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Gas chromatographic quantification of amino acid enantiomers in food matrices by their *N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl ester derivatives

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Abstract

Several amino acid enantiomer derivatives were prepared with different chloroformates and analysed by gas chromatography (GC) on a Chirasil-L-Val GC column, at a temperature below 200°C. Among them the *N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl esters presented the best compromise between short retention times, high yield responses and good resolution for almost all the tested amino acids. These derivatives proved to be suited for quantification of amino acids in aqueous media, with L-*p*-chlorophenylalanine as internal standard. The developed procedure was applied to several food samples for determination of their free amino acid profiles. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Enantiomer separation; Derivatisation, GC; Amino acids; *N*(*O*,*S*)-Ethoxycarbonyl heptafluorobutyl ester

1. Introduction

Over the past years, D-amino acids have been found in several species ranging from bacteria to humans [1–3], and significant amounts are commonly found in processed foods [3], especially in fermented beverages and dairy products, owing to the bacterial or starter cultures involved [4]. Heat treatments employed during food processing have also been shown to produce racemization of amino acids [3,5]. On account of their importance in biosciences and their possible nutritional implications, the occurrences, biological functions and metabolic fates of D-amino acids are the subject of an increasing number of studies.

In the past, the determination of amino acid (AA) enantiomers was a difficult and time-consuming task. In the present decade, gas–liquid chromatography employing chiral stationary phase such as Chirasil-Val (*tert*.-butylamide-linked polydimethylsiloxane) [6,7] or others [8–12] and, more recently high-performance liquid chromatography (HPLC) using either diastereomeric derivatives [2,3,13–16] or chiral stationary phases [17,18], have enabled the determination of amino acid enantiomers. Gas chromatographic methods have some inherent advantages over HPLC methods; gas chromatography (GC) gives shorter analysis times and needs less expensive equipment and maintenance.

In contrast to the procedures used for HPLC, for

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GC the amino acids must be converted into volatile derivatives, which are suitable for separation and elution within a reasonable time. Due to the wide differences in chemistry of the amino acid side chains it is not always easy to achieve reproducible sample preparation and derivatisation. The use of different internal standards is a common procedure and, among them, the enantiomer labelling technique has become increasingly popular with positive outcomes [19,20]. An additional problem in the analysis of amino acid enantiomers is the low thermal stability of the chiral stationary phases, with maximum operating temperatures allowed near 200°C. Therefore, the development of suitable derivatisation techniques for amino acids, in general, and their enantiomers, in particular, is considered to be a pre-requisite to obtain successful separations.

Although much progress has been made regarding this aspect, a procedure suitable for the quantitative and reproducible derivatisation of all amino acids in a single reaction remains to be developed. In general, the major derivatisation procedures for amino acid analysis by GC have been based on the preparation of *N*(*O*)-trifluoroacetyl alkyl esters, *N*-perfluoroacyl alkyl esters and *N*-alkyloxycarbonyl-trimethylsilyl derivatives [21–23]. These procedures involve such laborious multi-steps as the total removal of water from the sample and intense heating, from 60°C up to 150°C during 20 to 60 min. Temperature is known to cause conversions of amino acid enantiomers, hence a derivatisation method without heating steps would be the best approach for their analysis.

Another class of derivatives, the *N*-alkyloxycarbonyl alkyl esters, although time-consuming in the past have been, more recently, the subject of several improvements. In 1991, Hušek [24,25] described a fast method for GC analysis of amino acids in achiral phases with formation of *N*-ethoxycarbonyl ethyl esters. This derivatisation procedure took place within 1 min, at room temperature and used an aqueous medium. Wang et al. [26] extended this method to different chloroformates and alcohols in order to obtain a variety of different esters. An extensive review on chloroformates was recently published by Hušek [27]. To our knowledge, Abe et al. [28] were pioneers in applying this derivatisation procedure for the analysis of amino acid enantiomers; in their work they prepared methoxycarbonylmethyl and ethoxycarbonylethyl esters which were analysed in a Chirasil-Val column at maximum temperature of 220°C. In order to improve the volatility of the derivatives and achieve shorter analysis times and longer column lifetime, the same working group extended this derivatisation procedure by using 2,2,2-trifluoroethylchloroformate [29] and a wide variety of alcohols. Nevertheless, they assumed that the derivatisation method was more suitable for separation of enantiomers than for quantitative analysis.

