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Determination of enantiomeric amino acids with high-performance liquid chromatography and pre-column derivatisation with *o*-phthaldialdehyde and *N*-isobutyrylcysteine in seawater and fossil samples (mollusks)

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Abstract

After derivatisation with *ortho*-phthaldialdehyde and *N*-isobutyryl-D-cysteine or *N*-isobutyryl-L-cysteine, amino acid enantiomers and non-chiral amino acids, including the non-protein amino acids allo-isoleucine, α-amino-*n*-butyric acid, γ-aminobutyric acid and D-allo-threonine, were separated by high-performance liquid chromatography (HPLC) into their enantiomers and detected by fluorescence. For elution a ternary gradient was applied consisting of methanol and two sodium acetate buffers (pH 5.3 and 7.0). The use of two different reagents under the same HPLC conditions allows the control of the peak purity. Applications and results for dissolved and particulate amino acids in seawater and fossil Pleistocene samples (mollusks) are presented.

Keywords: Enantiomer separation; Derivatisation, LC; Geochronology; Water analysis; Amino acids

1. Introduction

The determination of amino acids is an important tool in biogeochemistry and often applied in terrestrial and aquatic investigations. While bulk parameters such as total carbon and nitrogen give only information on quantity, individual components describe also qualitative changes of the dissolved and particulate organic material. The abundance of D-amino acids in organic matter is a suitable tracer for the contribution of microbial biomers due to the high D-amino acid content in peptidoglycan, the main building block of bacterial cell walls [1].

In geochronology, amino acid analysis is widely used for dating soils and sediments. Abelson [2] found that amino acids are preserved in calcareous fossils, like mollusks, foraminifera and bones. While recent proteins consist exclusively of L-amino acids, fossil samples contain also D-enantiomers which are slowly converted from L-amino acids, finally leading to an equilibrium of L- and D-isomers. From the degree of racemisation the age of the sample can be estimated [3].

In biogeochemistry and geochronology, DL-amino acids are mainly analysed by gas chromatography (GC). Liquid ion-exchange chromatography (IC) with post-column derivatisation, often used in geochronology, only separates the diastereomeric L-isoleucine/D-allo-isoleucine pair, which is the most

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important pair for age determination of geological samples. This method is easier in handling, but fails to separate enantiomeric amino acids [4]. GC analysis requires time consuming sample clean up and desalting with high risk of contamination [5]. For analysis of amino acids in particulate and dissolved organic matter in seawater, the high-performance liquid chromatography (HPLC) method using *o*-phthaldialdehyde (OPA) was introduced by Lindroth and Mopper [6]. Amino acids and other primary amines form highly fluorescent isoindolyl derivatives after reaction with OPA and a thiol compound. For analysis of amino acid enantiomers a chiral thiol compound is necessary to form diastereomeric derivatives which can be separated by chromatography on a stationary phase. As thiols numerous compounds have been reported: *N*-acetyl-D-penicillamin (NAP) [7], thiosugars [8,9], several *N*-protected cysteine derivatives, e.g., *N*-acetyl-L-cysteine (NAC) [7,10–13] and *N*-isobutyrylcysteine (IBC) [14,15] from which IBC gives the best resolution [15]. A review on HPLC methods to separate amino acid enantiomers by derivatisation reagents, chiral stationary phases and the use of chiral mobile phase additives is given by Bhushan and Joshi [16].

For this study the OPA–IBC method was applied to unconcentrated seawater samples and samples of particulate material. Our aim was to obtain a better understanding of the pathways and the biogeochemistry of amino acids in aquatic environments, in particular about the contribution of D-amino acids. Due to the good separation of L-isoleucine/D-allo-isoleucine, samples used in an interlaboratory comparison study [17] were analysed in order to prove also the potential of the method in geochronology.

2. Experimental

2.1. Chemicals and reagents

N-Isobutyryl-L-cysteine (IBLC), *N*-isobutyryl-D-cysteine (IBDC), D-allo-isoleucine (D-allo-Ile), and L-allo-isoleucine (L-allo-Ile) were obtained from Fluka (Buchs, Switzerland). All other amino acids were from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA), fluoraldehyde reagent diluent from Pierce (Rockford, IL, USA), OPA for fluorescence analysis from Merck (Darmstadt, Germany). All other chemicals were of the highest available grade purchased from Merck. HPLC-grade water was produced by a Milli-Q Plus 185 system.

The OPA reagent was prepared by dissolving 200 mg OPA in 100 ml of fluoraldehyde reagent diluent (pH 10.4). For the thiol reagent 480 mg of IBDC or IBLC was dissolved in 100 ml of methanol. The OPA reagent was stored at 4°C, the thiol reagent at −18°C. Under these conditions the reagents were stable for about one week.

Ascorbic acid solution consists of 2 g ascorbic acid dissolved in 1 l of water, 0.5 *M* boric acid buffer was made by dissolving 30.91 g boric acid in 1 l of 32% sodium hydroxide. Pentasodium triphosphate solution was prepared by dissolving 10 g pentasodium triphosphate in 100 ml of water.

