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DETERMINATION OF D-AMINO ACIDS BY DEUTERIUM LABELLING AND SELECTED ION MONITORING

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SUMMARY

A method for the accurate determination of D-amino acids in protein/peptide samples has been developed. This method differentiates D-isomers formed during protein acid hydrolysis from the initial contribution of the sample. To this effect, the hydrolysis is carried out in deuterated hydrochloric acid. Any molecule that is inverted during this step becomes automatically labelled with deuterium. Amino acids are then separated by gas chromatography as N(O,S)-trifluoroacetyl-O-isopropyl esters on a chiral stationary phase and detected by mass spectrometry in selected ion monitoring mode. At present, twelve protein amino acids, including seven essential ones, are monitored in a two-run procedure. This technique allows the determination of the D/L ratio down to 0.1 %.

Based on this method, the inversion rate constants of fifteen amino acids have been determined under acid hydrolysis conditions (110°C, 6 *N* ^{2}HCl). Preliminary data are presented on the racemization induced in protein samples heated in the solid state in the presence of low amounts of water.

INTRODUCTION

Amino acid racemization has implications not only in pure chemistry, but also in biochemistry, nutrition, food technology and geology[1–4]. Research on racemization has been facilitated by the development of techniques for the separation of enantiomeric compounds. Methods based on liquid[5] or gas chromatography[6–8] have been proposed. Recently, a new optically active phase has been made available for gas chromatography[9,10]. Its remarkable thermal stability and wide range of application have provisionally given the advantage to this latter technique.

However, in spite of all these developments, the quantitative determination of amino acid enantiomers is liable to systematic errors. Acid hydrolysis, which is a common step in all analytical procedures, causes amino acids to racemize to various degrees[11,12]. Consequently, the enantiomer distribution in the analyzed sample cannot be identical with the initial distribution. The degree of racemization depends not only on the amino acid, but it may also be influenced by neighbours in the peptide chain[13,14]. Therefore, the procedure of taking as blank values the racemization rates of free

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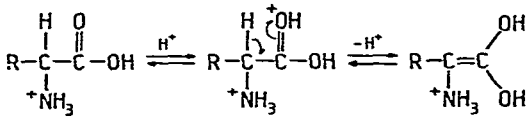
amino acids treated under the same conditions is not valid. A correct determination can be achieved only by measuring the hydrolysis-induced racemization in the sample itself.

A procedure can easily be designed to this effect. As shown in Scheme 1, amino acid inversion occurs concomitant with the removal of the α-hydrogen atom. Therefore, the amount of racemization can be estimated on the basis of deuterium or tritium incorporation. Some years ago, Manning[14] proposed a method in which tritium was used as a tracer for hydrolysis-induced racemization. Samples were hydrolyzed in tritiated HCl and amino acid enantiomers were separated by ion-exchange chromatography as dipeptidic diastereomers. A severe limitation of this approach, based on the determination of the global isotopic incorporation, stems from the fact that, in several amino acids, hydrogen exchange also takes place at positions other than the α-carbon.



Scheme 1.

We propose here a method based on the determination of deuterium incorporation by mass spectrometry. Following hydrolysis in deuterated hydrochloric acid, amino acids are converted into N(O,S)-trifluoroacetyl O-isopropyl esters and analyzed by gas chromatography–mass spectrometry (GC–MS) on a chiral stationary phase. The main advantage of this approach is that it allows selective measurement of the isotopic incorporation at the α-position, irrespective of hydrogen exchanges that could have taken place elsewhere in the molecule. A further interesting aspect of the method is the use of gas chromatography. At the present time, this technique makes it possible to separate most protein amino acid enantiomers in less than 40 min[9].

