60 m*M* dimethyl-β-CD). Electrophoreses were performed at 15 kV and 30°C. Other parameters are described in Section 2. L-enantiomers are shown as filled symbols and D-enantiomers as open symbols. Diamond: 3-(3-carboxyphenyl)alanine **1**; triangle: 3-carboxyphenylglycine **2**; square: 3-(3-carboxy-4-hydroxyphenyl)alanine **3**; circle: 3-carboxy-4-hydroxyphenylglycine **4**.

(t_m). In the currently used system, the t_m of **2** and **4** show much more pronounced sensitivity towards small changes in the buffer than seen for **1** and **3**. Hence, the compounds migrate in accordance with decreasing pK'_a values, thus implying that **1–4** have different pK'_{a1} values. Consequently, the phenylglycine compounds (**2** and **4**) are expected to migrate slower than the corresponding compounds with an additional CH_2 group between the amino acid group and the electronegative aromatic ring. The presence of a 4-hydroxy group is seen to increase the t_m of **3** and **4** compared to **1** and **2**, respectively (Fig. 3). Aromatic ring substituted hydroxy groups are para directors (activating groups) and therefore the presence of the hydroxy group could be expected to give an increase in the pK'_a value of the α-amino acid. However, the absence of this effect may be explained by the presence of the CH_2 group in **1** and **3** and the neighbouring *meta*-substituted carboxylic acid group in **3** and **4**, which is able to form hydrogen bonds with the hydroxy group giving 6-membered ring structure. This structure may then decrease the activating property of the hydroxy group and increase the deactivating property of the protonated carboxylic acid group. The additional negative charge at the applied buffer could also be contributions from the pK'_{a2} values for **3** and **4**, which thus need to be close to 3.

The three aromatic amino acids, Phe, Tyr and Try, were nicely separated in the same buffer system (Fig. 4). However, for optimal chiral separation of the aromatic amino acids, the run parameters (temperature and voltage) were slightly changed compared to Fig. 3. The three aromatic amino acids have previously been separated into their enantiomers using α-cyclodextrin [23]. The conditions found in the work presented here do, however, allow a better separation of tyrosine enantiomers from the other two aromatic amino acids. For the corresponding decarboxylated compounds (phenethylamine, tyramine and tryptamine) the phenethylamine and tyramine are separated from the peaks of the amino acids, whereas tryptamine co-migrated with the D-form of tyrosine.

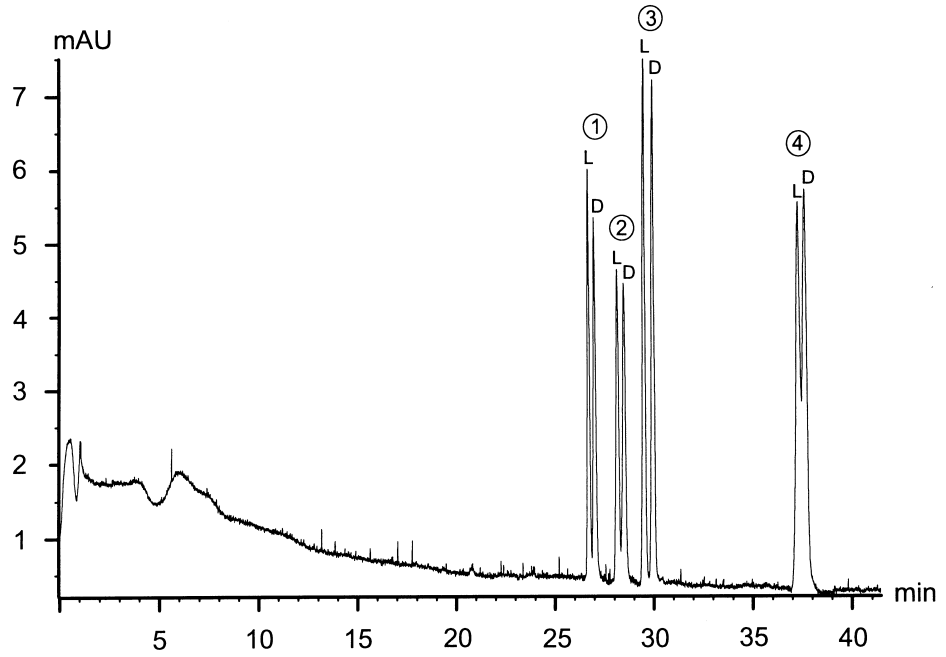

Fig. 3. Chiral separation of 3-(3-carboxyphenyl)alanine **1**; 3-carboxyphenylglycine **2**; 3-(3-carboxy-4-hydroxyphenyl)alanine **3**; 3-carboxy-4-hydroxyphenylglycine **4**; into D- and L-enantiomers. Separation conditions comprised a trizmaphosphate buffer (40 m*M*) with 60 m*M* dimethyl-β-CD and 25 μl 4 *M* (H_3PO_4) per ml buffer. Electrophoreses were performed at 15 kV and 30°C. Other parameters are described in Section 2.