The study presented herein investigated a more extended approach for the quantification of amino acid enantiomers using heptafluoro-1-butanol and a variety of chloroformates based on the knowledge that the introduction of fluoride atoms improves the volatility of the derivatives and that the flame ionisation detection (FID) response is proportional to the size of the alkyl group in the chloroformate [26]. Our objective was to achieve a reproducible derivatisation procedure suitable for separation of amino acid enantiomers in a Chirasil-L-Val column below 200°C, the maximum operating temperature suggested for this stationary phase, and for quantification of amino acid enantiomers.

2. Experimental

2.1. Chemicals

All the amino acids used in this study were obtained from Sigma (St. Louis, MO, USA). Ethylchloroformate (ECF), isobutyl chloroformate (iBuCF), ethanol (EtOH), isobutanol (iBuOH) and pyridine (Py) were from Fluka (Neu-Ulm, Germany) and 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB) was from Aldrich (Milwaskee, WI, USA). All other chemicals were analytical grade.

2.2. Amino acid solutions

The D- and L-amino acid solutions were prepared separately. The L-amino acid solution contained 16 L-amino acids plus glycine, 4 μmol/ml each in 0.1 *M* HCl. The D-amino acid solution was prepared with the corresponding 16 D-amino acids at the same concentrations. Solutions of the individual amino

acids were also prepared in order to allow for their identification. L-*p*-Chlorophenylalanine and D-norleucine were used as internal standards and prepared at 16 μmol/ml in 0.1 *M* HCl.

2.3. GC–FID

GC–FID analysis was carried out on a Chrompack CP-9001 gas chromatograph (Middelburg, The Netherlands).

The temperatures of the injector and detector were 250 and 280°C, respectively. Separation was achieved on a Chirasil-L-Val (25 m×0.25 mm I.D.) fused-silica capillary column with a 0.12 μm film coating (Chrompack), with a maximum operating temperature of 200°C. Helium was used as carrier gas at an initial inlet pressure of 60 kPa. Splitless injection was used with a purge time delay of 0.9 min.

The column temperature was 80°C, 1 min hold, and then programmed to increase at a rate of 5°C/min to 150°C, 7 min hold, and a further increase at a rate of 7°C/min to 200°C, 15 min hold. The carrier gas flow-rate was kept at 0.7 ml/min for the first 36 min and at this time it was increased to 1.7 ml/min until the end of the chromatographic run. This increase in the carrier gas flow-rate towards the end of the chromatographic run seems to avoid peak broadening and adsorption of the derivatives with high boiling points on the column.

2.4. Derivatisation procedure

Standard amino acid stock solutions (100 μl) plus internal standard solution (25 μl) were transferred into a silanised screw capped vial and 30 μl of alcohol–Py mixture (2:1, v/v) was added. After brief shaking 7 μl of the chloroformate was added. The vial was tightly capped, and vortexed for 15 s. Subsequently, 100 μl of chloroform and ca. 10 mg of NaCl were added and the vial thoroughly shaken for extraction of the derivatives into the organic layer. The vials were stored below 4°C until the chromatographic analysis. About 1.0 μl of the organic phase was injected directly into the gas chromatographic system.

2.5. Application to food samples

The derivatisation method was applied to the free amino acid determination in several food sample matrices following the classical approach of protein precipitation with acid solutions and ion-exchange clean-up [30]. For each matrix we have used the sample preparation methodology described by each of the authors as follow: UHT cow's milk, pasteurised fermented milk with *Bifibobacterium bifidum* and low-fat UHT yoghurt [31], black beer (4.3%, v/v) [32], balsamic vinegar from Modena (6°) [33] and *Coffea robusta* green coffee [34]. The internal standards were added to each sample proceeding any manipulation. The calibration curves were determined after subjecting standards to the same total procedure in order to compensate for the losses during protein precipitation, clean-up and derivatisation steps.