The standard mixtures were composed of 500 n*M* of each L-amino acid, glycine and γ-amino butyric acid (GABA), and 250 n*M* of each D-amino acid, dissolved in water. For storage, the standards were kept in a freezer at −30°C.

2.2. Chromatographic system

Derivatisation and HPLC were carried out on the Merck LaChrom HPLC system, consisting of the L-7100 quaternary low-pressure pump, L-7480 fluorescence detector, L-7250 intelligent autosampler and D-7500 integrator. The solvents were degassed by a Knauer membrane degasser (Berlin, Germany). The column used for the separation was a Superspher RP18 stainless steel cartridge, 4 μm particle diameter, 125 mm×4 mm I.D. (Merck) and a LiChrospher RP18 guard column, 5 μm, 4×4 mm (Merck). The column temperature was maintained at 20°C. The amino acid derivatives were monitored by fluorescence detection with excitation at 330 nm and emission at 445 nm.

For elution, a ternary gradient was used. Mobile phases A and B were 25 m*M* sodium acetate buffer (pH 7 and 5.3, respectively), mobile phase C was methanol. To prepare the buffer solutions, 2.05 g sodium acetate (anhydrous) were dissolved in 900 ml of water and adjusted to pH 7 by addition of 3% HCl or to pH 5.3 by 32% HCl. Then the final volume was made up to 1 l. The flow-rate of the gradient was 0.8 ml/min and the pressure did not exceed 200 bar. The

Table 1
Gradient profile for HPLC separation of OPA–IBC derivatives of amino acids

Time (min)	% A (25 m*M* sodium acetate, pH 7)	% B (25 m*M* sodium acetate, pH 5.3)	% C (methanol)
0	91	4	5
48	61	4	35
80	3	38	59
85	21	4	75
90	91	4	5
95	91	4	5

multi-step gradient (Table 1) started at pH 6.6 and ended at a final pH of 5.4 after 80 min. Thereafter, the column was flushed with methanol and equilibrated to starting conditions for 15 min.

2.3. Sample preparation

Hydrolysis of total dissolved amino acids (TDAAs): seawater samples were collected during cruises to the Siberian shelf regions (Arctic). Samples were filtered through Whatman GF/C glass fibre filters (precombusted at 500°C for 5 h) and stored in 50-ml glass ampoules at −30°C until analysis. For hydrolysis of the combined amino acids in the home laboratory, 10 ml of seawater were put into 50-ml glass ampoules and 10 ml of 32% hydrochloric acid and 100 μl of 11 m*M* ascorbic acid were added. The ampoules were flushed with nitrogen, melted down and kept for 24 h at 110°C. The solution was neutralised with 8.9 ml borate buffer, and the pH was adjusted to 8.5 with 32% NaOH.

Hydrolysis of particulate amino acids (PAA): 1 l of seawater was passed through precombusted glass fibre filters which were stored frozen (−30°C). The filters were put in 50-ml ampoules and after addition of 20 ml of 6 *M* HCl they were flushed with nitrogen and melted down. After 24 h at 110°C the solution was neutralised to pH 8.5 as described above for seawater samples.

Hydrolysis of fossil mollusks: the samples consisted of *Saxidomus* (ILC-A, approximated age of 50 000 years [17]) and *Mercenaria* (ILC-B, ca. 100 000 to 250 000 years and ILC-C, ca. 1 million years [17]). These samples have been used as reference and intercomparison material and were provided by John F. Wehmiller (Department of Geology, University of Delaware, DE, USA). For sample preparation and further information refer to Wehmiller [17]. One to 2 mg of powder were filled into a 100-μl glass microvial (Roth, Karlsruhe, Germany) and dissolved in 50 to 100 μl of 6 *M* HCl. Care must be taken to efferversence because of the CO_2-release. The liquid was decanted from the insoluble residue into another microvial, evaporated with nitrogen, and redissolved in 6 *M* HCl. The microvial was sealed with a blue silicone-PTFE lined septa (Chromacol, Welwyn Garden City, UK). Hydrolysis was performed under conditions as described above (110°C, 24 h, nitrogen atmosphere). The acid was removed with nitrogen, and the still acidic residue was redissolved in 200 μl of pentasodium triphosphate solution. The pH was controlled with Merck Alkalit pH strips to be in the range of 8–9.

2.4. Derivatisation procedure

Eight hundred μl of sample (hydrolysate from seawater or filters) was mixed with 100 μl of OPA reagent and 100 μl of the thiol reagent in a 1.5-ml standard vial (Merck). After 2 min of reaction time a volume of 100 μl was injected into the chromatographic system. The derivatisation procedure and injection was carried out automatically by the autosampler [18]. For the mollusk samples smaller volumes were used: 75 μl of hydrolysate are derivatised with 10 μl of each reagent and after 2 min of reaction time 50 μl of the sample was injected.

