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Liquid chromatographic determination of D- and L-amino acids by derivatization with *o*-phthaldialdehyde and chiral thiols

Applications with reference to biosciences

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ABSTRACT

A high-performance liquid chromatographic procedure was developed for the fully automated precolumn derivatization of amino acids by derivatization with *o*-phthaldialdehyde (OPA) together with the chiral thiols N-isobutyryl-L-cysteine (IBLC) and N-isobutyryl-D-cysteine (IBDC) [$(CH_3)_2CHCONHCH(CH_2SH)COOH$]. The derivatization is completed within 2 min at room temperature and the resulting diastereomeric isoindol derivatives exhibit linearity with respect to fluorescence in the range 25–1000 pmol. The derivatives are completely separated within 75 min on an octadecylsilyl stationary phase using a linear gradient generated by sodium acetate buffer (pH 5.95) and methanol–acetonitrile. Fluorescence detection at an excitation wavelength of 230 nm and an emission wavelength of 445 nm makes possible the detection of 1–2 pmol of an amino acid enantiomer. Derivatization with OPA–IBLC permits the separation of a 41-component standard containing seventeen protein L-amino acids and, in addition, glycine, DL-cysteic acid, the internal standard L-*homo*-arginine and the non-protein amino acids α-aminoisobutyric acid and DL-isovaline. Replacement of OPA–IBLC with OPA–IBDC (or *vice versa*) leads to a reversal in the elution of the derivatives of D- and L-amino acids. The applicability of the method to various fields of biosciences is demonstrated with the detection and determination of D-amino acids in bacteria, microfungi, higher plants, invertebrates and vertebrates, including man, and in the amino acid-containing Murchison meteorite.

INTRODUCTION

Although L-amino acids are of major importance in the biosciences, it is increasingly realized that free and peptide-bonded D-amino acids also play an important role in organisms. Probably as a result of post-translational modifications, peptide-bonded D-amino acids have been found in gene-encoded peptide antibiotics and neuropeptides [1], and D-Asp and D-Ala have been reported to occur in proteins possibly related to Alzheimer's disease [2,3]. Free D-amino acids have been detected in humans [4–7], rats and mice [4,8–10], bivalve molluscs [11], marine invertebrates [12] and insects [13]. Further, as a result of bacterial metabolism [14,15], free D-amino acids are common constituents of microbially fermented foods and beverages [16–19]. For chemical-induced racemization of amino acids in food proteins [20] and for the nutritional utilization and safety of D-amino acids [21], we

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refer to the literature and the references cited therein.

Further progress in the discovery of peptide-bonded D-amino acids and naturally occurring free D-amino acids and the elucidation of their biological functions are dependent on the availability of reliable, sensitive and rapid analytical methods. These methods should ideally be capable of separating in a single run all protein L-amino acids from each other and from their corresponding D-enantiomers and should make possible their determination.

We have systematically investigated the liquid chromatographic resolvability of diastereomers formed by the reaction of DL-amino acids with *o*-phthaldialdehyde (OPA) together with chiral N-acylated cysteines [22,23]. It was found that OPA and N-isobutyryl-L-cysteine (IBLC) as reagents and the use of an instrument allowing the fully automated precolumn derivatization made possible the satisfactory resolution of seventeen protein L-amino acids and their corresponding D-enantiomers [24]. We now demonstrate the performance of the method with the resolution of a 41-component standard and demonstrate the reliability and robustness of the procedure with reference to various applications in the biosciences.

EXPERIMENTAL

Instruments

An HP 1090 Series L instrument was used, consisting of a binary DR 5 solvent-delivery system, an autoinjector, autosampler, thermally controlled column compartment and an HP 1046 programmable fluorescence detector fitted with a xenon-arc flash lamp (lamp frequency 55 Hz) equipped with a cut-off filter (280 nm), operated at an excitation wavelength of 230 nm and an emission wavelength of 445 nm. The response time of the detector was set at 500 ms and the photomultiplier tube (PMT) gain was set at positions 8–10. For data processing a Series HP 79994A ChemStation computer was used in conjunction with a Model 7440A ColorPro plotter and Model 2225B ThinkJet printer (all instruments were supplied by Hewlett-Packard, Waldbronn Analytical Division, Waldbronn, Germany).

Chromatography

Stainless-steel columns (250 mm × 4 mm I.D.) and guard columns (20 mm × 2.1 mm I.D.) (Hewlett-Packard) packed with Hypersil ODS, particle size 5 μm (Shandon Scientific, Runcorn, UK), were used. The column temperature was maintained at 25°C. To prepare eluent A, 3.13 g (23 mmol of sodium acetate trihydrate) were dissolved in 990 ml of doubly distilled water and adjusted to pH 5.95 by addition of 10% (v/v) acetic acid. The pH was monitored using a glass electrode and the final volume was made up to 1 l using doubly distilled water. The eluent was filtered by means of a Millipore filter system fitted with a 0.45-μm filter (Sartorius, Göttingen, Germany). Eluent B consisted of 474 g (600 ml) of methanol and 39 g (50 ml) of acetonitrile. A stream of helium was permanently passed through the eluents to remove dissolved gases. A linear gradient was applied for 75 min at a flow-rate of 1 ml min^{-1} from 0 to 53.5% B and then equilibrated with 100% A for 10 min. Details of the derivatization device of the instrument can be found in ref. 24.

All chemicals and solvents were of analytical-reagent or chromatographic grade from Merck (Darmstadt, Germany). Dowex 50W-X8 cation exchanger (H^+ form; 200–400 mesh, particle size 0.037–0.075 mm, analytical-reagent grade) was used. DL- and L-cysteic acid and L-*homo*-arginine were from Serva (Heidelberg, Germany), D- and DL-amino acids and α-aminoisobutyric acid (Aib) from Sigma (St. Louis, MO, USA) and L-amino acids and DL-isoleucine ("free of *allo*-Ile") from Fluka (Buchs, Switzerland). DL-Isovaline (DL-Iva) (α-ethylalanine) was prepared in our laboratory according to the Strecker synthesis from methyl ethyl ketone, potassium cyanide and ammonium chloride. Sodium borate buffer, 0.4 *M* (pH 10.4), was obtained from Hewlett-Packard and potassium borate buffer, 1 *M* (pH 10.4), from Pierce (Rockford, IL, USA). *o*-Phthaldialdehyde (OPA) was supplied by Fluka. N-Isobutyryl-L-cysteine (IBLC) and N-isobutyryl-D-cysteine (IBDC) were synthesized in our laboratory [24] and are also available from

Calbiochem–Novabiochem (Läufelfingen, Switzerland) or Calbiochem (La Jolla, CA, USA). IBLC was of 99.91 ± 0.01% and IBDC of 99.78 ± 0.02% optical purity.

Amino acid standard solutions

Premixed standards of L-amino acids (1 nmol of amino acid in 1 μl of 0.1 *M* HCl) (Hewlett-Packard) were used.