The resolutions obtained for the different compounds shown in Figs. 3 and 4 are listed in Table 1 together with the number of theoretical plates (*N*) obtained. The reproducibility of the method and buffer system developed was investigated with respect to the standard deviation found for the resolution in experiments performed at different days (Table 1), and it was found that the method gave reproducible resolution between enantiomers (RSDs less than 8% for $n=10$).

The migration orders of the D and L forms were established by different methods comprising spiking with pure D- and L-enantiomers of the compounds and use of stereospecific enzymes (Table 2).

L-Tyrosine decarboxylase catalyses the decarboxylation at the α-amino acid group. This enzyme is particularly effective for decarboxylation of L-tyrosine [24], where complete decarboxylation was observed. However, L-phenylalanine was also decarboxylated to a minor degree at the reaction conditions employed. With respect to the aromatic amino acids such decarboxylation results in the amine derivatives of the amino acids [10,25] (Fig. 1). This reaction can now be followed by the currently developed FZCE method where a decrease in the L-form of the amino acid results in an emerging peak corresponding to the amine. This method did, however, not work on compounds **1–4** under the applied conditions. Instead, the use of amino acid oxidase was investigated. These enzymes catalyse oxidation of the amino acid group to the corresponding α-ketocarboxylic acid and reduced flavine co-factor which reoxidise in the presence of oxygen resulting in hydrogen peroxide. If this is not removed by catalase or peroxidase, decarboxylation will occur. Due to the removal of the positively charged amino group the result will be prolonged migration time of the reaction product. The reaction product is therefore not seen in the electropherogram (Fig. 5B). The L-amino acid oxidase employed reacted more readily with **1**, **3** and **4** than with **2** but this was overcome by increasing the incubation temperature to 37°C and increase the incubation time to give an observable degradation of the L-**2**.

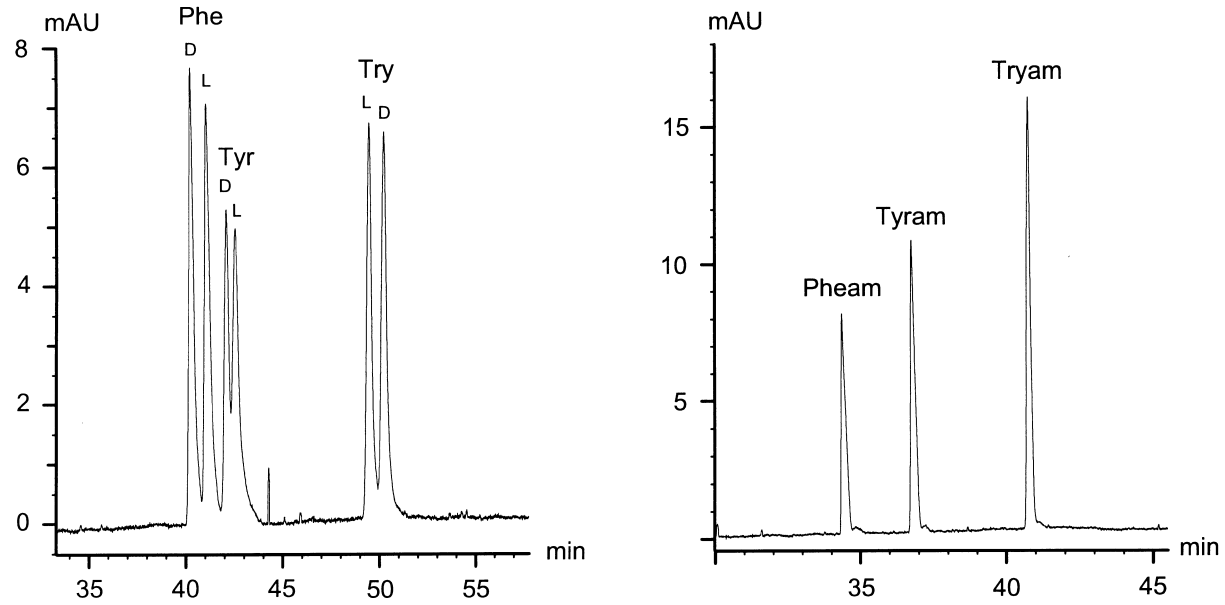

Fig. 4. Chiral separation of phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Try) and separation in the same system of their corresponding decarboxylated forms phenethylamine (Pheam), tyramine (Tyram) and tryptamine (Tryam). Electrophoreses were performed at the conditions described in Fig. 3 except that the run conditions were changed to 20 kV and 12°C.