3. Results and discussion

3.1. Methodological aspects

Two HPLC chromatograms of amino acid standard mixtures are shown in Fig. 1, derivatised with IBLC (a) and IBDC (b). The standard mixture in Fig. 1a contains 39 and in Fig. 1b 40 amino acids, including the non-protein amino acids GABA, D-allo-threonine (D-allo-Thr), D- and L-α-amino-*n*-butyric acid (D-ABA, L-ABA) and D- and L-allo-isoleucine (D-allo-Ile, L-allo-Ile). All amino acid pairs could be separated in their enantiomers. Only few peaks interfered, depending on the chiral reagent used for derivatisation: With IBLC, the peaks of D-glutamine and L-threonine, glycine and D-threonine were interfering; with IBDC the peaks of L-glutamine and D-threonine, glycine and L-threonine, L-alanine and D-allo-threonine overlapped. Secondary amino acids such as proline or hydroxy proline are not derivatised with OPA and thiols, and cysteine and cystine give a very low fluorescence signal.

The retention times t_R, peak resolutions R_S, the standard deviations of retention times and the relative standard deviations (R.S.D.s) of peak areas are given in Table 2. Valine and tryptophan showed the

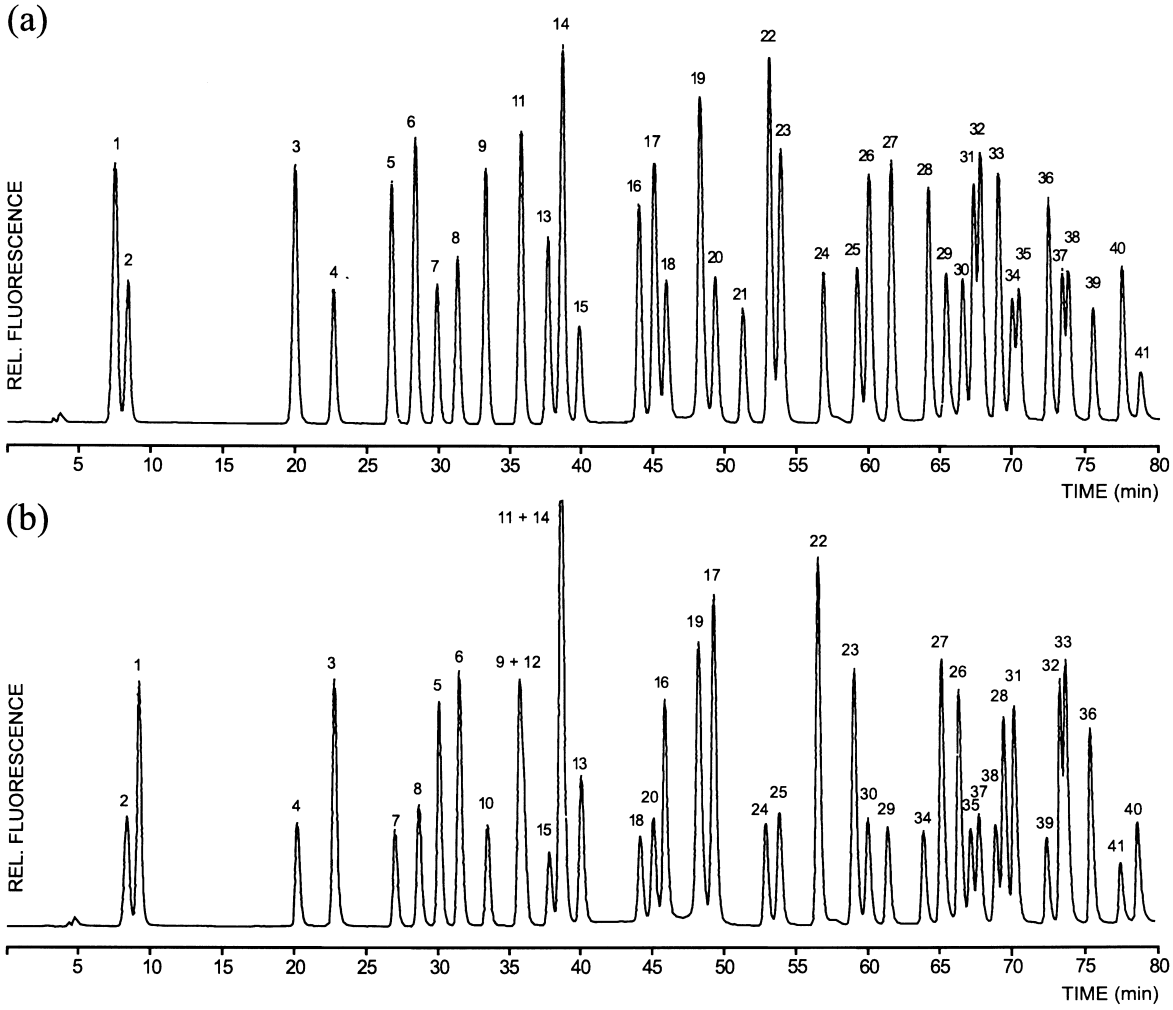

Fig. 1. Chromatograms of standards of OPA–IBLC (a) and OPA–IBDC (b) derivatives of free amino acids. For chromatographic conditions refer to Section 2.2 and Table 1, peak numbers as in Table 2.