For preparing individual DL-amino acid standard solutions, 20 mmol of DL-amino acid were dissolved in 0.1 *M* HCl (with the exceptions of hydrolysis-sensitive DL-Asn and DL-Gln, which were dissolved in doubly distilled water). Analogously, standards of L-*homo*-Arg, Aib, DL-Iva and DL-cysteic acid were prepared. The above solutions were stored at −18°C; after thawing, aliquots were diluted with 0.1 *M* HCl in order to prepare the desired multi-AA standard.

Samples

Lactobacillus acidophilus. A freeze-dried culture (VISBYVAC series 50; Laboratorium Wiesby, Niebüll, Germany) was used and grown in a sterilized culture broth according to De Man *et al.* [25]. Growth in 10 ml of fermentation broth was conducted statically at 30°C for 3 days. The bacteria were centrifuged at 2400 *g* and the sediment was washed three times with 0.85% NaCl solution. The free amino acids were extracted by treatment with 70% aqueous ethanol (3 × 2 ml) and sonication for 20 min at 60–70°C. The combined ethanolic extracts were evaporated to dryness *in vacuo* and the residue was dissolved in 0.1 *M* HCl (2 ml) and filtered. The solution was passed through a column packed with Dowex 50W-X8 cation exchanger (bed size 5 cm × 1.0 cm). The ion exchanger was washed with doubly distilled water, after which the amino acids were eluted with 4 *M* aqueous ammonia (30 ml). The effluent was evaporated to dryness, the residue was dissolved in 0.1 *M* HCl (2 ml) and aliquots of 2 μl were analysed.

Nectria ochroleuca. A lyophilized culture of *N. ochroleuca* CBS 194.57 was obtained from the Centraalbureau voor Schimmelcultures (Baarn, Netherlands). The fungus was grown in petri dishes (9 cm diameter) for 7 days at 25°C on autoclaved malt extract–peptone agar [30 g of malt extract from Serva, 3 g of soy peptone from Oxoid (Wesel, Germany) and 15 g of agar agar from Merck were dissolved in 1 l of distilled water and adjusted to pH 6.5]. The mycelium was extracted with dichloromethane–methanol (1:1, v/v; 2 × 5 ml), the organic extracts were combined, evaporated to dryness, transferred into 1-ml Reacti-Vials (Wheaton, Milleville, NJ, USA) and hydrolysed in 6 *M* HCl at 110°C for 24 h. The hydrolysate was evaporated to dryness and subjected to the cation-exchange treatment and analysed by HPLC as described for *Lactobacillus acidophilus*.

Meteorite sample. A 600-mg portion of a 3-g fragment of the Murchison meteorite (for which we are obliged to Mr. R.W. Bühler, Swiss Meteorite Laboratory, Glarus, Switzerland) was ground and then extracted in a 5 ml Reacti-Vial under nitrogen in 3 ml of doubly distilled water (15 h, 110°C). The aqueous extract was evaporated to dryness *in vacuo*, the residue was dissolved in 0.5 ml of 0.01 *M* HCl and aliquots of 2 μl were analysed. Analogously a blank was prepared from doubly distilled water and analysed.

Apple juice. A freshly harvested apple (Golden Delicious, from an orchard in the Stuttgart area, Germany) was washed with 70% aqueous ethanol, peeled, the core was removed and the apple was cut into pieces. Apple juice was obtained by use of an automatic juicer based on the centrifugal principle (Multipress MP 50, Type 4154, from Braun, Frankfurt, Germany). The juice was filtered by means of a folded filter-paper (18.5 cm diameter; product No. 311 6347 from Schleicher & Schüll, Dassel, Germany) and the filtrate was centrifuged at 1650 *g*. To 1-ml aliquots of the juice, 31.3 μl of 1.6 m*M* L-*homo*-Arg in 0.1 *M* HCl were added as an internal standard and 2 *M* HCl was added in order to adjust the pH to 2.0. The solution was subjected to the Dowex 50W-X8 procedure as described above, the resulting residue was dissolved in 1 ml of 0.1 *M* HCl and aliquots of 2 μl were subjected to HPLC.

Worms. Amounts of 500 mg of purified and freeze-dried tubifex worms (*Tubifex tubifex*) (Sera, Heinsberg, Germany) were ground and extracted with 70% aqueous ethanol (3 × 10 ml)

at 70°C with sonication. The mixture was centrifuged at 1600 *g*, the supernatants were combined and evaporated to dryness, the residue was dissolved in 0.1 *M* HCl (3 ml) and the solution was filtered and subjected to Dowex 50W-X8 treatment and HPLC as described above.

Animal and human blood. Blood serum from a dog (*Canis lupus* var. *domesticus*; 6-year-old male of a German shephard bastard) was provided by a veterinary clinic. Human blood serum was provided by a healthy volunteer (German white male, 27 years old). To 400-μl aliquots of serum, 50 μl of 30% (w/v) aqueous 5-sulphosalicylic acid and 50 μl of 0.52 m*M* L-*homo*-Arg (human serum) were added, the mixture was centrifuged at 6000 *g* and 2-μl aliquots of the supernatants were subjected to HPLC.

Human urine. The urine sample was provided by a healthy volunteer (white male, 34 years old). An aliquot of 400 μl was mixed with 50 μl of 30% (w/v) aqueous 5-sulphosalicylic acid and 50 μl of 0.52 m*M* L-*homo*-Arg and the mixture was centrifuged and analysed as described for blood serum.

RESULTS AND DISCUSSION

Chromatography

The resolution of a 37-component standard of amino acids, derivatized with OPA–IBDC, is shown in Fig. 1a. A chromatogram of this standard with the addition of L-*homo*-Arg and the non-protein amino acid Aib and DL-Iva, derivatized with OPA–IBLC, is shown in Fig. 1b; 41 components were resolved. Both chromatograms display a satisfactory resolution. The synthetic L-*homo*-Arg (Fig. 1b) serves as an internal standard. When IBDC is used as reagent the derivative of L-*homo*-Arg co-elutes together with that of D-Tyr. Proline is not derivatized with OPA and thiols, and cysteine gives a very low fluorescence. D- and L-cysteic acid are well resolved and elute first in the chromatogram; cysteine and cystine are determinable after oxidation to cysteic acid [26].

It should be noted that the method used is an indirect method for the determination of amino acids enantiomers: derivatizations of DL-amino acids with OPA together with the L-reagent (IBLC) results in the formation of the diastereomeric pairs D–L and L–L, which are separable on the achiral stationary phase used. Using the IBLC reagent, the stereoisomer derived from a certain L-amino acid elutes prior to those from the corresponding D-amino acid, *i.e.*, L–L before D–L (with the exception of diastereoisomers formed from DL-Iva). Reaction of DL-amino acids with OPA and the D-reagent (IBDC) gives the diastereomeric pairs D–D and L–D, which are also resolved on the stationary phase. The enantiomeric pairs L–L, D–D and D–L, L–D, respectively, have identical retention times using the same achiral column (see Fig. 1a and b). Therefore, subsequent derivatization of DL-amino acids with the L- and D-reagent leads to an apparent reversal of the elution order of D- and L-amino acids, although actually the elution order of diasteromeric dipeptides has been reversed. This behaviour is shown schematically in Fig. 2.