The peak identification were also performed with use of the available, pure enantiomers of the investigated compounds for spiking (Fig. 5C) and these results supported the findings using enzymes.

The method currently developed was used for investigation of the relative distribution of **1–4** and of the L- and D-forms of **1–4** in inflorescence of Reseda species. The crude extracts contained various interfering compounds, but **1–4** had t_m values greater than most of the compounds (Fig. 6, middle). A gentle group separation by ion exchange chromatography [5–8] resulted in appreciable purification and

Table 1
Names and numbers/abbreviations of analysed compounds together with the number of theoretical plates and resolution between enantiomers obtained from the electropherograms shown in Figs. 3 and 4 (for compounds **1–4** standard deviations (SDs) were calculated with $n=10$)

Compound	Number/ abbrev.	Theoretical plates $(N/m)\times10^{-3}$ ($\pm$SD)		Resolution between enantiomers ($\pm$SD)
		L-form	D-form	
3-(3-Carboxyphenyl)alanine	1	457±73	351±74	1.32±0.10
3-Carboxyphenyl-glycine	2	333±22	299±26	1.26±0.05
3-(3-Carboxy-4-hydroxyphenyl)alanine	3	416±28	393±39	1.79±0.09
3-Carboxy-4-hydroxyphenylglycine	4	181±29	182±23	0.70±0.05
Phenylalanine	Phe	219	184	1.67
Tyrosine	Tyr	252	158	0.90
Tryptophan	Try	332	290	1.61
Phenethylamine	Pheam	134		
Tyramine	Tyram	168		
Tryptamine	Trpam	307		

Table 2
Elution order of D- and L-enantiomers in the developed FZCE method determined by specific enzyme conversion or spiking with pure enantiomers. (n.t.: not tested)

Compound	L-Tyrosine decarboxylase	L-Amino acid oxidase	Spiking with enantiomer
3-(3-Carboxyphenyl)alanine	–	L–D	
3-Carboxyphenyl-glycine	–	L–D	
3-(3-Carboxy-4-hydroxyphenyl)alanine	–	L–D	L–D[a]
3-Carboxy-4-hydroxyphenylglycine	–	L–D	L–D[b]
Phenylalanine	D–L	n.t.	D–L[a]
Tyrosine	D–L	n.t.	D–L[a]
Tryptophan	–	n.t.	L–D[a]

[a] Spiking performed with L-enantiomer.
[b] Spiking performed with D-enantiomer.

concentration of **1–4** and spiking with reference compounds resulted in the peak identifications shown in Fig. 6. Compounds **1** and **3** were present only as the L-enantiomer forms as has previously been described [12,13]. Compound **2** was not detectable and compound **4** was present as the D-enantiomer. Previously, compound **4** has been found as both D- and L-enantiomers [12,13] but with the currently used gentle extraction, purification and methods of analyses, the native form of compound **4** is found to be the D-enantiomer. It is thus possible to use the method described here for chiral identification of the amino acids considered, either by using spiking with reference compounds or by enzymatic treatment with amino acid oxidase. In addition, the method gives an efficient and specific assay technique for aromatic amino acid decarboxylase or amino acid oxidases.

Acknowledgements

This work was financially supported by the Commission of European Union (Contract Nos. FAIR CT 95-0260 and FAIR CT 98-3778).