Table 2
Retention times (t_R), peak resolutions (R_S) and relative standard deviations (R.S.D.s) of peak areas for OPA–IBLC derivatives of amino acid standards

Peak No.	Amino acid	Retention time, t_R (min) ±standard deviation		Peak resolution (R_S)	R.S.D. of peak areas (%)	
		L-Form	D-Form		L-Form	D-Form
1, 2	DL-Aspartic acid	7.4±0.1	8.3±0.2	1.5	2.1	0.6
3, 4	DL-Glutamic acid	19.9±0.5	22.5±0.5	4.4	2.8	3.1
5, 7	DL-Asparagine	26.6±0.4	29.8±0.4	5.3	3.6	3.7
6, 8	DL-Serine	28.2±0.4	31.2±0.4	4.9	3.5	3.5
9, 10	DL-Glutamine	33.1±0.5	35.6±0.5	4.1	3.2	n.d.
11, 12	DL-Threonine	35.6±0.4	38.5±0.4	4.9	3.3	n.d.
13, 15	DL-Histidine	37.5±0.5	39.8±0.5	3.8	8.1	10.2
14	Glycine	38.5±0.6		–	3.1	
16, 18	DL-Arginine	44.0±0.5	45.9±0.5	3.1	3.4	2.6
17, 20	DL-Alanine	45.0±0.5	49.3±0.6	7.1	2.6	3.7
19	GABA	48.2±0.6		–	0.5	
21	D-allo-Threonine		51.2±0.5	–		3.3
22, 24	DL-Tyrosine	53.1±0.6	56.8±0.7	6.3	3.0	4.2
23, 25	DL-ABA	53.8±0.5	59.2±0.6	8.9	3.4	2.7
26, 30	DL-Valine	60.0±0.4	66.5±0.5	10.8	2.9	4.4
27, 29	DL-Methionine	61.5±0.5	65.3±0.5	6.3	3.0	2.8
28, 34	DL-Tryptophan	64.1±0.6	69.9±0.7	9.6	3.5	5.2
31, 35	DL-Phenylalanine	67.2±0.5	70.3±0.5	5.2	1.6	6.7
32, 37	DL-Isoleucine	67.7±0.5	73.3±0.4	9.4	5.4	2.5
33, 38	DL-allo-Isoleucine	68.9±0.5	73.7±0.4	8.0	3.8	3.0
36, 39	DL-Leucine	72.4±0.4	75.4±0.4	5.0	2.3	2.3
40, 41	DL-Lysine	77.4±0.5	78.7±0.4	2.1	7.2	7.9

Chromatographic conditions as in Section 2.2. Abbreviations: GABA=γ-aminobutyric acid, ABA=α-aminobutyric acid, n.d.=not determined. Number of replicates $n=4$.

best resolution factors with 10.8 and 9.8, respectively, while aspartic acid and lysine had the lowest R_S values. The best separation was achieved at a column temperature of 20°C, at higher temperatures the separation decreased rapidly. At lower temperature (5°C), the column back pressure increased over the limit of 300 bar. With ageing of the column, double peaks of aspartic acid may appear, possibly representing the dissociated and the non-dissociated form. This problem may be solved by varying the starting proportion of eluent B between of 2 and 8%.

The fluorescence signal was found to be linear over the range from 0.5 to 200 pmol per injection. The detection limits for each amino acid were in the lower picomol range and are comparable to those obtained by HPLC with other methods [10]. It should be mentioned that the derivatives of D- and L-amino acid pairs differ in their flourescence intensity (as diastereomers in general). Thus the D/L-ratio can not be calculated directly by the peak areas but by the concentrations obtained by calibration with external standards.

The excitation spectrum of the OPA–IBLC derivative of L-alanine shows two maxima at 230 nm and 330 nm [19]. For detection, the use of both wavelengths is possible. Sensitivity was on average five-times higher by using the excitation wavelength of 230 nm instead of 330 nm. However, at the shorter wavelength more interfering peaks were detected in biological samples and the noise level was higher. The R.S.D. of the peak areas calculated from four runs was 0.6–5.4% for standards, except for histidine and lysine derivatives. Lysine is known to form unstable OPA–IBC derivatives [14] which are destroyed due to the high retention and the low pH at the end of the run, as also reported by Umagat et al. [20] from the OPA–mercaptoethanol derivative of lysine. The standard deviations of the retention times

were between 0.1 and 0.7 min. The samples were injected directly without prior neutralisation. Addition of 20 μl of 7.5% HCl caused a decrease in signal response of 20% on average. To prevent rapid column degradation by the high pH, the injection volume should not exceed 100 μl.