EXPERIMENTAL

Hydrolysis

Equimolar mixtures of free amino acids (each 1 μmole) or protein samples (1–2 mg) were placed in vacuum reaction tubes (Pierce, Rockford, IL, U.S.A.) together with 2 ml 6 *N* ^{2}HCl, made up from 1 ml concentrated ^{2}HCl and 1 ml ^2H_2O (Fluka, Buchs, Switzerland). Each sample was degassed several times under vacuum to remove oxygen. Ethylmercaptan (50 μl) was then added as a reducing agent for converting cystine into cysteine and protecting methionine from oxidation[10]. The tubes were sealed under vacuum and heated for various periods of time at 110°C in a heating block. The hydrochloric acid was then removed on a rotary evaporator. The amino acid dry residue was dissolved in distilled water and transferred into a conical reaction vial where it was again taken to dryness under a stream of nitrogen. The elimination of water was completed by azeotropic entrainment with methylene chloride.

Amino acid derivatization

The preparation of amino acid N-trifluoroacetyl (N-TFA) O-isopropyl esters

was performed in two steps. First, 250 μl acetyl chloride–isopropanol (20:100, v/v) and 50 μl ethylmercaptan were added to the dry residue and the whole was heated for 30 min at 110°C. The excess of reagent was removed by evaporation under a stream of nitrogen. Then 50 μl trifluoroacetic anhydride (TFAA) were added to the sample which was heated again for 10 min at 110°C. After evaporating the excess of anhydride under nitrogen, the sample was finally dissolved in 200 μl ethyl acetate.

GC–MS analysis

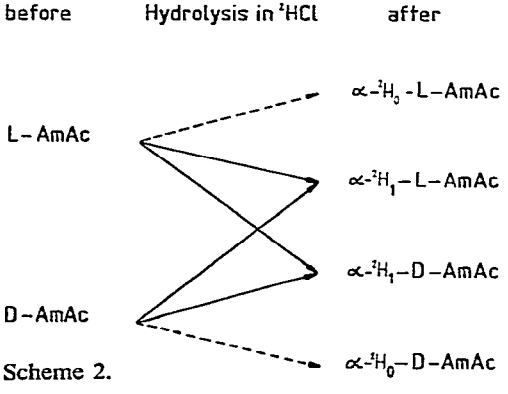
The conditions for the GC separation of amino acid enantiomers and their quantitative determination by MS were as follows: Hewlett-Packard 5992 gas chromatograph–mass spectrometer–data system equipped with two floppy disc units; glass capillary column (20 m × 0.2 mm I.D.) coated with Chirasil-Val (Applied Science Labs., State College, PA, U.S.A.); injector temperature, 200°C; column temperature, programmed from 70 to 200°C, at 4°/min; carrier gas (helium) flow-rate, 3 ml/min; inlet split ratio, 20:1; sample, 1 μl ethyl acetate solution of amino acid derivatives.

For quantitative determinations, the mass spectrometer was run in multiple ion detection mode. The whole amino acid range was covered by analyzing every sample twice. During each run, 15–17 ion masses were monitored simultaneously, with a 25 msec dwelling time on each. Peak surface areas were measured by post-run integration by means of the routine provided within the HP 5992 software package.

METHOD

As shown in Scheme 1, the hydrolysis of a protein or peptide in concentrated acid can induce racemization of each amino acid. In the presence of deuterated hydrochloric acid, this reaction mechanism leads to the formation of labelled species. An illustration of the possible reaction pathways is presented in Scheme 2. It shows that, following ^{2}HCl treatment, any amino acid may be found under four different forms, the relative concentrations of which are functions of the initial D- and L-isomer distribution (I) and the hydrolysis-induced racemization rate (R).

Providing the amino acids have been converted into suitable volatile derivatives, the concentrations can be determined by GC–MS. To this effect, three characteristic



Scheme 2.

fragment ions, F — 1, F and F + 1, were selected from the mass spectrum of each amino acid derivative. These fragments necessarily included the α-hydrogen. Combined GC separation and multiple ion detection MS gave for each amino acid mass fragmentograms of the type shown in Fig. 1. The abundances of F — 1, F and F + 1 for both enantiomers were evaluated by measuring the surface areas of the corresponding peaks.