The reversal of the elution order of the stereoisomers of D- and L-amino acids of a sample derivatized separately with OPA–IBLC and OPA–IBDC and analysed under the same chromatographic conditions is very suitable in order to cross-check the presence of D-amino acids in complex matrices (for examples, see Figs. 7 and 10).

The time dependence of the relative fluorescence of selected L- and D-amino acids by derivatization with OPA–IBLC is shown in Fig. 3a and b, respectively. The formation of the diastereomeric isoindole derivatives is completed after 78 s; they are stable for at least 18 min (longer reaction times have not been tested) with the exceptions of Lys and Gly, which show a rapid decline of their fluorescence. As a result of the fast reaction, no kinetic discrimination could be observed.

The linearity of the fluorescence of amino acids derivatized with OPA–IBLC in the range 25–1000 pmol is shown in Table I.

The reproducibility of the peak areas and retention times of a DL-amino acid standard (100 pmol per amino acid enantiomer) derivatized with OPA–IBLC is shown in Table II. The relative fluorescence under these conditions was 40% for most amino acids (full-scale = 100%) at

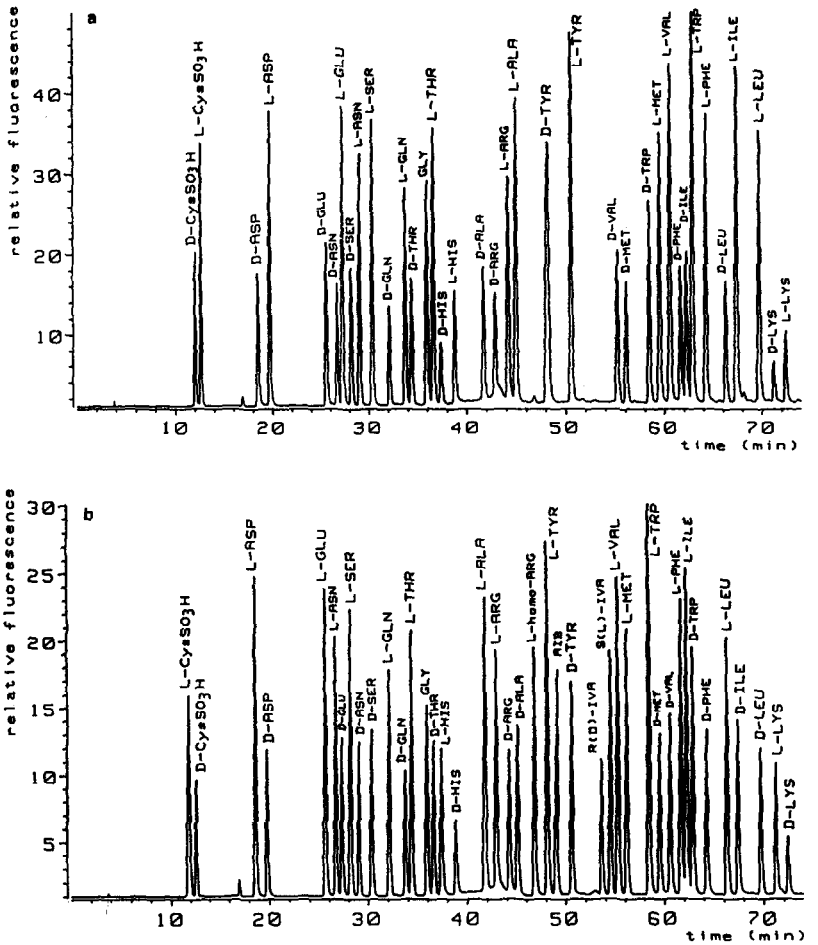

Fig. 1. Elution profiles of standards of amino acids derivatized with (a) OPA–IBDC and (b) OPA–IBLC. Amounts of 100 pmol of L-amino acids and of 50 pmol of D-amino acids and Gly. For the elution conditions for all chromatograms and abbreviations of amino acids, see Experimental.

a PMT gain of 10. This sensitivity was also used for most of the samples investigated. The relative standard deviation (R.S.D.) of the peak areas was ≤2.7% for Lys and His and ≤2.5% for the other amino acids. The R.S.D. for the retention times was ≤0.9% for Asp and Glu and ≤0.5% for the other amino acids.

The resolution (R_s) of the different amino acids was >1.2, with few exceptions, where pairs of amino acids had $R \approx 1.15$. The peak symmetry of all amino acid derivatives was >0.7 with the exceptions of D-Ile and L-Lys with a peak symmetry of approximately 0.55.

When the relative amounts of amino acid enantiomers are calculated from the relative fluorescence of their diasteromers (measured as peak areas), fluorescence factors have to be used (Table III). Using derivatization with the L-reagent the areas of the respective D–L diasteromers have to be multiplied using the fluorescence factors in Table III and with derivatization with the D-reagent the areas of the respective L–D diastereomers have to be multiplied with the fluorescence factors listed in Table III (for illustration see also Fig. 2). Relative amounts of D-amino acids were calculated from the equation $\%D = 100 \cdot D/(D + L)$.

Alternatively, for the quantification of amino

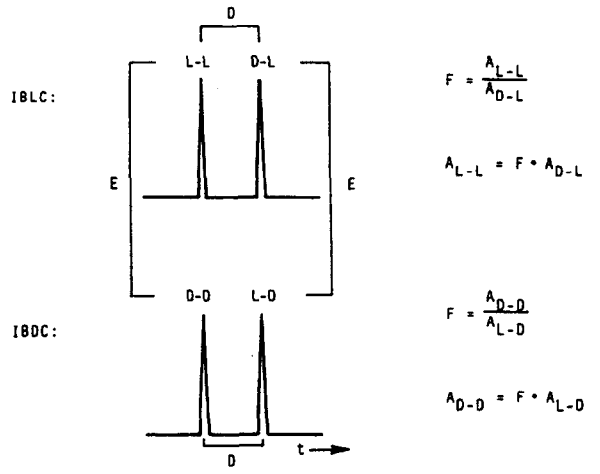

Fig. 2. Scheme of the elution order on a non-chiral stationary phase of diastereomeric derivatives, obtained by derivatization of D- and L-amino acids with OPA–IBLC and OPA–IBDC. Abbreviations: D and E on a bracket refer to diastereomeric and enantiomeric derivatives respectively; t = relative elution time; F = fluorescence factor, used for the correction of peak areas (A) as a result of the differing fluorescence of the diastereomers A_{L-L}, A_{D-L}, A_{D-D}, A_{L-D}; the first letter of the subscript refers to the amino acid enantiomer derivatized with the L- or D-reagent (second letter of subscript).

acid enantiomers an external standard consisting of D- and L-amino acids can be used, from which peak areas may be compared directly.