The polarity of ionic compounds like amino acids and their interaction with the solid-phase in the chromatographic process is strongly dependent on the pH. To obtain an optimal separation, two buffers of different pH can be mixed for the formation of a pH gradient instead of using one buffer with constant pH. For our method we used this technique to combine the good separation behaviour of the early eluting amino acids at pH 6.6 with the improved separation of the aliphatic and aromatic amino acids (in particular L-isoleucine, L-phenylalanine and L-allo-isoleucine) at pH 5.4. By using different pH values the peak resolution for L-Ile and L-Phe can be increased from $R_S=0.4$ at pH 6.6 to $R_S=1.1$ at pH 5.3 with an isocratic buffer systems and with a pH gradient system to $R_S=0.8$.

All L-enantiomers, derivatised with OPA–IBLC, elute before their corresponding D-enantiomers (Fig. 1a). This elution order is changed (D before L), if the IBDC-reagent is used (Fig. 1b). The derivatisation of a DL-amino acid pair with the L-reagent forms the diastereomeric IBLC-D-amino acid and the IBLC-L-amino acid derivatives. Using the IBDC-reagent, the IBDC-L-amino acid and the IBDC-D-amino acid derivatives are formed. Because the IBLC-L- and the IBDC-D-forms as well as IBLC-D- and IBDC-L-forms are enantiomers, they have the same retention times with achiral stationary and mobile phases. Achiral amino acids like glycine or GABA form enantiomeric derivatives, which could not be separated. This characteristic makes it possible to detect coeluting impurities. While the amino acid derivatives change their order of elution, interfering peaks remain at the same position if the impurity is not chiral. Thus, with two runs both enantiomers of an amino acid can be quantified reliably even in biological matrices.

Racemisation occurs also during hydrolysis, catalysed by the high temperature and the acidic pH [21]. Treatment of free amino acids with hot acid has been used as a control of the racemisation of amino acid residues during acid hydrolysis, but this may not always be adequate. In some peptides increased racemisation has been observed due to chemical and sterical reasons. During hydrolysis the L-phenylalanyl moiety of the dipeptide L-Phe–L-Ser is racemised stronger than the free L-phenylalanine [22]. Racemisation is essentially dependent on the chemical environment and bonding of the amino acids. We found that enantiomeric ratios in hydrolysates of commercial L-amino acid standards varied within a range of 5%. Thus, the derivatisation ratios of standard mixtures can only give an estimate of racemisation [22], and ratios for TDAA, PAA and mollusk samples were not corrected for the racemisation blank.

3.2. Applications

The application of the OPA–IBC method to marine samples is shown in Figs. 2–4. The chromatogram of TDAAs from hydrolysed seawater samples of the Arctic Laptev Sea (Fig. 2) had a very similar composition to that reported for Greenland Sea samples [23]. Glycine represented 26% (mol%) of the total dissolved amino acids, followed by alanine (15%), aspartic acid (10%), serine (8%) and glutamic acid (7%). High relative amounts of D-alanine (30.8%), D-aspartic acid (17.5%), D-glutamic acid (14.4%) and D-serine (7.7%) were found which were calculated by the equation $\%\ D=100\cdot D/(D+L)$. PAAs (Fig. 3) were characterised by the dominance of glycine (13%), alanine (13%), glutamic acid (9%) and aspartic acid (8%) with significant proportions of D-aspartic acid (11.9%), D-arginine (9.2%), D-alanine (6.2%) and D-glutamic acid (6.0%). The amount of non-protein amino acids, GABA and DL-ABA, was low.

Although most organisms only contain L-enantiomers of the amino acids, there are several biological and chemical explanations for the occurrence of D-amino acids in aquatic environments. D-Aspartic acid, D-alanine and D-glutamic acid are known to be major components of bacterial cell wall proteins, which may explain the high ratios of these amino acids found in PAA and TDAA samples [24]. Another suggested mechanism is the formation of glycine and racemic mixtures of alanine and ABA by dehydration reactions of serine and threonine [25].