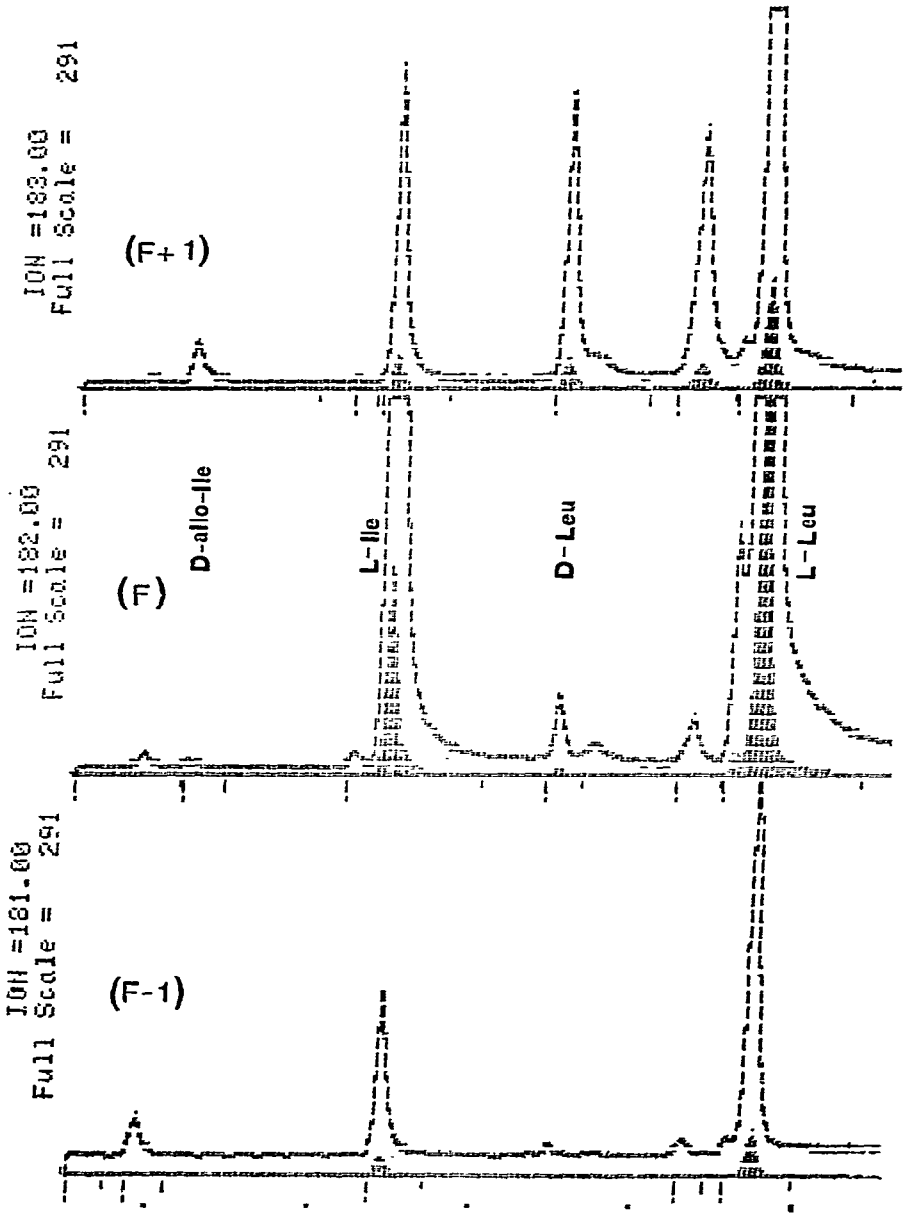


Fig. 1. Mass fragmentogram showing the separation of Leu and Ile stereomers.