3. Results and discussion

In order to find the best amino acid derivatives, characterised by short retention times and high detectability, for separation in the Chirasil-L-Val column at a temperature below 200°C, several derivatives were prepared using a series of ethyl and isobutyl esters with the corresponding alcohols (EtOH and iBuOH) and HFB.

Table 1 shows the retention times (t_R) and the relative retention times (RRTs) of all L-amino acid derivatives. The RRTs were calculated in relation to the ECF–EtOH derivatives. The t_R values of all amino acids increased with the size of the alkyl group, both in the chloroformate and in the alcohol, and the introduction of fluoride atoms decreased, in general, the t_R. With the iBuCF–iBuOH derivatives the last peaks (associated with His, Lys, Tyr and Trp) presented intense peak broadening. Except for Glu, all the D-amino acid derivatives eluted faster in this column than the corresponding L-derivatives.

The FID response increased with the increasing sizes of the alkyl group and, was enhanced with the introduction of the fluoride atoms as represented in Table 2 for some L-AAs. As already described by Hušek in his initial work with chloroformates [24,25], not all the reactive groups in the amino acids

Table 1
Retention times and relative retention of L-amino acid derivatives[a]

Amino acid	Derivative			
	ECF–EtOH	ECF–HFB	iBuCF–iBuOH	iBuCF–HFB
Ala	9.80	9.70 (0.99)	16.26 (1.66)	12.64 (1.29)
Gly	10.35	10.38 (1.00)	17.29 (1.67)	13.56 (1.31)
Val	11.82	11.01 (0.93)	18.42 (1.56)	13.82 (1.17)
Pro	13.12	11.37 (0.87)	21.10 (1.61)	14.34 (1.09)
Ile	13.72	12.66 (0.92)	21.74 (1.58)	15.40 (1.12)
Leu	14.25	13.42 (0.94)	22.80 (1.60)	16.23 (1.14)
Asp	19.10	16.06 (0.84)	27.21 (1.42)	19.86 (1.04)
Met	23.42	21.46 (0.92)	29.83 (1.27)	25.88 (1.11)
Fen	25.82	23.52 (0.91)	31.47 (1.22)	27.00 (1.05)
Cys	27.24	24.99 (0.92)	33.93 (1.25)	30.02 (1.10)
His	36.02	32.42 (0.90)	52.77 (1.47)	40.65 (1.13)
Lys	39.48	35.26 (0.89)	55.38 (1.40)	43.90 (1.11)
Tyr	41.40	37.42 (0.90)	59.88 (1.45)	46.96 (1.13)
Trp	55.20	46.23 (1.02)	67.46 (1.49)	54.10 (1.19)

[a] The data represent retention time (min); data in parentheses represent relative retention time to ECF–EtOH.

are altered by the action of the reagent. The guanidine group of arginine remains untouched. For the determination of arginine an additional reaction step would be necessary or, alternatively, its conversion into ornithine by arginase before derivatisation. Asn and Gln are converted to Asp and Glu during derivatisation. In most cases, AAs with one hydroxyl group, namely serine or threonine, only gave one small peak that was difficult to identify in some occasions. In fact, serine had to be excluded from our studies owing to its extremely low FID response. For all the other AAs the highest yields were achieved when HFB was used. Other perfluoroalcohols could also have been tested as well but it has been well established from studies by Wang et al. [26] that the detectability increases with the size of the perfluoroalcohol.