Biosamples

In order to demonstrate the general applicability of the method we have given several examples of the detection of D-amino acids in various areas of biosciences. Investigated were microorganisms (the bacterium *Lactobacillus acidophilus* and the mould *Nectria ochroleuca*), higher plants (apples, *Malus domesticus*), invertebrates (the worm *Tubifex tubifex*), vertebrates (dog, *Canis lupus* var. *domesticus*, and man, *Homo sapiens*) and the carbonaceous Murchison meteorite.

Lactobacillus acidophilus. The elution profile of amino acids, derivatized with OPA–IBLC, from a 70% ethanolic extract of the bacterium *Lactobacillus acidophilus* is shown in Fig. 4. High amounts of D-Asp (42.2%), D-Ala (25%) and D-Glu (12.8%) were found in addition to significant amounts of D-Ser (2.7%), D-Val

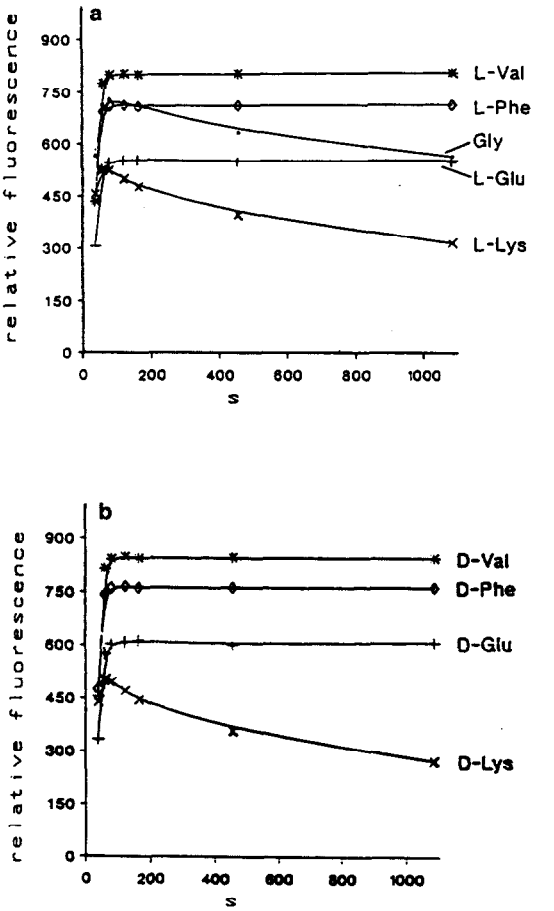

Fig. 3. Time dependence of the relative fluorescence of selected (a) L-amino acids and Gly, derivatized with OPA–IBLC, and (b) D-amino acids derivatized with OPA–IBLC.

(1.7%), D-Leu (1.1%) and D-Lys (2.2%). It is of interest that these extractable D-amino acids occur in their free state in the bacterial cytoplasma. Racemization of amino acids under the mild conditions experienced during extraction and derivatization is not possible. It is worth noting that free extractable D-amino acids have also been determined using chromatographic methods in various bacteria which are used as so-called starter cultures in food biotechnology [15] and, consequently, in fermented foods [17].

Moulds and Murchison meteorite. The determination of amino acid enantiomers obtained in a hydrolysed extract of the mycelium of the mould *Nectria ochroleuca* is shown in Fig. 5.

TABLE I

LINEAR REGRESSION ($y = ax + b$) AND CORRELATION COEFFICIENTS (r) FOR AMINO ACID ENANTIOMERS DERIVATIZED WITH OPA–IBLC AND RELATIVE STANDARD DEVIATIONS (R.S.D.) CALCULATED FROM THE RELATIVE FLUORESCENCE OF AMINO ACID DERIVATIVES (x = 1000, 250, 100 AND 25 pmol OF AMINO ACID INJECTED, y = RELATIVE FLUORESCENCE MEASURED AS PEAK AREA, n = 3)

Amino acid	1000, 250, 100 and 25 pmol			R.S.D. (%)			
	a	*b*	*r*	1000 pmol	250 pmol	100 pmol	25 pmol
L-Asp	0.691	−3.02	1.000	1.1	1.1	0.7	2.2
D-Asp	0.659	−3.19	1.000	1.1	1.1	0.5	2.5
L-Glu	0.630	−2.86	1.000	1.2	1.2	0.5	1.9
D-Glu	0.695	−4.07	1.000	1.2	1.2	0.6	2.0
L-Asn	0.638	−0.82	1.000	1.0	1.0	0.6	1.0
D-Asn	0.721	−1.18	1.000	1.0	1.0	0.7	1.2
L-Ser	0.701	−1.27	1.000	1.0	1.1	0.8	1.1
D-Ser	0.768	−2.60	1.000	1.0	1.0	0.7	1.4
L-Gln	0.638	1.32	1.000	0.1	0.4	1.9	0.5
D-Gln	0.719	1.44	1.000	0.2	0.5	1.8	0.7
L-Thr	0.712	−1.76	1.000	1.1	0.8	0.6	1.5
D-Thr	0.822	−1.13	1.000	1.1	1.2	0.4	1.3
Gly	0.826	−9.88	0.999	0.9	1.4	0.9	1.5
L-His	0.482	−3.00	1.000	1.2	1.4	0.6	1.4
D-His	0.472	−3.41	1.000	1.2	2.5	3.1	1.4
L-Ala	0.784	0.59	1.000	1.0	1.4	0.5	1.8
D-Ala	0.899	−0.49	1.000	1.0	0.9	0.3	1.2
L-Arg	0.770	−0.75	1.000	1.0	0.9	0.1	1.3
D-Arg	0.844	−1.73	1.000	1.1	1.1	0.2	1.5
L-*homo*-Arg	0.767	−1.94	1.000	1.0	1.1	0.1	1.1
L-Tyr	0.971	0.15	1.000	1.0	1.0	0.2	0.8
D-Tyr	1.080	−0.93	1.000	1.0	0.9	0.1	1.0
L-Val	0.930	−0.99	1.000	1.0	1.0	0.1	0.8
D-Val	0.973	−0.61	1.000	1.0	1.0	0.1	1.0
L-Met	0.750	−0.94	1.000	1.0	0.9	0.1	1.0
D-Met	0.855	−1.67	1.000	1.1	1.0	0.1	1.0
L-Trp	1.250	−0.78	1.000	1.0	0.9	0.1	1.0
D-Trp	1.290	−0.61	1.000	0.9	0.9	0.4	1.0
L-Phe	0.820	−0.76	1.000	1.0	1.0	0.3	1.0
D-Phe	0.884	−0.91	1.000	1.0	0.9	0.9	1.3
L-Ile	0.938	−0.98	1.000	1.0	1.1	0.2	1.0
D-Ile	0.982	−0.83	1.000	1.2	0.9	0.3	1.6
L-Leu	0.722	−1.09	1.000	1.1	1.1	0.4	1.4
D-Leu	0.808	−2.59	1.000	1.0	1.1	0.2	0.8
L-Lys	0.613	−8.15	0.999	1.1	2.3	0.6	1.2
D-Lys	0.574	−9.11	0.999	1.1	2.5	0.8	1.8