TABLE I

EQUATIONS FOR THE CALCULATION OF D-ISOMER CONTENT I AND HYDROLYSIS-INDUCED RACEMIZATION R

$[F+1]_D$, $[F]_D$, etc. are the measured abundances of ions F + 1,F, F − 1 for D- and L-isomer; X is the contribution (%) due to racemization during hydrolysis, Y that due to D-isomer present in the sample before hydrolysis; f_{+1}, f_{-1} are factors relating the formation probability of fragment ion F + 1 or F − 1 to that of F; I is the D-isomer content (%) in the sample before hydrolysis; R is the hydrolysis-induced racemization rate (%).

$$\frac{[F+1]_D}{[F]_L}\cdot 100 = X + Y\cdot f_{+1} + \frac{X\cdot Y}{100 - X\cdot f_{-1}} \tag{1}$$

$$\frac{[F]_D}{[F]_L}\cdot 100 = X\cdot f_{-1} + Y \tag{2}$$

$$\frac{[F+1]_L}{[F]_L}\cdot 100 = 100\cdot f_{+1} + X - Y\cdot f_{+1}\cdot f_{-1} + \frac{X\cdot Y}{100 - X\cdot f_{-1}} \tag{3}$$

$$\frac{[F-1]_L}{[F]_L}\cdot 100 = 100\cdot f_{-1} - X\cdot f^2{}_{-1}\cdot\frac{X\cdot Y}{100 - X\cdot f_{-1}} \tag{4}$$

$$I = \frac{Y + 2\cdot X\cdot Y/(100 - X\cdot f_{-1})}{100 + Y + X\cdot(2 - f_{-1}) + X\cdot Y\cdot(2 - f_{-1})/(100 - X\cdot f_{-1})}\cdot 100 \tag{5}$$

$$R = \frac{X}{100 + X\cdot(1 - f_{-1}) - X\cdot Y\cdot f_{-1}/(100 - X\cdot f_{-1})}\cdot 100 \tag{6}$$

The measured abundances were then entered into eqns. 1–4 of Table I, where they are expressed in terms of the contribution of the reaction pathways shown in Scheme 2 and the mass spectral fragmentations resulting in the formation of ions F − 1, F and F + 1. Solving this system allows the possibility of calculating the D-isomer initial contents I and the hydrolysis-induced racemization rate R, by means of eqns. 5 and 6. These two quantities are defined for each acid as:

$$I = 100 \times \frac{\text{D-isomer concentration before hydrolysis}}{\text{D- + L-isomer concentration before hydrolysis}}$$

$$R = 100 \times \frac{\text{D-isomer formed during hydrolysis}}{\text{remaining initial L-isomer after hydrolysis}}$$

Because of its complexity, the calculation had to be carried out by a computer program. The simultaneous eqns. 1–4 were first simplified by neglecting all higher degree terms and the solutions of the resulting system were calculated. In a second step, these values served as a base for evaluating the solutions of the complete system by an iterative procedure. A convergence test indicated when a satisfactory set of values had been obtained. The last part of the computation consisted in calculating I and R according to eqns. 5 and 6 (Table I).

RESULTS AND DISCUSSION

Practical considerations

Following the proposition of Bayer and co-workers[9,10] who developed the Chirasil-Val stationary phase, amino acids were converted into N-perfluoroacylated alkyl esters. From a survey of the chromatographic behaviour of the various possible homologues, N-trifluoroacetyl O-isopropyl esters were found to be the best compromise[15]. Thanks to the GC–MS combination, the separation of the different amino acids could be disregarded in favour of a better enantiomer resolution. However, a good separation could not be obtained for proline and histidine (N^{im}-isobutyloxycarbonyl-N-TFA-O-isopropyl histidine) enantiomers. Arginine had also to be left out of this study because of the instability of the derivative.

A further limitation was apparent when selecting the characteristic fragment ions. The fragmentation pathways of tyrosine and tryptophan derivatives do not lead to the formation of α-hydrogen-containing ions. For these two compounds it was possible to measure only the overall D- and L-isomer distribution after hydrolysis. Consequently, the complete determination procedure described in the previous section was restricted to the twelve protein amino acids listed in Table II, together with their characteristic ion masses.