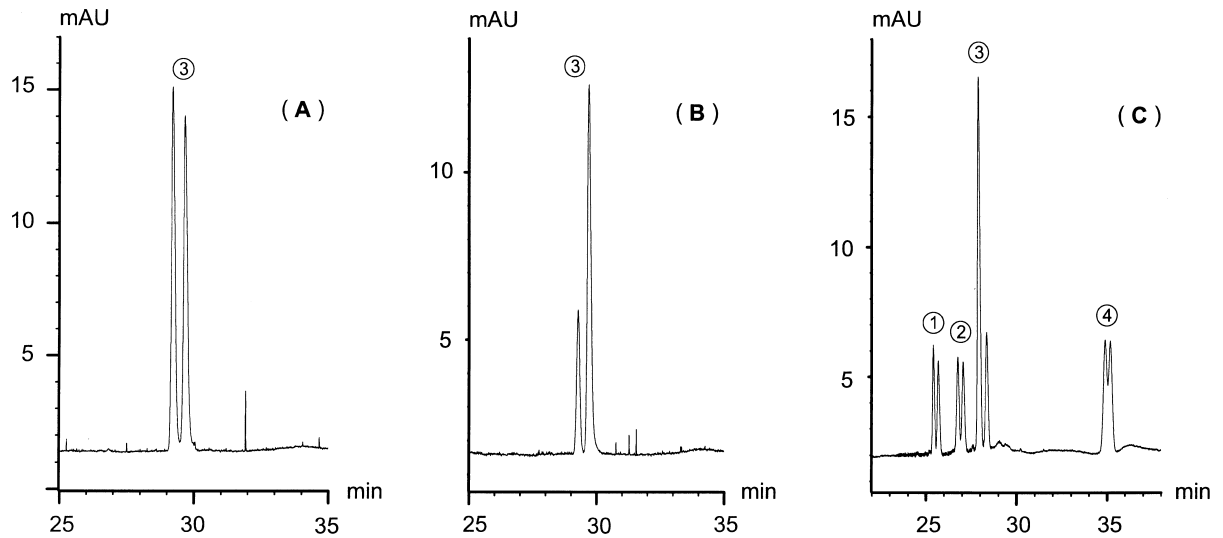

Fig. 5. Chiral separation of **3** before (A) and after (B) addition of L-amino acid oxidase. Spiking with pure L-**3** is shown in (C). Electrophoreses were performed at the conditions described in Fig. 3 except that the run conditions were changed to 20 kV and 12°C.

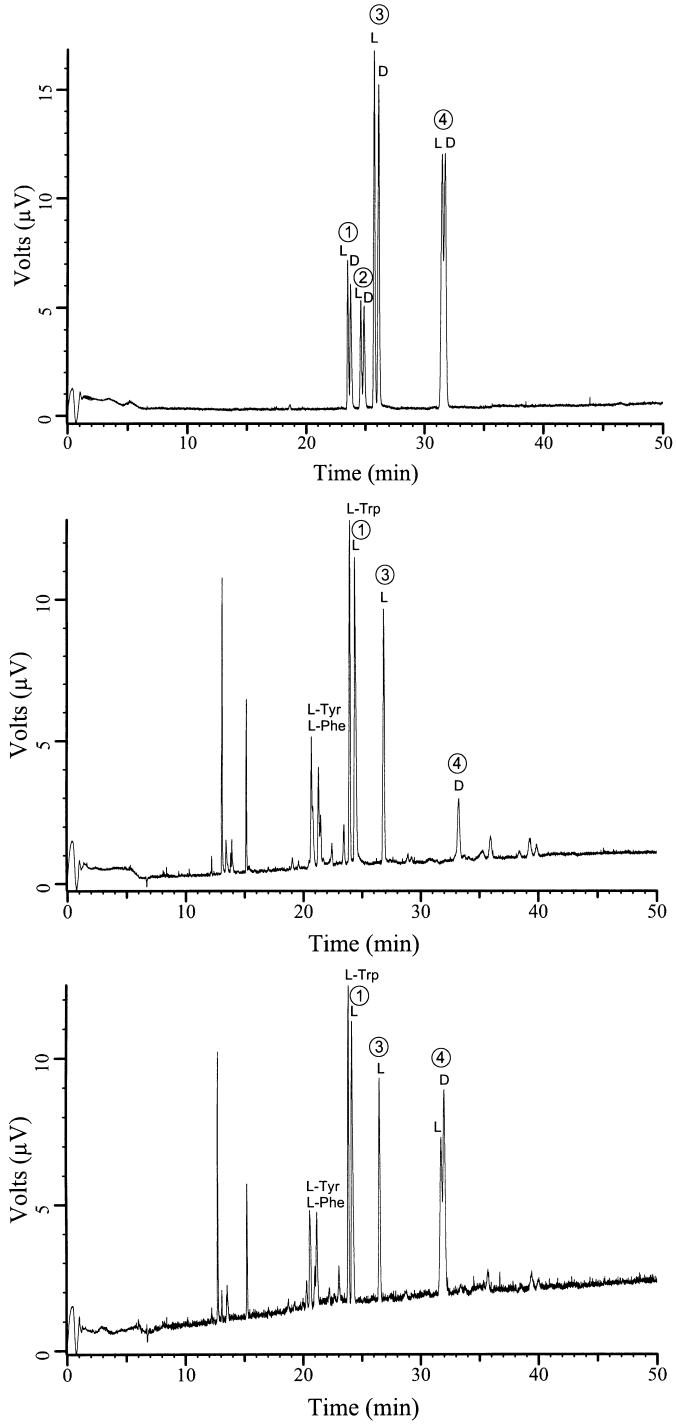

Fig. 6. Electropherograms of standard mixture (top), extracts of *Reseda lutea* inflorescence (middle) purified by ion exchange chromatography and the *Reseda lutea* sample spiked with compound **4** with equal amounts of the D- and L-forms (bottom). Electrophoreses were performed with the conditions described in Fig. 3.

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