Table 3 shows the separation factors and resolution of all the derivatives tested. Except for proline, all eluted amino acids were almost completely

Table 2
Ratio of peak area[a] of indicated L-amino acid derivatives relative to those prepared with EtCF–EtOH

Amino acid	Derivative		
	ECF–HFB/ECF–EtOH	iBuCF–iBuOH/ECF–EtOH	iBuCF–HFB/ECF–EtOH
Ala	2.5	1.9	2.6
Gly	2.5	2.9	ns[b]
Val	5.3	1.5	3.1
Pro	3.0	1.7	3.5
Ile	5.5	1.5	4.1
Leu	2.7	2.1	2.5
Asp	9.0	1.0	6.5
Met	4.8	2.7	3.8
Fen	4.5	2.8	6.0
His	2.0	1.2	6.5
Lys	1.3	1.4	1.1
Tyr	3.8	0.8	4.3
Trp	6.8	6.5	8.4

[a] FID responses are the average of results from triplicate analyses.
[b] ns: Not separated.

Table 3
Resolutions of amino acid derivatives

Amino acid	Derivative			
	ECF–EtOH	ECF–HFB	iBCF–iBOH	iBCF–HFB
Ala	1.74	1.50	2.92	2.27
Val	1.56	1.52	2.49	ns[a]
Ile	1.94	1.70	2.38	1.67
Leu	3.41	2.42	2.78	3.40
Asp	1.40	1.03	1.74	1.27
Met	3.51	2.83	2.31	7.34
Fen	2.26	1.91	1.95	1.93
His	3.01	1.70	2.45	1.90
Lys	1.80	1.81	1.65	1.91
Tyr	1.43	1.58	1.77	1.66
Trp	1.41	1.40	1.91	1.33

[a] ns: Not separated.

separated into their enantiomeric pairs, especially those derivatives obtained from treatment with ECF. The iBuCF derivatives of the AAs eluted in the first half of the chromatographic run showed a better resolution than their ECF counterparts; unfortunately, owing to the already mentioned low vapour pressures of the AAs eluted in the second half of the chromatographic run, after the end of the temperature program, a broadening of their peaks was observed. Temperatures higher than 200°C would be needed for the correct elution of these derivatives but these would lead to loss of stationary phase and decrease in column lifetime.

Based on these results the ECF–HFB derivatives [*N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl esters] were adopted for further studies, since they present the best compromise between short retention times, high yield responses and good resolution for almost

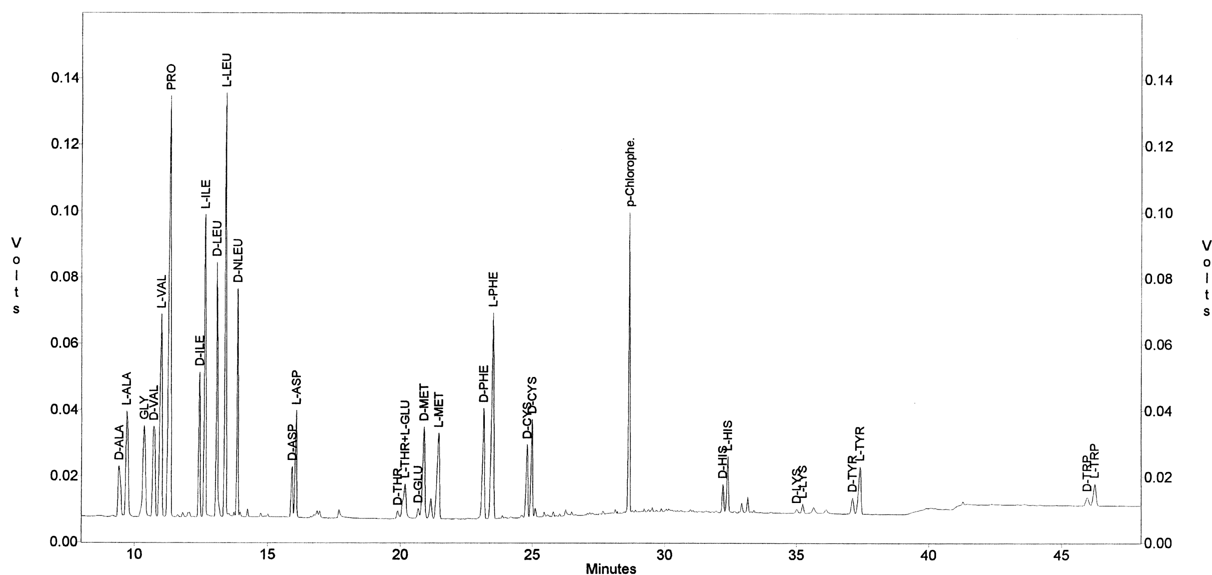

Fig. 1. Gas chromatogram of *N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl ester derivatives of standard L-amino acids (4 μmol/ml each) and D-amino acids (2 μmol/ml each) with *p*-chlorophenylalanine as internal standard (I.S.). For chromatographic conditions see Experimental.