TABLE II

MASS AND ABUNDANCE OF MAIN CHARACTERISTIC FRAGMENT ION F OF N-TRIFLUOROACETYL O-ISOPROPYL AMINO ACIDS

Amino acid	*m/e*	*Abundance**
Ala	140	100
Val	168	100
Leu	140	60
Ile	182	60
Cys	268	20
Met	287	15
Phe	216	25
Lys	180	100
Asp	186**	75
Glu	228**	60
Ser	139	75
Thr	266	10

* Given as percentage of base peak.

** Includes two deuterium atoms on the carbon adjacent to the ω-carboxyl group.

For some amino acids, H/^{2}H exchange in hot ^{2}HCl was not limited to the α-hydrogen and lead to the formation of molecules containing more than one deuterium atom. This phenomenon was particularly important for aspartic acid, glutamic acid, tyrosine and tryptophan, and occurred to a lesser extent with phenylalanine. However, it could be assumed that the presence of deuterium on the α-carbon had no influence on the rate of H/^{2}H exchange at other positions in the same molecule. It was, therefore, relatively easy to cope with those side reactions. High exchange rates resulting in the complete labelling of one or several positions were taken into ac-

count when selecting the characteristic fragment ions. The ion masses were incremented by the number of labelled positions. On the other hand, partial labelling due to lower exchange rates was automatically taken into account by the caculation procedure. This was the reason for treating the quantities f_{+1} and f_{-1} as variables rather than constant factors. Consequently, the present determinations were not affected by multiple H/^{2}H exchange processes. This is an important advantage over the technique based on tritium incorporation[14].

Method evaluation

A test was designed to evaluate the degree of reliability and accuracy of the method described. To this effect, a mixture of D-amino acids was added in various proportions to a standard solution of L-amino acids. The relative concentration of the D-isomers ranged from 0 to 16%. These samples were heated at 110°C in 6 *N* ^{2}HCl for time periods varying between 12 and 160 h. Thereafter, they were analyzed according to the above procedure. From the experimental data, plots of the type shown in Fig. 2 were drawn and evaluated by a linear least-squares fitting. The resulting data are presented in Table III. Plots of measured *versus* nominal D-isomer initial concentration gave in all cases a straight line with a slope equal to 1.0 within a 10% deviation range and *y*-axis intercept close to zero.

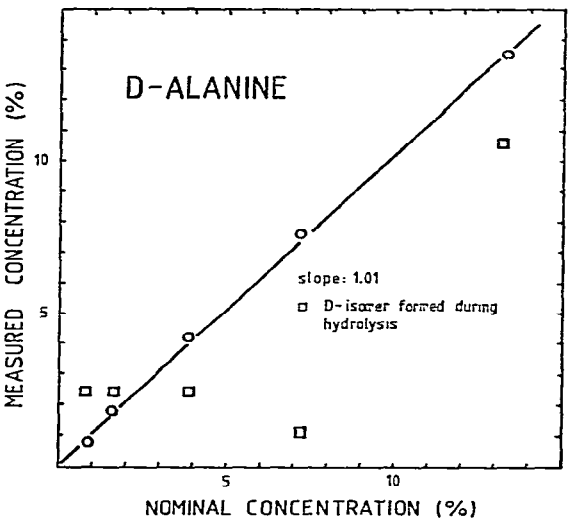


Fig. 2. Calibration curve for the determination of D-alanine in amino acid mixtures heated at 110°C in 6 *N* ^{2}HCl. ○, Initial D-alanine; □, D-alanine formed during hydrolysis.

These results confirmed the validity of the method for measuring the D-isomer content in amino acid samples independently from the racemization occurring during the hydrolysis. It was found that the accuracy of the determinations remained good even when the amount of hydrolysis-induced D-isomer was several times higher than the initial concentration. Under the usual conditions of hydrolysis, *i.e.*, 12–48 h,

TABLE III

DATA FROM METHOD EVALUATION TEST

Results from least-squares fittings of plots of the type shown in Fig. 2.