High amounts of the non-proteinogenic α-aminoisobutyric acid (α-methylalanine, Aib) were found in addition to roughly equal amounts of D- and L-isovaline (2-ethylalanine, Iva). Remarkably, Aib and approximately equal amounts of D- and L-Iva were also found to occur in the *free* state in the mycelium of this mould. This is of particular interest because for the first time the simultaneous biosynthesis of the D- and L-enantiomers of Iva by a microorganism has been observed. Aib and D-Iva could also be detected by the method in hydrolysed extracts of the microfungus *Hypocrea muroiana* IFO 31288 and the entomopathogenic fungus *Metarhizium*

TABLE II

PEAK AREAS (*A*) OF L- AND D-AMINO ACIDS (100 pmol) USED FOR THE CALCULATION OF THE RELATIVE FLUORESCENCE FACTORS IN TABLE III, AND ABSOLUTE RETENTION TIMES (*t*) OF DERIVATIVES

S.D. = Standard deviation and R.S.D. = relative standard deviation ($n = 5$). Derivatization with OPA–IBLC.

Amino acid	*A*			*t* (min)		
	Mean	S.D.	R.S.D. (%)	Mean	S.D.	R.S.D. (%)
L-Asp	419	9.6	2.3	18.89	0.162	0.9
D-Asp	401	7.2	1.8	20.09	0.155	0.8
L-Glu	439	8.0	1.8	25.87	0.166	0.6
D-Glu	481	8.5	1.8	27.54	0.175	0.6
L-Asn	402	7.8	1.9	26.95	0.115	0.4
D-Asn	459	7.9	1.7	29.30	0.132	0.5
L-Ser	433	7.2	1.7	28.46	0.117	0.4
D-Ser	472	7.3	1.5	30.61	0.138	0.5
L-Gln	409	7.0	1.7	32.41	0.112	0.3
D-Gln	461	8.1	1.8	34.01	0.112	0.3
L-Thr	440	6.2	1.4	34.59	0.116	0.3
D-Thr	478	6.6	1.4	36.78	0.120	0.3
Gly	479	6.7	1.4	36.21	0.120	0.3
L-His	272	5.6	2.1	37.76	0.091	0.2
D-His	271	6.1	2.3	39.15	0.088	0.2
L-Ala	497	8.5	1.7	41.95	0.118	0.3
D-Ala	533	9.6	1.8	45.27	0.117	0.3
L-Arg	522	11.3	2.2	43.29	0.102	0.2
D-Arg	577	10.9	1.9	44.66	0.119	0.3
L-Tyr	599	7.9	1.3	48.29	0.126	0.3
D-Tyr	685	8.3	1.2	50.87	0.135	0.3
L-Val	552	7.0	1.3	55.36	0.144	0.3
D-Val	589	7.7	1.3	60.71	0.147	0.2
L-Met	450	7.9	1.8	56.30	0.153	0.3
D-Met	508	8.1	1.6	59.65	0.158	0.3
L-Trp	628	9.0	1.4	58.53	0.169	0.3
D-Trp	678	10.4	1.5	63.08	0.205	0.3
L-Phe	448	9.2	2.1	61.80	0.144	0.2
D-Phe	499	8.5	1.7	64.39	0.159	0.2
L-Ile	547	8.9	1.6	62.33	0.192	0.3
D-Ile	580	9.3	1.6	67.56	0.160	0.2
L-Leu	426	4.8	1.1	66.45	0.180	0.3
D-Leu	471	4.8	1.0	69.87	0.174	0.2
L-Lys	354	9.1	2.6	71.41	0.169	0.2
D-Lys	322	8.7	2.7	72.60	0.181	0.2

anisopliae CBS 597.80 (chromatograms not shown). It should be emphasized that, owing to the lack of an α-hydrogen atom, the acid-catalysed racemization of Iva, and also α-alkyl-α-amino acids, is in general not possible.

These findings are of the greatest interest as the detection of racemic Iva in sediment samples close to the cretaceous–tertiary (K–T) boundary has been used as evidence for the impact of a carbonaceous meteorite about 65 million years ago [27,28]. The determination of amino acid enantiomers in a hot-water extract of the Murchison meteorite (amino acid-containing, carbonaceous chondrite type CM 2) is shown in Fig. 6. The non-protein, achiral Aib and both of the Iva enantiomers were detectable. As to whether

TABLE III

RELATIVE FLUORESCENCE FACTORS (*F*) CALCULATED FROM PEAK AREAS FOR AMINO ACIDS DERIVATIZED WITH OPA–IBLC ($n = 5$)

For use of factors, see Fig. 2.

Amino acid	*F*		
	Mean	S.D.	R.S.D. (%)
Asp	1.04	0.011	1.06
Glu	0.91	0.013	1.43
Asn	0.87	0.003	0.34
Ser	0.92	0.010	1.09
Gln	0.89	0.007	0.79
Thr	0.92	0.006	0.65
His	1.01	0.025	2.48
Ala	0.90	0.017	1.89
Arg	0.90	0.021	2.33
Tyr	0.87	0.007	0.80
Iva	1.11	0.010	0.90
Val	0.92	0.012	1.30
Met	0.89	0.006	0.67
Trp	0.93	0.004	0.43
Phe	0.91	0.007	0.77
Ile	0.96	0.013	1.35
Leu	0.90	0.005	0.56
Lys	1.11	0.024	2.16

Iva in this meteorite sample is completely racemic could not be determined with certainty by application of either IBLC or IBDC. The occurrence of non-proteinogenic amino acids such as Aib and of racemic Iva in certain carbonaceous meteorites is considered as proof for the abiotic synthesis of these amino acids [29,30]. The large amounts of L-Asp and L-Ser in the meteorite extract shown in Fig. 6 are attributed to terrestrial contamination.

Fruit juices. The elution profile of free amino acids isolated from the freshly squeezed juice of an apple (Golden Delicious), determined with IBLC and IBDC is shown in Fig. 7a and b. Significant amounts of D-Ala (2.7%), D-Asp (0.4%), D-Asn (0.7%), D-Glu (0.5%) and D-Ser (1.7%) were found. These results were also confirmed by GC–MS (data not shown). It is concluded that these D-amino acids occur in their free state in the cytoplasma of apple, as fresh fruit was used and contamination through the growth of microorganisms in the juice was prevented. The same D-amino acids were also found in other kinds of apples analysed by HPLC and GC–MS (Granny Smith, Jonathan, Jonagold) and pears, (Alexander Lucas), hence there is little doubt that low but significant amounts of certain free D-amino acids occur naturally in the cytoplasma of certain fruits, as has been assumed previously [31].