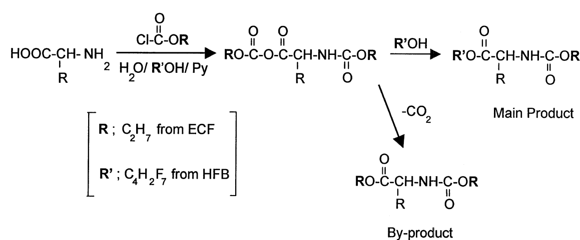

Fig. 2. Reaction mechanism of amino acids with ethylchloroformate and heptafluorobutanol.

all amino acids. A chromatogram of a mixture at 4 μmol/ml of L-AAs and at half concentration for D-AAs is shown in Fig. 1.

3.1. N(O,S)-ethoxycarbonyl heptafluorobutyl ester derivatives

The reaction mechanism of an amino acid with alkyl ethylchloroformate in a reaction medium containing HFB can be described as depicted in Fig. 2 based on work by Wang et al. [26], where it is described in detail. The double derivative formation prevents the correct determination of the peak area and the rapid identification of the correct signal, especially when using a FID system. For the complete suppression of by-product formation the authors suggested that the electronegativity of the alcohol must be larger than that of the chloroformate donor group.

In this particular case, when using HFB, it was found that the excess of chloroformate contributed to by-product formation. Thus, careful adjustment of the quantity of ethylchloroformate added was needed for derivatisation with single peaks, although in some cases complete elimination of the by-product was not achieved.

The yield of some amino acids can also be partially influenced by the water–alcohol ratio used in the derivatisation and the total HFB should be kept at ca. 16%. The pyridine proportion, provided that it was present in molar excess, did not influence the yields of the amino acid derivatives.

These observations clearly demonstrate that the proportion of the reagent used in the derivatisation procedure have to be stringently controlled.

The addition of NaCl slightly improved the relative molar response (RMR) of some amino acid derivatives, especially of the most hydrophilic. The addition of carbonate/hydrogencarbonate solutions, in order to eliminate the HCl dissolved in the chloroformic and hydroalcoholic layers did not improve the chromatographic baseline or the yields; instead a decline of the RMR was observed for some amino acids.

A tentative answer to the query on whether the reaction of the amino acids with chloroformates is

Table 4
Relative molar responses (RMRs) of *N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl ester derivatives with different internal standards

Amino acid	L-*p*-Chlorophelylalanine			D-Norleucine			Enantiomer labelling method RSD (%)
	RMR	SD[a]	RSD (%)	RMR	SD[a]	RSD (%)	
Ala	0.88	0.05	5.5	0.55	0.02	4.1	1.5
Gly	0.90	0.06	6.6	0.56	0.03	5.1	2.3
Val	1.49	0.06	3.8	1.00	0.03	3.1	2.2
Pro	1.82	0.07	4.0	1.21	0.04	3.6	3.2
Ile	1.52	0.09	5.9	1.02	0.05	4.5	5.0
Leu	1.56	0.11	7.0	1.01	0.02	2.0	2.3
Asp	0.82	0.05	6.3	0.49	0.04	8.4	1.0
Met	0.62	0.00	1.9	0.39	0.03	7.7	4.7
Phe	1.24	0.01	0.9	0.81	0.07	8.6	1.0
Cys	0.62	0.01	1.7	0.39	0.05	12.9	1.8
Hys	0.11	0.01	7.3	0.07	0.01	13.7	0.8
Lys	0.03	0.00	4.6	0.02	0.00	11.8	1.3
Tyr	0.34	0.00	5.5	0.21	0.02	11.4	0.7
Trp	0.14	0.01	4.2	0.08	0.01	13.7	1.3

[a] $n=6$.