The use of pentasodium triphosphate as com-

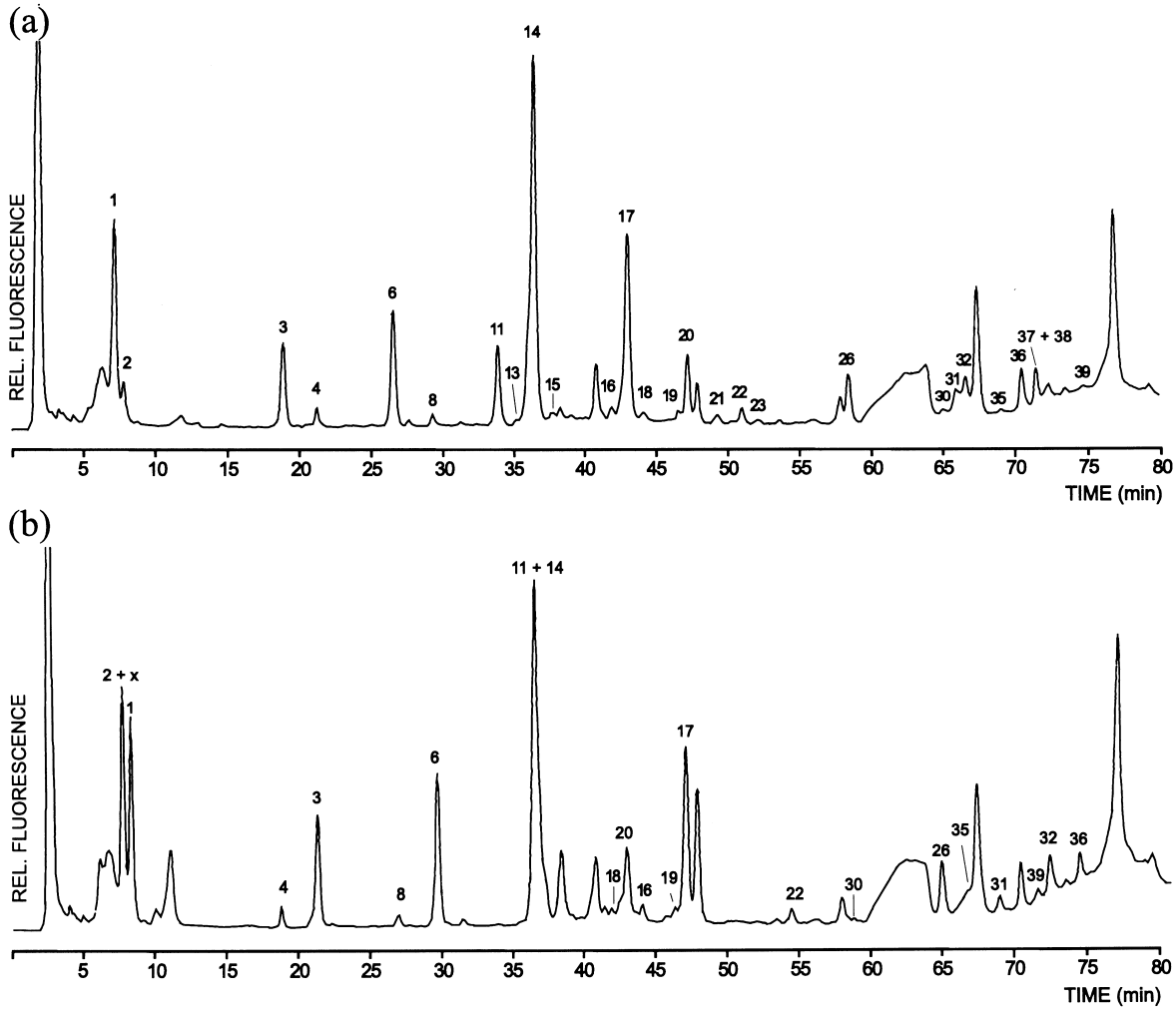

Fig. 2. Chromatograms of amino acids in a hydrolysed seawater sample, derivatisation with OPA–IBLC (a) and OPA–IBDC (b). Peak numbers as in Table 2, ×=unknown impurity.

plexation reagent enables the application of the OPA–IBC method for fossil samples for the first time, with the advantages of fast derivatisation and good peak separation as compared to other chromatographic methods. The high calcium carbonate content in matrices such as fossil shells and bones always afforded time consuming sample preparations such as desalting procedures prior to amino acids analysis [17]. With the OPA method calcium hydroxide precipitates at pH 9.5 during derivatisation. This was prevented by complexation of calcium with pentasodium triphosphate forming a soluble complex. Thus, problems like column blocking can be avoided without disadvantages for the derivatisation reaction.

Two chromatograms of amino acids of the hydrolysed mollusks samples ILC-A and ILC-C are shown in Fig. 4a and b. In all samples glycine and aspartic acid dominated, followed by glutamic acid and alanine. D- or L-ABA, suggested as decomposition products of threonine [25], was only present in traces in all samples. Since L-isoleucine/D-allo-isoleucine, aspartic acid and leucine are mainly taken for age determinations the IBDC reagent should be used; by