Worms. The chromatogram of amino acids found in an ethanolic extract of the worm *Tubifex tubifex*, the habitat of which is in the mud of riverbeds and lakes, is shown in Fig. 8. Large amounts of D-Ser (25%) and D-Ala

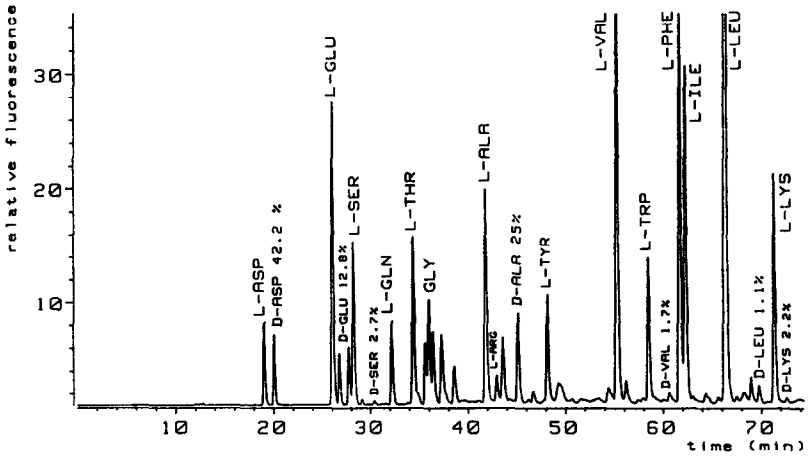

Fig. 4. Elution profile of amino acids from an ethanolic extract of the bacterium *Lactobacillus acidophilus*; derivatization with OPA–IBLC. Relative amounts of D-amino acids were calculated from the equation $\%D = 100 \cdot D/(D + L)$.

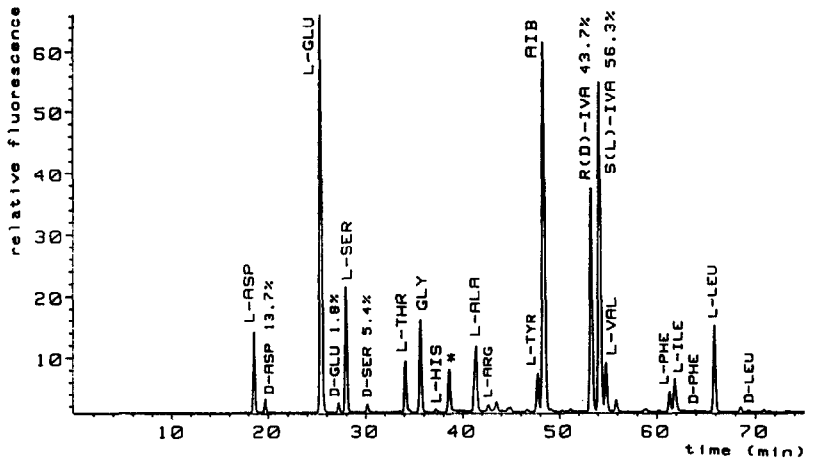

Fig. 5. Elution profile of amino acids from a totally hydrolysed organic extract of the mould *Nectria ochroleuca*; derivatization with OPA–IBLC. Aib = α-aminoisobutyric acid; Iva = isovaline; asterisk = unknown.

(9.5%) were detected. These results are in general agreement with reports in which the presence of D-Ser in the earthworm *Lubricus terrestris* [32] and of various D-amino acids in the lungworm *Dictyocaulus viviparus* [33] have been reported.

Blood serum of mammals including man. Fig. 9a shows a chromatogram of amino acids found in the blood serum of a dog; D-Asp (6.0%; 0.97 nmol/ml) and D-Ser (1.9%; 4.16 nmol/ml) were found. In the blood plasma of a stallion, D-Asp (7.5%; 1.23 nmol/ml) and D-Ser (1.9%; 3.49 nmol/ml) were detected by HPLC (chromatogram not shown). Fig. 9b shows that D-Asp (4.0%) and D-Ser (1.0%) were also detected in the blood serum of a human male. This confirms results reported recently, in which the blood of healthy volunteers and patients suffering from renal diseases were investigated for D-amino acids [5,7]. Taking these data and reports as to the occurrence of D-amino acids in guinea pigs [8], rats and mice [4,6,9,10,34] into account, it is concluded that significant amounts of D-amino acids are permanently present in certain physiological fluids and tissues of mammalia.

Human urine. That D-amino acids are excreted in urine is demonstrated in Fig. 10a and b. Using IBLC and IBDC, in the urine of a healthy male

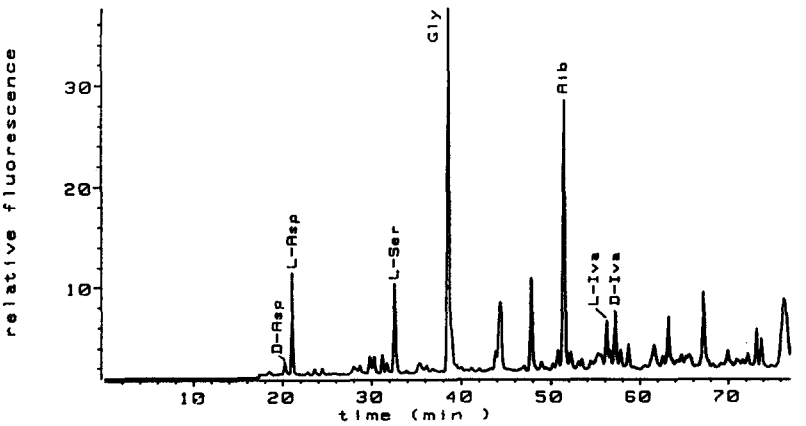

Fig. 6. Elution profile of amino acids from a hot water extract of the carbonaceous Murchison meteorite derivatized with OPA–IBDC.