Amino acid	*Measured concentration/nominal concentration*		
	Slope	*Y-intercept*	r^2
Ala	1.01	0.19	0.9994
Val	0.97	0.26	0.9954
Leu	1.05	−0.23	0.9992
Ile	1.11	−0.06	0.9989
Cys	0.97	0.01	0.9968
Met	0.98	−0.24	0.9929
Phe	1.01	0.04	0.9956
Lys	1.11	0.42	0.9930
Glu	0.96	−0.33	0.9796
Ser	1.02	−0.21	0.9927
Thr	1.04	0.20	0.9954

110°C, the lower limit for a correct determination of D-isomer content in amino acid mixtures was 0.2–0.4%. In samples containing only one amino acid, concentrations down to 0.1% could be measured accurately (see Fig. 3). Below this limit the determinations were impaired by the residual interference of hydrolysis-induced racemization.

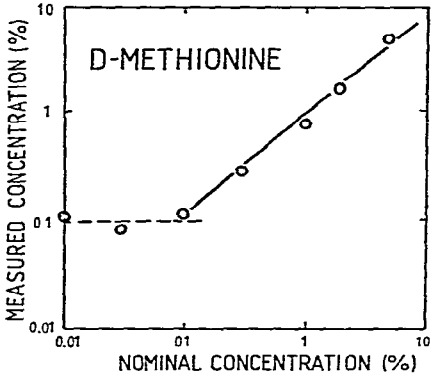


Fig. 3. Calibration curve for the determination of D-methionine in D- and L-methionine mixtures heated at 110°C in 6 N ^{2}HCl.

Hydrolysis-induced racemization

Free amino acids. The general amino acid racemization reaction can be written as

$$\text{L-amino acid} \underset{k}{\overset{k}{\rightleftarrows}} \text{D-amino acid}$$

where k is the first-order rate constant for the interconversion of L- and D-enantiomer. The integrated kinetic equation for this reaction is

$$2t \cdot k = \ln\left[\frac{1 + \text{D/L}}{1 - \text{D/L}}\right] \qquad (7)$$

where t = time and D/L is the amino acid enantiomeric ratio. For an amino acid with more than one asymmetry centre, *e.g.*, isoleucine, the inversion reaction is more properly termed epimerization. For isoleucine, epimerization can be written as:

$$\text{L-isoleucine} \underset{k_{alleu}}{\overset{k_{Ile}}{\rightleftharpoons}} \text{D-alloisoleucine}$$

The corresponding integrated kinetic equation is:

$$(1 + K)\, t \cdot k_{Ile} = \ln\left[\frac{1 + (\text{alleu/iso})}{1 - K(\text{alleu/iso})}\right] \tag{8}$$

The quantity K is the iso/alleu equilibrium ratio, which for isoleucine has the value 0.79 (ref. 16).

In the case of racemization occurring during the hydrolysis, the ratio D/L or iso/alleu in eqns. 7 and 8 is the quantity R determined by the present method. Therefore, on the basis of measurements made in the course of the evaluation test it was possible to calculate the inversion rate constants of free amino acids placed under the conditions of acid hydrolysis. The resulting values are listed in Table IV. To our knowledge, this is the first time that racemization rate constants have been measured under acid hydrolysis conditions for as many as fifteen protein amino acids. The majority of the amino acids have rate constants ranging from $10 \cdot 10^{-8}$ to $20 \cdot 10^{-8}$ sec^{-1}. Aspartic acid, cysteine and proline are characterized by significantly higher values, while serine and threonine do not racemize to any detectable level under the chosen conditions. Of interest in this respect is the considerable difference in behaviour between serine and cysteine, in spite of their structural similarity. Another interesting observation is the low rate constants of the β-substituted valine and isoleucine. Some of these results confirm earlier findings[16].