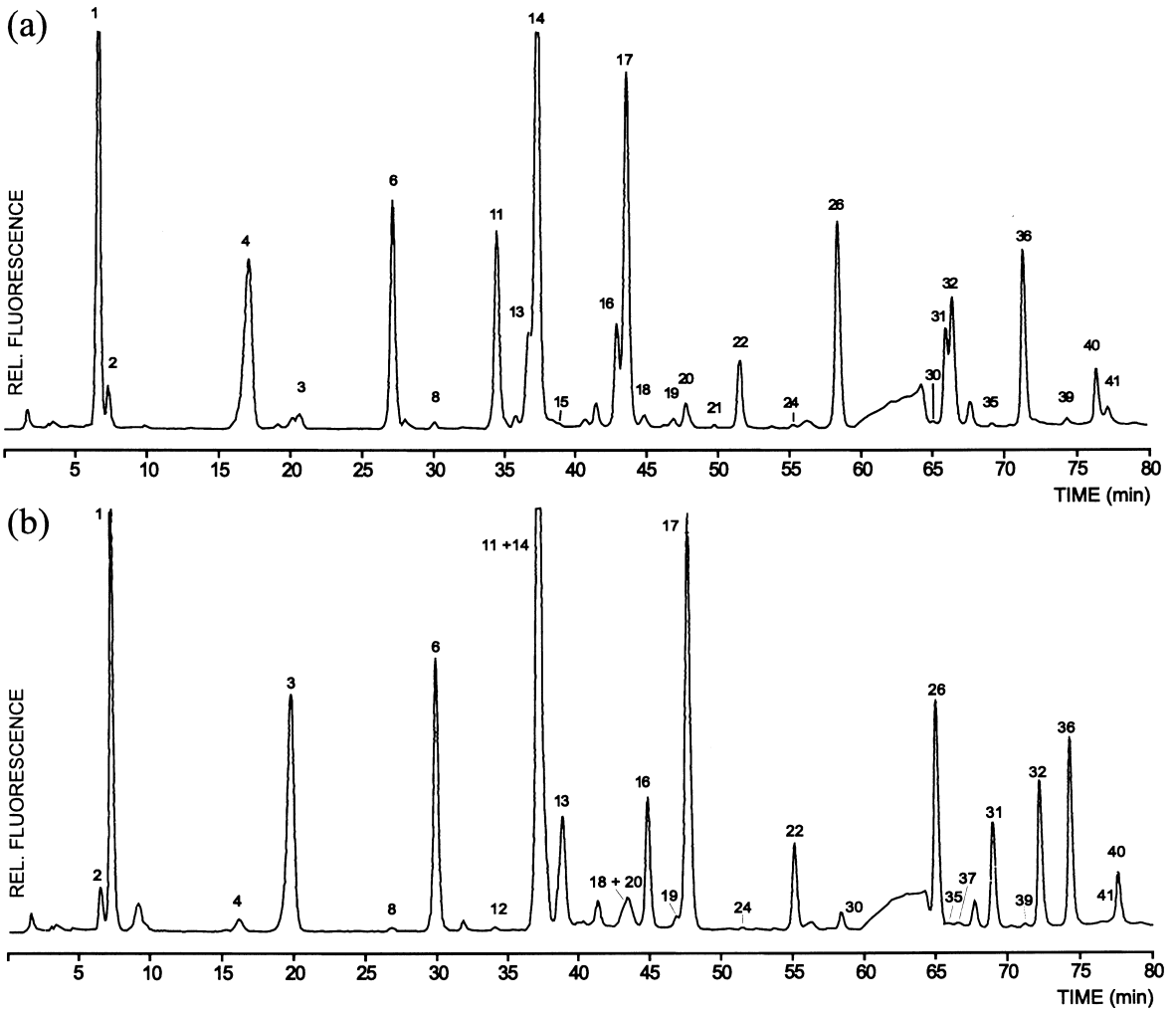

Fig. 3. Chromatograms of amino acids in hydrolysed particulate material from a seawater sample, derivatisation with OPA–IBLC (a) and OPA–IBDC (b). Peak numbers as in Table 2.

this derivatisation no interference of the L-phenylalanine and L-isoleucine peaks occurred (Fig. 4a). The later eluting valine enantiomer could not always completely separated from a broad unknown impurity. This impurity peak occurred in the hydrolysates in the area of methionine and tryptophan. Since these amino acids are not stable under hydrolysis conditions, this interference has no influence on the quantification. Tryptophan is degraded completely and methionine is partially and non-reproducible oxidised to the corresponding sulfone and sulfoxide [26].

In fossil samples racemisation occurs as a time dependent process, which is often used for dating of sediments or soils. If proteins are embedded in calcareous matrices like shells or bones, they are preserved for thousands of years like in a closed system and racemise slowly until the thermodynamic equilibrium as a racemic mixture (enantiomeric amino acids: 1:1, except for allo-isoleucine 1:1.3 and threonine which are diastereomers) is reached [3]. The degree of racemisation allows an estimate of the age of samples. The ratios obtained with the method presented (Table 3) were similar to those determined