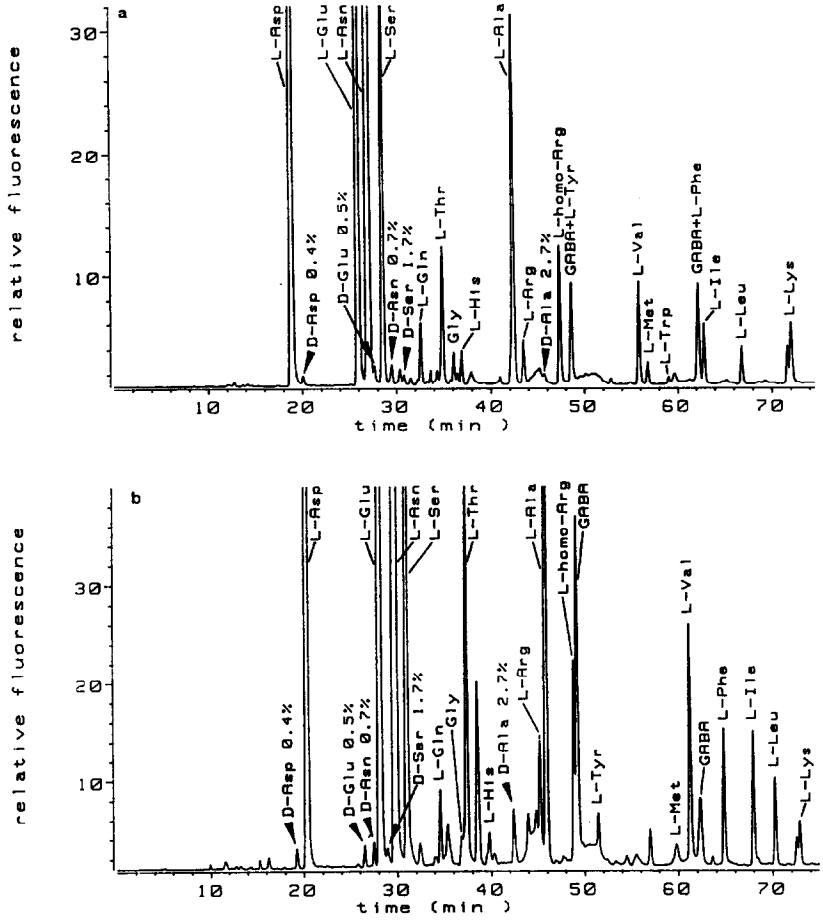

Fig. 7. Elution profile of amino acids from freshly pressed apple juice (Golden Delicious); derivatization with (a) OPA–IBLC and (b) OPA–IBDC.

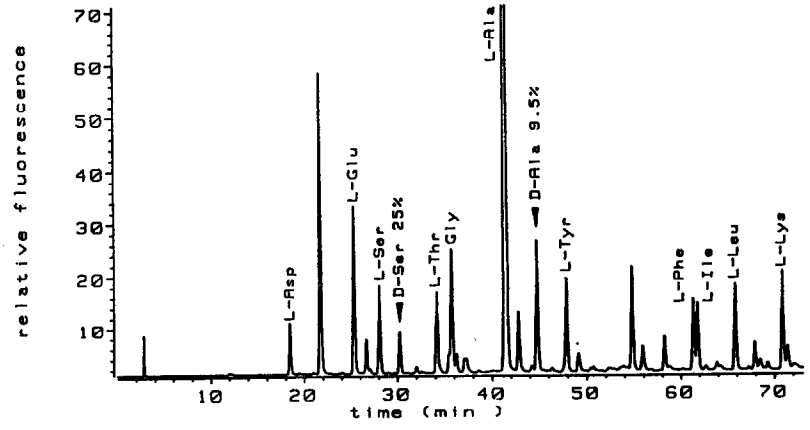

Fig. 8. Elution profile of amino acids from an ethanolic extract of the worm *Tubifex tubifex*; derivatization with OPA–IBLC.

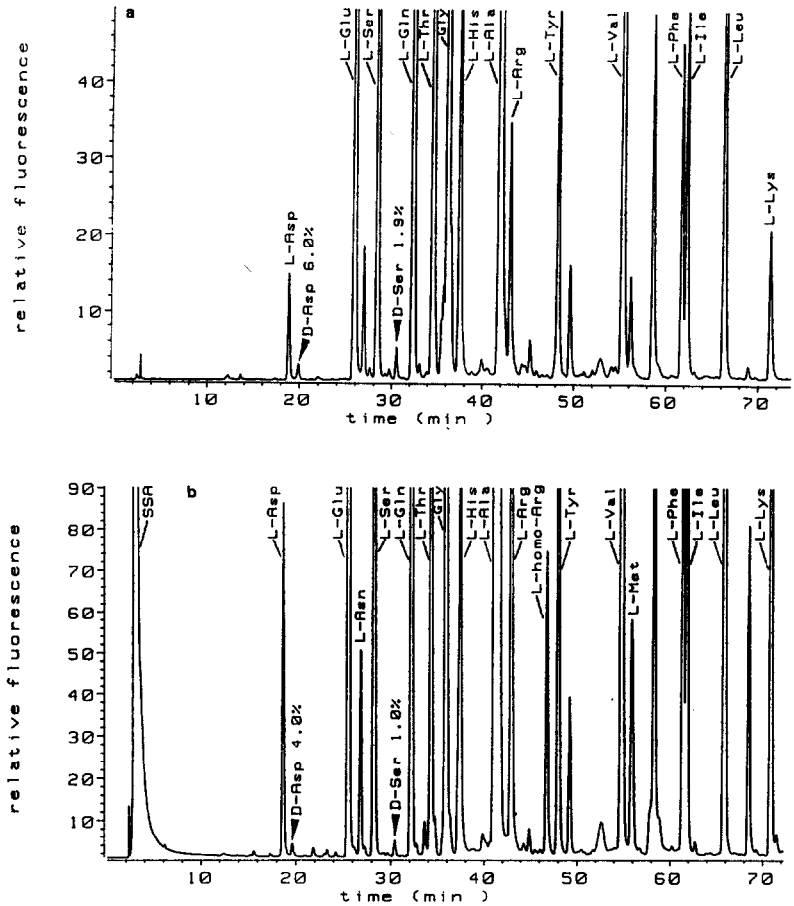

Fig. 9. Elution profiles of amino acids from the blood serum of (a) a dog and (b) a human male; derivatization with OPA–IBLC. SSA = Sulphosalicylic acid (with reference to (a), the detector was switched on after 10 min and therefore no SSA can be seen).

volunteer large amounts of D-Ser (60.3%; 224 nmol/ml) and D-Ala (20.6%; 82 nmol/ml) and significant amounts of D-Gln (7.6%; 44 nmol/ml) were found. D-Ser and D-Ala, among other D-amino acids, have also been detected in relatively large amounts and as the most abundant D-amino acids in the urine of other volunteers [35]. This is of interest as the liquid chromatographic detection of only low or trace amounts of certain D-AA (D-Phe, D-Tyr, D-Trp, D-Phe and D-pipecolic acid) in human urine has been reported [6,9,37]. From the results it is evident that significant amounts of various D-amino acids are renally excreted and that the D-amino acid oxidase system of humans [36] obviously does not completely oxidize these D-amino acids.

The results demonstrate that the chromatographic method described makes possible the efficient detection and determination of D-amino acids in biomatrices and hence the screening of biological samples including organisms for the presence of D-amino acids. For examples from the fields of peptide chemistry [38,39] and food science [19,24] we refer to the literature.

Practical considerations

After having performed several hundred analyses of various biomatrices, certain practical considerations should be discussed.