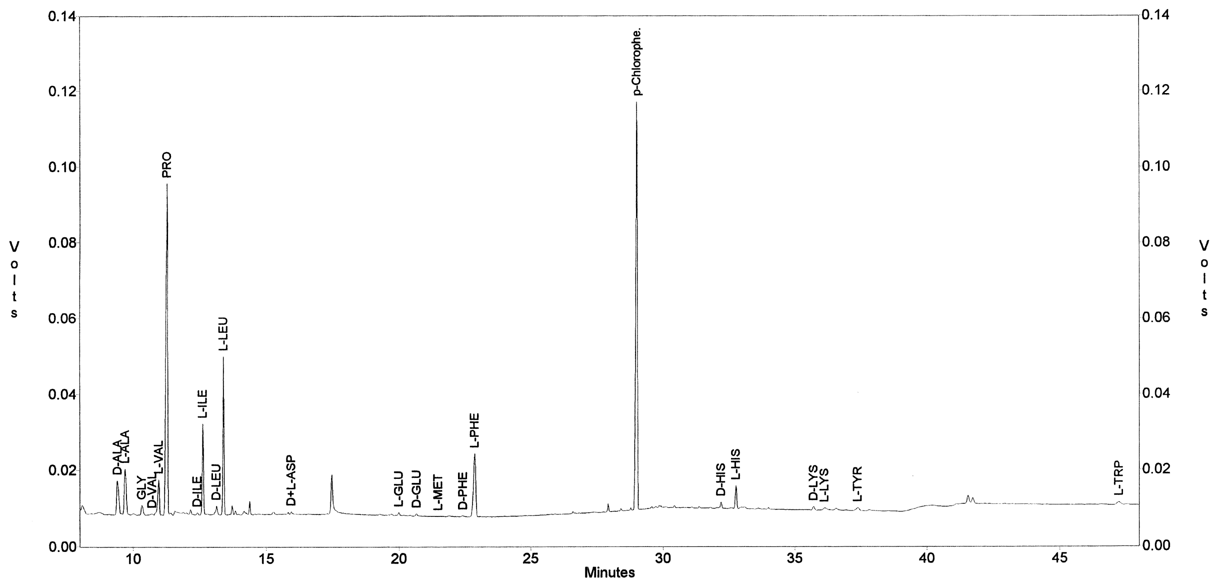

Fig. 3. Gas chromatogram of $N(O,S)$-ethoxycarbonyl heptafluorobutyl ester derivatives of a fermented milk sample. For chromatographic conditions see Experimental.

instantaneous [24,25,27] or, on the other hand, whether it is not rapidly completed [29], was pursued. In order to study the reaction time the relative peak area of some derivatives was determined from 0 up to 4 h upon the addition of ECF and before the extraction with chloroform derivatives. No significant alterations ($P<0.05$) were found in the peak areas with the reaction time provided that the