TABLE IV

INVERSION RATE CONSTANTS OF FREE AMINO ACIDS AT 110°C IN 6 N ^{2}HCl

Amino acid	$k \cdot 10^{-8}$* (sec^{-1})
Asp	58 ± 5
Cys	54 ± 3
Pro**	30 ± 2
Glu	17.6 ± 1.4
Met	17.6 ± 1.1
Leu	15.4 ± 0.8 (15)
Lys	13.4 ± 1.4
Ala	13.2 ± 0.3 (14)
Phe	11.8 ± 0.6
Tyr	10.8 ± 0.8
Trp	8.9 ± 0.3
Ile***	4.6 ± 0.1
Val	2.8 ± 0.2 (2.9)
Ser	<1
Thr	<1

* Values in parentheses measured at 105°C; ref. 16.

** Determination made by measuring the deuterium incorporation rate.

*** Rate constant for the conversion L-Ile → D-allo-Ile.

Protein-bound amino acids. It has already been mentioned that, under acid hydrolysis conditions, protein-bound amino acids may not racemize at the same rate as free acids. For example, cysteine could promote the complete racemization of a neighbouring isoleucine[11,12]. However, the generalization of this type of phenomenon was still to be verified. For this purpose, samples of three purified proteins, namely α-lactalbumin B, β-lactoglobulin A and bovine plasma albumin (BPA), were hydrolyzed for 24 h in 6 *N* ^{2}HCl at 110°C and analyzed by GC–MS. The amounts of D-isomer measured for every amino acid in these protein samples are reported in Table V, and compared with the values determined in a mixture of free amino acids heated under the same conditions. It appears that there are significant differences in racemization not only between protein-bound and free amino acids, but also between the various proteins. Striking examples are valine, isoleucine and methionine. These data illustrate the type of error which can be made when determining D-enantiomers in a protein or peptide if the hydrolysis-induced racemization is not also measured in the same sample.

TABLE V

RACEMIZATION INDUCED BY 24 h ACID HYDROLYSIS OF PROTEIN-BOUND AND FREE AMINO ACIDS

Values of the D/L ratio (%) are given, with mean standard deviations in parentheses

Amino acid	*Free acids*	*α-Lactalbumin B*	*β-Lactalbumin A*	*BPA*
Ala	1.1 (0.1)	1.2 (0.2)	1.4 (0.2)	2.7 (0.1)
Val	0.24 (0.02)	2.6 (0.2)	0.9 (0.2)	1.14 (0.01)
Leu	1.3 (0.1)	3.7 (0.5)	2.2 (0.2)	3.3 (0.2)
Ile	0.40 (0.05)	3.7 (0.5)	1.2 (0.5)	4.9 (0.3)
Cys	4.0 (0.3)	5.4 (1.0)	5.1 (0.5)	8.4 (0.3)
Met	1.0 (0.1)	9 (2)	1.1 (0.1)	—
Phe	0.9 (0.1)	1.0 (0.3)	3.5 (0.5)	2.4 (0.2)
Lys	0.5 (0.1)	1.2 (0.2)	1.3 (0.3)	2.0 (0.3)
Asp	3.1 (0.3)	5.3 (0.2)	4.9 (0.5)	7.2 (0.3)
Glu	1.0 (0.1)	1.9 (0.3)	3.4 (0.3)	3.6 (0.2)

Racemization in heated protein

As an example of application, samples of bovine plasma albumin (BPA) were submitted to prolonged heating at 121°C in the presence of 15% water. The amount of racemization induced was measured for each amino acid by means of the proposed method. The resulting data (Table VI) emphasize again the importance of distinguishing between the true enantiomer distribution in a sample and the modifications caused by the hydrolytic treatment. The present determinations on heat-induced racemization in protein confirm and complete the earlier observations of Bjarnason and Carpenter[17], and the more recent determinations of Hayase *et al.*[3] who found no detectable racemization in casein after heating for 20 min at 120°C.

CONCLUSION

It has been confirmed that racemization occurring during acid hydrolysis should not be neglected in accurate determinations of D-amino acid contents in a

TABLE VI

D-AMINO ACIDS FOUND AFTER HYDROLYSIS OF BOVINE PLASMA ALBUMIN HEATED AT 121°C IN THE PRESENCE OF 15% WATER

I is the concentration of D-isomer formed in protein by heating, *R* the rate of racemization induced by 24 h hydrolysis at 110°C. Mean standard deviations are given in parentheses. n.d. = Below detection level.