Eluents are best prepared by weighing the constituents and not by volumetric measurements. The pH of 5.95 is determined by use of a glass electrode calibrated with standard buffers. It was found to be helpful to measure the pH of

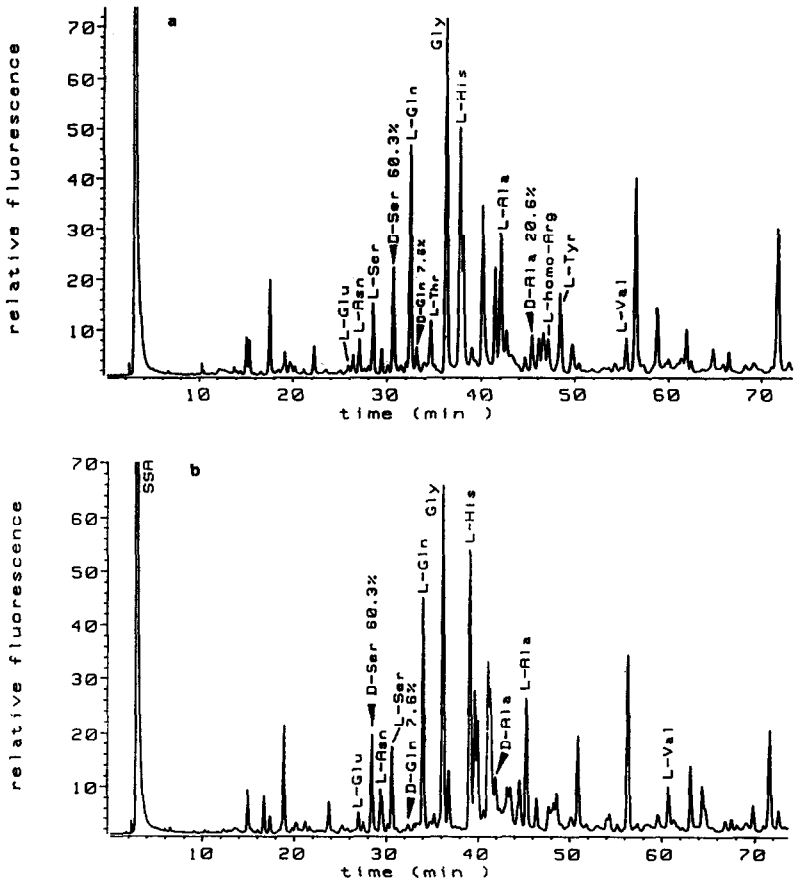

Fig. 10. Elution profile of amino acids from the urine of a human male; derivatization with (a) OPA–IBLC and (b) OPA–IBDC.

the remaining eluent (A) and to compare and adjust the pH of the freshly prepared buffer accordingly. It is important to note that sodium acetate buffer is a good growth medium for microorganisms and thus a source of L- and D-amino acids. If not consumed within a few days, the buffer should be stored at 4°C in the dark. The ODS phase used was found to resolve the standard reproducibly, also when different batches of the stationary phase were used. A single column is able to resolve approximately 700 injections of standard; a change of the precolumn is necessary after approximately 70 injections. The removal of protein in biosamples by the use of picric acid or sulphosalicylic acid is necessary. A further clean-up step by the use of a cation exchanger can be advantageous with complex samples. Approximately 400 analyses of biological samples are possible when the precolumn is changed regularly. When proteins are precipitated but no ion-exchange procedure has been carried out, it might be necessary to change the precolumn every 30–40 analyses.

The guard column should be renewed when a decrease in the resolution of the amino acid standard is observed. Blanks with reference to the ion exchanger and chemicals should be run in parallel with the analysis.

Particular care should be given to the purity of the doubly distilled or deionized water, used for all procedures, and the aqueous ammonia, used for the elution of amino acids from the ion exchanger should be tested for the absence of amino acids. Further, when trace amounts of D-amino acids have to be determined, in agreement with the literature [40], the use of heat-cleaned tools and glassware is strongly recommended. It should also be kept in mind that

bacterial presence in samples or reagents will with certainty lead to contamination with D-amino acids. For the principles concerning the preparation of biosamples for subsequent chromatographic amino acid analysis, we refer to the literature [41,42].

Sulphosalicylic acid, used for deproteinizing samples, is eluted at the very beginning of the chromatogram (see Figs. 9 and 10) and therefore does not influence the chromatographic resolution. It is worth noting that we have observed a strong memory effect of this compound within the HPLC system. The crystalline reagents, IBLC and IBDC, are stable but should be stored at −18°C under nitrogen. The prepared reagent solutions should be stored at −18°C and small aliquots removed for the analyses as required. The optical purity of the reagents should be checked by reaction with an L-amino acid of highest optical purity. We used L-Asp; it may be necessary, however, to recrystallize this or other reference amino acids once or twice in order to obtain the required purity [43]. It is also recommended to check the optical purity of this standard AA by use of a direct GC or HPLC method. For measuring the relative amounts of D- and L-amino acids, the fluorescence factors of the derivatives (see Table III) have to be taken into account. For determining the recovery of the enantiomers from biological samples the use of L-*homo*-Arg as an internal standard is recommended. Other frequently used internal amino acid standards such as norvaline, norleucine, cycloleucine or α-amino-β-guanidinopropionic acid co-elute with amino acids of the standard mixture and are therefore less suitable for complex samples. As no standard with suitable amounts of L- and D-amino acids is commercially available, to the best of our knowledge, it has to be prepared by the analyst. In this event the optical purity of the amino acid enantiomers used have to be checked. In agreement with others [37,43], we have found that certain purchased amino acids are contaminated with relatively large amounts of the opposite enantiomer.

As the verification of D- and L-amino acids using the procedure described is based solely on a comparison of the retention times of the diastereomers formed, the reversal of the elution order of the derivatives formed from amino acid enantiomers by derivatization with the L- or D-reagent has been found to be most valuable (see Figs. 1, 7 and 10).

In comparison with the gas chromatographic determination of amino acid enantiomers, the liquid chromatographic method has the advantage that the analysis is fully automated and that those amino acids which require special attention in GC [44] such as Asn, Gln, Arg and His are determinable. Further, the absolute quantification of enantiomers is much easier to achieve with HPLC in contrast to capillary GC. In instances where the elegant method of enantiomer labelling [44] is used for the quantification of amino acids by GC, the amounts of D-amino acids already present in the sample have to be determined. Reversal of the elution order of amino acid enantiomers (or diastereomers) does not require a change of the stationary phase simply a change of the L- or D-reagent under otherwise identical chromatographic conditions. In GC the availability of an L- and D-stationary phase is necessary. On the other hand, GC together with mass spectrometry [35] is unrivalled with respect to resolution and reliability.

The diastereomeric approach is an indirect method for the determination of amino acid enantiomers and requires reagents of the highest optical purity. The secondary DL-amino acids Pro and Hyp are not yet determinable.

In comparison with other chiral reagents for the analysis of DL-amino acids [45], and according to published results, only (+)- and (−)-1-(9-fluorenyl)ethyl chloroformate (FLEC) seem to offer an alternative method with reference to the liquid chromatographic resolution of complex mixtures of amino acid enantiomers [46,47]. It should be noted that the results reported using FLEC do not appear to be easily reproducible. This may be attributable to the very delicate chromatographic conditions required, *i.e.*, a multi-step gradient, starting with large amounts of THF in conjunction with unsatisfactory matched stationary phases. Further, several amino acids in addition to DL-Pro are not resolved by FLEC using the standard analysis. DL-Pro appears, however, to be resolvable under specific chromatographic conditions [46]. Further, FLEC

has the disadvantage that an intensive reagent peak elutes in the chromatogram. It is also worth noting that the (+)- and (−)-FLEC reagents are much more expensive than the IBLC and IBDC reagents, which are easily synthesizable in two steps from L- and D-cystine using standard laboratory equipment [24].

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