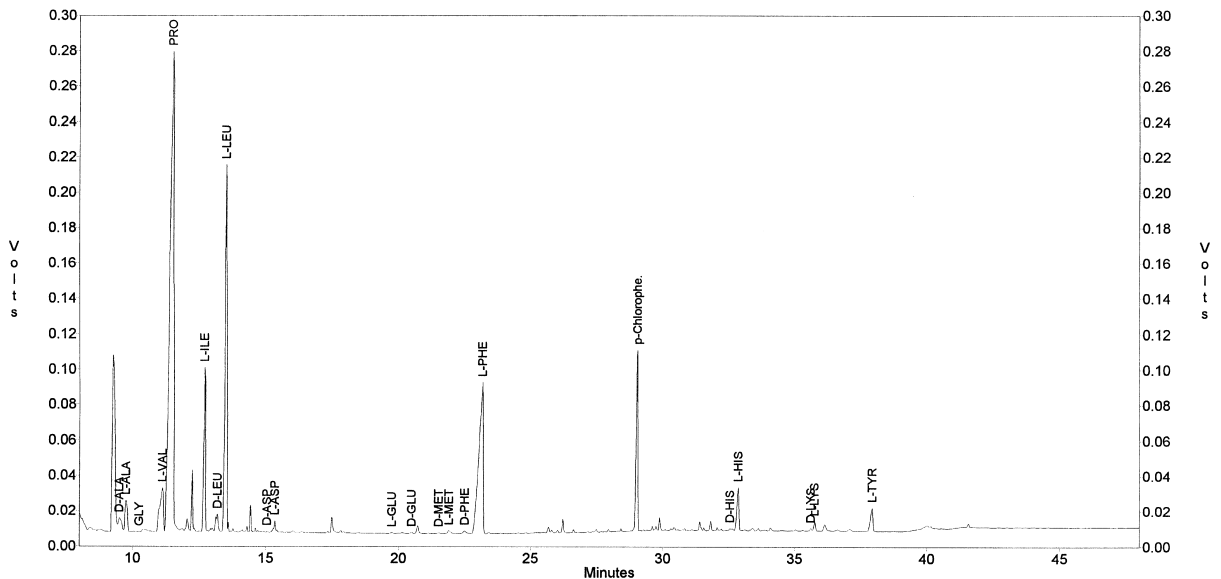

Fig. 4. Gas chromatogram of $N(O,S)$-ethoxycarbonyl heptafluorobutyl ester derivatives of a balsamic vinegar from Modena. For chromatographic conditions see Experimental.

Table 5
Free amino acid content in several food samples

Amino acid	UHT cow's milk		Fermented milk		Yoghurt		Black beer		Balsamic vinegar		*Robusta* green coffee	
	D (mg/100 ml)	L (mg/100 ml)	D (mg/100 g)	L (mg/100 g)	D (mg/100 g)	L (mg/100 g)	D (mg/100 ml)	L (mg/100 ml)	D (mg/100 ml)	L (mg/100 ml)	D (mg/100 g)	L (mg/100 g)
Alanine	0.01 (3.4%)	0.28	2.86 (45.9%)	3.37	0.62 (60.2%)	0.41	NC[a]	1.51	1.46 (28.3%)	3.70	–	38.60
Glycine	0.18		0.99		0.56		0.81		1.82		2.79	
Valine	–[b]	0.29	0.04 (5.0%)	0.76	0.01 (2.9%)	0.33	0.02 (9.5%)	0.19	–	3.35	–	14.56
Proline	0.36		5.02		1.61		30.17		33.70		38.30	
Isoleucine	–	0.16	0.02 (2.7%)	0.72	0.03 (20.0%)	0.12	0.01 (5.3%)	0.18	–	2.83	–	12.11
Leucine	0.04 (17.4%)	0.19	0.09 (9.6%)	0.85	0.04 (12.1%)	0.29	–	0.38	0.38 (5.2%)	6.98	–	17.03
Aspartic acid[c]	–	0.49	0.68 (43.9%)	0.87	0.74 (9.6%)	6.99	0.37 (20.9%)	1.40	0.54 (16.2%)	3.12	0.2 (0.4%)	53.15
Glutamic acid[c]	–	4.67	0.12 (10.0%)	1.08	0.33 (44.6%)	0.41	0.21 (18.3%)	0.94	0.75 (37.7%)	1.24	–	NC
Methionine	–	0.36	–	0.10	–	0.08	0.01 (8.3%)	0.11	0.10 (16.1%)	0.52	–	–
Phenylalanine	–	0.08	0.01 (0.9%)	1.09	0.06 (15.8%)	0.32	0.04 (10.8%)	0.33	0.13 (1.1%)	11.38	0.31 (0.9%)	35.38
Histidine	–	0.03	0.22 (26.8%)	0.60	0.03 (7.7%)	0.36	0.03 (12.0%)	0.22	0.09 (1.8%)	4.98	–	4.40
Lysine	–	1.46	1.16 (36.3%)	2.04	0.75 (29.3%)	1.81	–	0.69	1.77 (28.8%)	4.38	1.42 (9.3%)	13.80
Tyrosine	–	0.25	–	0.04	0.01 (11.1%)	0.08	–	0.26	–	4.56	–	14.67
Tryptophan	–	–	–	0.07	–	0.05	0.08 (12.9%)	0.54	–	–	–	23.59
Free D-AAs	0.05		5.20		2.62		0.77		5.22		1.93	
Total free AAs	8.85		22.80		16.04		38.50		87.78		270.31	