Amino acid	*Heating time (h)*					
	0		*8*		*27*	
	I (%)	*R (%)*	*I (%)*	*R (%)*	*I (%)*	*R (%)*
Ala	0	2.7 (0.1)	0.8 (0.04)	2.8 (0.1)	4.1 (0.04)	2.4 (0.1)
Val	0	1.14 (0.01)	0.3 (0.03)	1.0 (0.2)	0.4 (0.1)	0.8 (0.1)
Leu	0	3.3 (0.2)	0	3.3 (0.1)	1.2 (0.1)	2.8 (0.1)
Ile	1.8 (0.1)	4.9 (0.3)	1.9 (0.3)	2.3 (0.8)	2.7 (0.2)	1.8 (0.6)
Cys	0	8.4 (0.3)	29.1 (0.4)	13 (1)	32 (1)	10 (3)
Phe	0	2.4 (0.2)	0.6 (0.1)	2.6 (0.2)	2.2 (0.1)	2.2 (0.2)
Lys	0	2.0 (0.3)	0.6 (0.1)	2.5 (0.1)	3.0 (0.1)	2.1 (0.1)
Asp	0	7.2 (0.3)	28.6 (0.5)	7.4 (0.9)	40 (1)	12 (3)
Glu	0	3.6 (0.2)	0.96 (0.01)	3.5 (0.1)	4.71 (0.02)	2.9 (0.1)
Ser	0	0	4.2 (0.5)	0	15 (1)	0
Thr	0	0	0	0	0	0
Met	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

protein/peptide sample. Depending on the amino acid and on the sample, racemization degrees of up to 10% have been measured after heating for 24 h at 110°C in 6 *N* hydrochloric acid. The analytical method presented has proved to be a valuable tool for distinguishing D-amino acid molecules initially present in the sample from those formed during the hydrolysis. It is believed that this technique will be a useful complement to the recent developments in gas chromatographic separation of amino acid enantiomers.

REFERENCES

1 P. M. Masters, J. L. Bada and J. S. Zigler, *Nature (London)*, 268 (1977) 71.
2 J. L. Bada and R. A. Schroeder, *Naturwissenschaften*, 62 (1975) 71.
3 F. Hayase, H. Kato and M. Fujimaki, *Agr. Biol. Chem.*, 43 (1979) 2459.
4 C. P. Berg, *Physiol. Rev.*, 33 (1953) 145.
5 J. M. Manning and S. Moore, *J. Biol. Chem.*, 243 (1968) 5591.
6 S. Nakaparksin, P. Birrell, E. Gil-Av and J. Oro, *J. Chromatogr. Sci.*, 8 (1970) 177.
7 W. A. Koenig, W. Parr, H. A. Lichtenstein, E. Bayer and J. Oro, *J. Chromatogr. Sci.*, 8 (1970) 183.
8 W. A. König, W. Rahn and J. Eyem, *J. Chromatogr.*, 133 (1977) 141.
9 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr. Sci.*, 15 (1977) 174.
10 H. Frank, G. J. Nicholson and E. Bayer, *J. Chromatogr.*, 167 (1978) 187.
11 A. Neuberger, *Advan. Protein Chem.*, 4 (1948) 297.
12 N. Radulescu and S. Iacobescu-Cilianu, *Stud. Cercet. Chim.*, 31 (1973) 131.
13 L. C. Craig, W. Hausmann and J. R. Weisiger, *J. Amer. Chem. Soc.*, 76 (1954) 2839.
14 J. M. Manning, *J. Amer. Chem. Soc.*, 92 (1970) 7449.
15 R. Liardon and S. Ledermann, *J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1980)*.
16 S. Nakaparksin, E. Gil-Av and J. Oro, *Anal. Biochem.*, 33 (1970) 374.
17 J. Bjarnason and K. J. Carpenter, *Brit. J. Nutr.*, 25 (1970) 313.