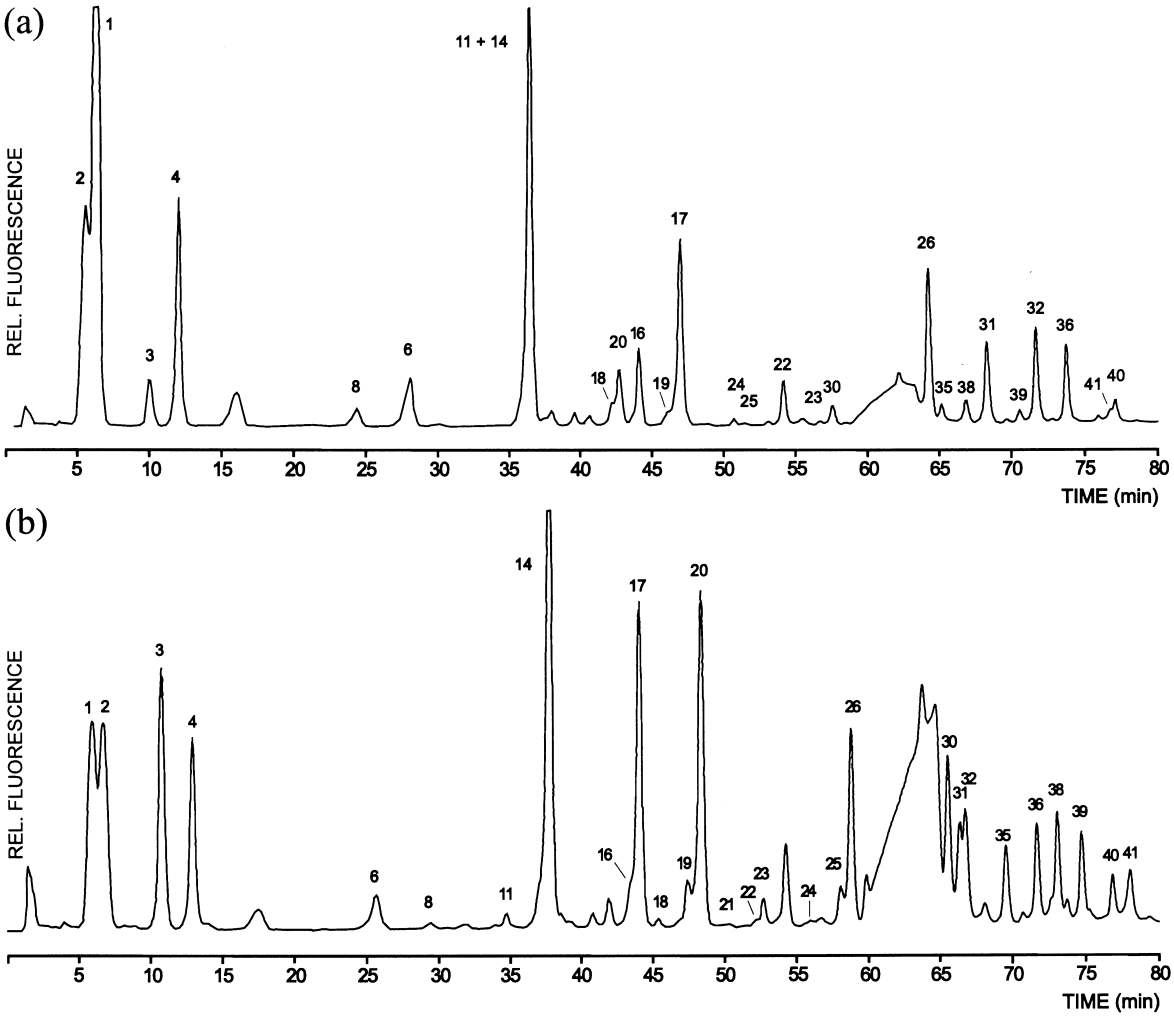

Fig. 4. Chromatograms of amino acids in the hydrolysed fossil mollusks sample ILC-A (a) derivatised with OPA–IBDC and in sample ILC-C (b) derivatised with OPA–IBLC. Peak numbers as in Table 2.

Table 3
Enantiomeric ratio data of the fossil ILC samples obtained by the OPA–IBC method

	Asp	Glu	Ala	Val	Phe	Ile/Allo-Ile	Leu
ILC-A	0.36[a]	0.24±0.02	0.36±0.06	0.14±0.03	0.30±0.03	0.24±0.09	0.28±0.08
ILC-B	0.57[a]	0.36±0.004	0.73±0.04	0.33±0.02	0.59±0.05	0.53±0.06	0.47±0.13
ILC-C	0.94±0.11	0.77±0.12	0.90±0.04	0.76±0.03	1.00±0.01	1.23±0.03	0.86±0.08

Ratios were calculated from the equation % $D=100\cdot D/(D+L)$; (D, L: concentrations of the D- or L-enantiomer). Mean values are presented for one analysis with IBLC and one with IBDC, except for [a]: determination only with IBDC. Chromatographic conditions as in Section 2.2, for sample preparation and further information see Wehmiller [17].

with gas chromatography and ion chromatography. Compared with published data [17], our L-isoleucine/D-alloisoleucine ratios were in the same range, glutamic acid and valine tended to be slightly lower while phenylalanine was higher.

4. Conclusions

The applications for the OPA–IBC method presented offer new opportunities for biological and geochemical research. All reagents used are commercially available. The sensitivity and specificity and the simple pretreatment of samples make the method rapid and inexpensive. With detection limits in the nanomolar range it is possible to analyse even small amounts of material or matrices with low concentrations like foraminifera or seawater. In comparison to established methods the ease of sample preparation and therefore the low risk of contamination, the good resolution of L-isoleucine/D-allo-isoleucine with the simultaneous determination of several amino acid ratios in one run are important improvements. The method is highly reliable due to the possibility of controlling the data by the use of the two different reagents.

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