[a] NC=Not calculated due to incomplete separation from interference peak.
[b] –=No significant amounts found.
[c] Aspartic acid=Asp+Asn; glutamic acid=Glu+Gln.

proportion of the reagents were correct and in sufficient quantity, as described previously.

The prepared ECF–HFB derivatives were stable compounds. Their stability was tested over a period of 2 weeks with the chloroformic solution kept below 4°C in contact with the hydroalcoholic phase (upper phase). No significant ($P<0.05$) alterations were observed.

The enantiomer conversion during derivatisation treatment and during storage period was also tested. After derivatisation of a solution of L-AAs no D-forms were observed. The same was observed after storage, for 2 weeks, under 4°C.

From the chromatographic point of view improvement of separation of glutamic acid, threonine and methionine pairs is not possible simultaneously as a better separation of one of those pairs is accompanied by worsening in separation of others.

The results for the RMRs for both *p*-chlorophenylalanine and norleucine are represented in Table 4 and the corresponding relative standard deviations (RSDs) were, on average, 4.5% and 7.5%, respectively. The inter-day reproducibility of derivative formation was somehow higher with RSDs near 12%. The RSDs were also calculated by the enantiomer labelling method and proved to be much smaller.

The derivatisation was examined for the linearity of the calibration plots using *p*-chlorophenylalanine as internal standard. The peak areas of AAs were measured and the peak-area ratios of AA against I.S. were calculated. A linear response relationship was obtained with the correlation coefficients being above 0.99 in the range 0.4–6.0 nmol of injected solution (three injections of five standard solutions). The minimum detectable amounts of the AAs to give a signal-to-noise ratio of 3 under our GC conditions were 0.04–4 ng injected.

3.2. Food samples

Typical chromatograms obtained with a fermented milk sample and with balsamic vinegar are represented in Figs. 3 and 4, respectively. The results of the free amino acid composition of all the samples studied are represented in Table 5. These are, in general, in good agreement with those reported in literature, although some authors only quantified the D/L ratio. The dairy products results were comparable to those reported by Tamine and Robinson [35], Palla et al. [31] and Brückner and Hausch [3], the beer results with Brückner and Hausch [32,36], the balsamic vinegar results with Erbe and Brückner [33] and Chiano et al. [37] and the green coffee results with Arnold et al. [34] and Clarke and Macrae [38].

The separation of proline enantiomers is not possible with this type of derivatives but it would be of great importance in the analysis of balsamic vinegars, since D-Pro is directly related with their ageing. As their major AA, proline is also important in beers. Therefore it is suggested that, when the determination of proline enantiomers is in order other type of derivatives should be employed; the same reasoning may be applied for serine and threonine analysis.

4. Conclusions

A convenient and reliable method for the determination of amino acid enantiomers has been extensively developed. A significant advantage of the method presented herein is that the amino acid *N*(*O*,*S*)-ethoxycarbonyl heptafluorobutyl ester derivatives are rapidly prepared without the use of high temperature and are eluted below 200°C, with good resolutions. The peak response for threonine and serine is somehow low, especially for the latter.

The rapid derivatisation in aqueous media justifies this method suitability for routine amino acid profiling analysis and quantification. This method was successfully applied to various food samples in order to determine their free amino acid profiles. It is important to highlight that although food sample preparation is still time-consuming, the derivatisation step is significantly shorter.

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